A STUDY OF CELL FUSION AND POSSIBLE GENETIC RECOMBINATION IN THE PARASITIC PROTOZOAN *LEISHMANIA*

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biology Program

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YOUNGSTOWN STATE UNIVERSITY

February, 1993

Y-10-4
(9)

THESIS APPROVAL FORM

THESIS TITLE: A Study of Cell Fusion and Possible Genetic Recombination in the Parasitic Protozoan Leishmania.

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DATE 2-18-93

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ABSTRACT

A STUDY OF CELL FUSION AND POSSIBLE GENETIC RECOMBINATION IN THE PARASITIC PROTOZOAN *LEISHMANIA*

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Master of Science

Youngstown State University, 1993

Microspectrophotometric quantitative DNA measurements were made on cells of the promastigote stage and the amastigote stage of species in the genus *Leishmania,* for the purpose of determining the period of the life cycle where a ploidy change may occur.

The results of this study indicate that cellular fusion occurs during the amastigote stage in the genus *Leishmania*. These may subsequently undergo mitotic division, while the promastigotes exhibited an accelerated growth stage followed my mitosis, but no cell fusion. Mitosis and cell fusion of the amastigotes occurs within the macrophage cell. Results also indicate that cell fusion increases with the duration of infection in the macrophages. Cell fusion, rather than a single cell doubling its own DNA, is confirmed in isozyme data from what appears to be hybrid isolates of *L. panamensis* and *L. braziliensis*. Cell fusion in the amastigote presents a possible mechanism for genetic exchange.

ACKNOWLEDGMENTS

I would like to express my gratitude and appreciation for the guidance and assistance given by Dr. John J. Yemma and Dr. Richard D. Kreutzer for his assistance in this investigation. I would also like to thank members of the National Institutes of Health and Walter Reed Army Institute of Research for providing the parasites used in this study.

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INTRODUCTION

Recent advances in molecular and cellular biology techniques have provided breakthroughs in all areas of scientific research. Parasitology, for example, has branched into many new directions. Scientists in this field are now placing an emphasis on molecular techniques for identification, life cycle kinetics, and classification, while systematics based on morphology and geographic location play a less important role. In addition to taxonomic purposes, molecular methods are now employed in most laboratories, because they not only provide information for taxonomic purposes but also provide insight regarding life cycle kinetics. This information in turn often provides answers to medical questions concerning mode of infection for many parasitological diseases. One of these diseases, leishmaniasis, is of considerable concern to members of the scientific and medical community, for it is a life-threatening disease for the inhabitants of countries where the disease is endemic.

Leishmaniasis is caused by various parasitic protozoan species and subspecies of the genus *Leishmania*. Since the year 1900 when Leishman (Leishman, 1903) discovered the etiologic agent associated with visceral leishmaniasis, the disease has been identified in many parts of the world and has spread to epidemic proportions. It has been estimated that twelve million new cases of leishmaniasis occur each year, and compared with other human protozoal diseases ranks second only to malaria (Chang et al., 1985).

Classification and Clinical Manifestations

Protozoa of the genus *Leishmania* belong to the family Tripanosomatidae, order Kinetoplastida, and generally have been classified based on morphology and apparent life

cycle (Table 1). Because these protozoa present characteristics not found in higher organisms, they are believed to have diverged from the main eukaryotic lineage before plants, animals, and fungi (Sogin et al., 1986). Nomenclature of species of this genus have been assigned dependent on the geographic location and clinical manifestations of the disease (Table 2).

Leishmaniasis is a disease of the reticulo-endothelial system, which is usually expressed in one of two ways: as 1) visceral leishmaniasis or 2) cutaneous leishmaniasis. Of the different species of *Leishmania* identified, each has a bias to express one of the two disease states.

After its first discovery, cases of leishmaniasis appeared in regions of Africa, Asia, and the Mediterranean. Occurrence of the disease from these regions is now commonly referred to as Old World Leishmaniasis. With the advent of discoveries of the disease in regions of South America and Central America such as Mexico, Panama, Brazil, and most recently Ecuador (Mimori et al., 1989), the disease state in this area of the world is referred to as New World Leishmaniasis (Lainson, 1983). Citings of leishmaniasis in Texas (McHugh et al., 1990) indicate that problems with the disease in the North American region could occur. A growing concern in the United States about leishmaniasis is occurring due to the fact that military personnel in Operation Desert Storm returned home with the disease.

Cutaneous leishmaniasis is a public health problem in all areas where it has been described. Initially classified according to clinical and dermatological aspects, it is now grouped based on the biochemical aspects (Kreutzer and Christensen, 1980).

Cutaneous leishmaniasis is described as two forms, one with localized lesions (cutaneous), and one with invasion of nasal mucous tissue (muco-cutaneous). Of the two divisions described, the muco-cutaneous form is overwhelmingly caused by *L.braziliensis*, whereas the cutaneous form is caused by species such as *L. mexicana, L. panamensis* (New World), and *L. tropica* (Old World), (Shaw and Lainson, 1981).

The localized cutaneous lesion is less a concern of the two, for in most cases the patient's immunity is able to combat the disease and subsequently display resistance to reinfection by the same agent (Shaw, 1981). It is common place in some regions to purposely inoculate children with the parasite in a non-visible location of their body, hoping to later avoid infection that would cause a more apparent malformation (Schmidt and Roberts, 1989).

Muco-cutaneous leishmaniasis or diffuse cutaneous leishmaniasis (D.C.L.) is characterized by massive invasion of the skin and mucous tissue of the nasopharynx, as well as certain lymph nodes (Schmidt and Roberts, 1989). This form of the disease is severely malforming. Patients with D.C.L. often exhibit no response to conventional treatment (Bray and Lainson, 1967), and it is proposed that this form of the disease is due to an immunological defect in the host rather than a special strain of parasite (Convit et al., 1972).

Cutaneous leishmaniasis is observed when parasites invade macrophage cells under the dermis to produce a localized ulcer. If the parasites enter the bloodstream and invade macrophages of the viscera, the result is a sever and potentially lethal form of the disease referred to as visceral leishmaniasis.

TABLE 1: CLASSIFICATION AND CHARACTERISTICS OF *LEISHMANIA

TAXONOMY	CHARACTERISTICS
Phylum: Protozoa	
Subphylum: Sarcomastigophora	Flagella present, single type of nucleus, spore formation absent, sexuality present as syngamy.
Superclass: Mastigophora	Colonal, assexual reproduction by binary fission.
Class: Zoomastigophora	Chromatophores absent, sex in some forms, predominantly parasitic.
Order: Kinetoplastida	One to four flagella, kinetoplast present as mitochondrion.
Suborder: Trypanosomatina	
Family: Trypanosomatidae	One flagellum, all species parasitic.

*Adapted from Lumsden, 1974.

TABLE 2: NOMENCLATURE OF SPECIES OF *LEISHMANIA

PARASITE	LOCALITY	DISEASE STATE
Section **SUPRAPYLARIA**		
Leishmania tropica	Urban areas of Middle East and India	Cutaneous
Leishmania major	Africa, Middle East, Soviet Asia	Cutaneous
Leishmania donovani	China, India, and Africa	Visceral
Leishmania amazonensis	Amazon Basin	Cutaneous/Muco-cutaneous
Section **PERIPYLARIA**		
Leishmania braziliensis	Brazil	Cutaneous/Muco-cutaneous
Leishmania panamensis	Panama, Costa Rica	Cutaneous

* Species presented here are representative of the isolates used in this study and do not present the full spectrum of species identified.

Adapted from Schmidt and Roberts, 1989.

Visceral leishmaniasis has its most severe effects reflected in such organs as the spleen, liver, bone marrow, lymph glands, and intestines. These organs become grossly enlarged due to congestion of the parasites within the host's macrophage cells. Death occurs when the patient's organ malfunctions or most commonly when the bone marrow is affected. Destruction of bone marrow tissue results in the weakening and eventual disappearance of the host's immunity (Lainson et al., 1981).

The Life Cycle of *Leishmania*

Parasites of the genus *Leishmania* have a digenetic life cycle, alternating their life between two multicellular hosts (Bard, 1989). The parasites are transmitted by insect vectors to the vertebrate host. Insect vectors for the disease are from the genus *Phlebotomus* (Old World), commonly known as the sandfly (Alder and Ber, 1941). Vectors for New World Leishmaniasis are of the genus *Lutzomyia* (Walters et al., 1989). In most cases man is an accidental host for the parasite. Canids, rodents, and lizards are the most affected vertebrates, serving as reservoirs for the disease (Lainson and Shaw, 1979).

One potential vector, the phlebotomine sandflies, are "pool-feeders" (Alder and Ber, 1941). The bite of the insect first cuts the host's skin to form a tiny pool of blood, which is then sucked into the gut of the fly. If the vertebrate host on which it feeds is infected with leishmaniasis, parasites may be taken up in the bloodmeal.

While living inside the sandfly the microorganisms display one of its two distinct morphological forms, the promastigote. The promastigote is an extracellular, flagellated,

and elongate cell measuring between 12 and 16 um in length and 1.5 to 3.5 um in width (Bard, 1989). The nucleus is centrally located, while the kinetoplast, containing mitochondrial DNA, and the flagellum are situated at the anterior end. At this stage the flagellates are highly motile and undergo profuse multiplication and development within the gut of the sandfly (Walters et al., 1989).

Species of *Leishmania* are segregated into three classes; Hypopylaria, Peripylaria, and Suprapylaria. Classification is dependent upon location of development within the gut of the insect vector. Parasites of the class Hypopylaria develop in the hindgut region of the sandfly. Migration of the promastigotes to the biting mouthparts does not occur, and transmission of the disease only occurs through ingestion of the sandfly. In the class Peripylaria, development of the promastigote occurs in the hindgut region, followed by migration of the flagellates to the midgut and foregut. Transmission of the parasites of this class is by bite. Representatives are *L. braziliensis* and *L. panamensis*.

L. donovani, L. major, L. tropica, and *L. amazonensis* belong to the class Suprapylaria. Members of this class develop in the midgut of the vector and migrate anteriorly, where they are then transmitted by the biting parts of the mouth (Alder and Theodor, 1957).

Interestingly, one study which followed the development of *L. panamensis* in its natural sandfly host, *Lutzomyia gomezi* (Walters et al., 1989), showed eight distinct morphological forms of the parasite development within the insect. Subsequent research in this area reported that development within the sandfly appears to transform non-infective promastigotes to the infective stage (Sacks and Perkins, 1984). It has been reported that promastigotes taken from stationary phase cultures had a more rapid rate of infectivity

than that of promastigote cultures in logarithmic phase. Infective promastigotes are now termed "metacyclics" (Mallinson and Coombs, 1989). The exact biochemical methods by which the change in infectivity occurs remains undetermined.

Transmission of infective promastigotes to the vertebrate host results in a morphological change of the parasite to the amastigote form. Amastigotes are commonly referred to as L-D bodies or Leishman-Donovan bodies (Lumsden, 1974), named for the first two individuals to isolate them from patients suffering from leishmaniasis. The amastigote is a rounded or oval body which invades the macrophage cells of its host. This morphological stage of the parasite can have dimensions from 1.5 to 6.5 um, depending on the species of the parasite. Many of the organelles found in the promastigote are also found in the amastigote form, with the exception of the flagellum. Important characteristics of the amastigote are its well-defined nucleolus and the "flagellar pocket" which contains a rudimentary flagellum (Lumsden, 1974).

On a biochemical level amastigotes differ from their promastigote partners in that the former posses metabolic reactions occurring at a pH near 5, while the later proceed optimally at a pH near 7 (reviewed by Glew et al., 1988). It seems that the amastigote has adapted to survive and multiply in an acid environment. Differences in rate of tubulin synthesis and in surfaces proteins between the two forms have also been observed (Ramamoorthy et al., 1992). The exact trigger that causes this shift in morphology is unclear; however, researchers have noted that *in vitro* cultures of promastigotes can convert themselves to amastigote-like forms when incubated with

hexose sugars (Darling and Blum, 1990), and at elevated temperatures (Eperon and McMahon-Pratt, 1989); (Stinson et al., 1989).

Spread of infection within the host occurs when the amastigote invaded macrophages divide or these cells rupture. Amastigotes are often able to lyse the cells they inhabit, causing release of the protozoa into the extracellular fluid, leading to invasion of other areas of the body.

The Genetics of *Leishmania*

Members of the genus *Leishmania* have no recognizable condensed chromosomes during their life cycle; therefore, it is impossible to establish a karyotype by classical means. Other methods using pulsed field gradient gel electrophoresis (PFGGE) (Schwarz and Cantor, 1984) and orthogonal field alternation gel electrophoresis (OFAGE) (Carle and Olson, 1984) have attempted to determine a karyotype for the genus based on separation of chromosome sized DNA molecules.

Of the studies performed, 22 to 33 bands were identified in different *Leishmania* species (Giannini et al., 1986). Studies of this type also indicate that *Leishmania* could be diploid (Bastien et al., 1992). This is at least true for some genetic loci, such as the mini-exon (Iovanisci and Beverly, 1989), and at the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene loci (Coderre et al., 1983). Data also show that chromosomes bearing essential housekeeping genes are diploid as well (Iovanisci and Beverly, 1989); (Giannini et al., 1986). Scientists predict that *Leishmania* could be aneuploid for some sequences such as the alpha- and beta-tubulin gene loci (Spithill and Samaras, 1985).

Experiments using these techniques also indicate that *Leishmania* has a very plastic genome. Karyotype analysis demonstrate that there are differences in banding patterns between species and subspecies, and to a lesser extent within a species itself (Bishop and Miles, 1987). Some unique characteristics of *Leishmania* genetics are the de novo appearance of small linear DNAs (Beverly and Coburn, 1990), and the findings of circular extrachromosomal DNA elements (Hightower et al., 1988).

Leishmania belong to the order Kinetoplastida, a name that is derived from an organelle that is located near the flagellum; the kinetoplast. The kinetoplast represents the sole mitochondrion of the organism. It contains two forms of kinetoplast DNA (kDNA); maxicircles and minicircles. The two forms are meshed together in a giant network consisting of about 10,000 minicircles and 25-50 maxicircles (Simpson, 1987). Maxicircle DNA is actively transcribed; however, there is no definite proof that minicircle DNA is transcribed, and its function remains unknown. During *in vitro* cultivation, it has been noted that minicircle DNA goes through minor alterations in its sequences (Spithill et al., 1984). Also, a rapid and dramatic shift in the proportion of kDNA circles of different sequences has been found; a phenomenon now termed "transkinetoplastidy" (Lee et al., 1992). Findings in the area of *Leishmania* genetics indicate that the organism is unique in its molecular structure. The nature of the kDNA and the ploidy of the organism still remain open questions.

Is There Sex in *Leishmania*?

With the advent of new molecular techniques for identification, several observations strongly support the occurrence of sexual genetic exchange in *Leishmania*.

Videomicroscopy has recorded cell fusion of promastigote forms in several species (Lanotte and Rioux, 1985). Further support comes from electrophoretic data indicating hybrids with heterozygous isoenzyme (Kelly et al., 1991); (Kreutzer , unpublished data); and DNA patterns (Kelly et al., 1991).

Kelly (1991) provides evidence of genetic recombination in *Leishmania*. The study based on three criteria: 1) opportunity for interaction of parental strains, 2) evidence for a recombinant genotype, and 3) independent transmission of mitochondrial and nuclear DNA sequences showed that some form of sexual exchange occurs in this organism. It was also noted that the inheritance of minicircle kDNA is uniparental. Another study reports recombination in *Leishmania* occurring in as high as twenty-five percent of the products (Tobin et al., 1991).

Mixed infections of *Trypanosoma brucei* within its natural insect vector, the tsetse fly demonstrate that genetic exchange occurs in this related organism (Jenni et al., 1986). The progeny observed presented restriction patterns that fit a Mendelian model of meiosis. Similar experiments with *Leishmania major* (Panton et al., 1991) demonstrate the frequency of genetic exchange to be too low to indicate sexuality as a normal part of the life cycle.

Of sexual processes observed in other organisms (Tait, 1983), two main events must occur; karyogamy and meiosis. Findings so far cannot hold up to these two criteria. Studies seem to indicate that a clonal model of reproduction best represents *Leishmania* (Panton et al., 1991); (Tibayrenic et al., 1991), with sexual exchange occurring to a lesser extent.

Kreutzer (unpublished) found evidence supporting a sexual process in *Leishmania*. Using isoenzyme analysis to identify isolates of the parasite, it was determined that a number of promastigote samples displayed heterozygous isoenzyme allomorphs for a number of enzymes examined. It is proposed that these isolates are hybrids of *L. panamensis* and *L. braziliensis*. Upon examining the data, it was concluded that one of two phenomena occurred; either a type of mating between the two strains produced a heterozygous intermediate, or there was fusion of two cells to produce a heterokaryon.

A primary objective of this study was to examine the DNA content of different species of *Leishmania*, both in the promastigote and amastigote form. Using a Feulgen staining technique combined with the two wavelength method of quantitative microspectrophotometry (Patau, 1957); (Ornstein, 1957); (Yemma, 1975), it is possible to quantify the DNA content of individual cells. This method also allows the detection of differences in mitotic activity and ploidy levels. It is also useful in determining the presence of a sexual cycle. Data from this study will attempt to determine whether meiosis is occurring in either of the two morphological stages, and it will also detect a difference in ploidy, if it exists, between "normal" isolates and the proposed hybrids.

This study will not only provide information on the characteristics of the organism itself but will hopefully aid in the development of treatment against the disease which it causes. The presence of sexuality is important here since strategies for treatment are different for clonal organisms than sexual organisms.

MATERIALS AND METHODS

Leishmania Strains

Thirteen strains of *Leishmania spp.* were obtained from Walter Reed Army Institute of Research (WRAIR), National Intitutes of Health (NIH),and Yale Arbovirus Research Unit isolate banks. These isolates had been previously identified by enzyme analysis (Table 3). Prior to the study each isolate was reidentified by enzyme analysis performed by Dr. Richard Kreutzer at Youngstown State University. Some of the isolates subject to electrophoresis could not be identified as a single species and were thought to be hybrids of *L. panamensis* and *L. braziliensis*. Isolates in this category are noted in Table 3 as *Leishmania sp.*

Cultivation of promastigote isolates

These isolates were cultivated following previously described procedures (Hendricks et al., 1978); (Childs et al., 1978). The particular method chosen for growth of the promastigotes was use of Schneider's Drosophila Medium. Stocks of the promastigote isolates were stored in a cryochamber with liquid nitrogen. When needed, the isolates were removed from the chamber, fast thawed, and pelleted at 1000xg for 10 minutes. The pellet was washed in with 1 ml of growth medium and centrifuged at 1000xg for 10 minutes (two repititions). The sample was then placed in fresh growth medium. The growth medium consisted of 80% Schneider's Drosophila Medium purchased from GIBCO, Grand Island, New York, mixed with 20% fetal bovine serum (deactivated at 57^{o}C for 30 minutes). Gentamycin, purchased from Sigma Chemical, was

added to the medium as 0.1ml of gentamycin/100ml of growth medium.

Cultivation of amastigote isolates

The macrophage cells were kept in medium containing 10% fetal bovine serum in RMPI (1640) with gentamycin. The culture was kept at 35°C in 5% CO_2. Before infection, the cells were counted by making a 1:10 dilution. Using a six well plate, 2.5ml of the macrophage/media mixture was placed in each of the wells. Parasites were added to each of the wells so that the number of parasites exceeded the number of macrophages by 3:1. The parasites and the macrophages were mixed and then replated by adding 2.5ml of the mixture to each of the six wells. The plate was then covered and allowed to incubate for 24-48 hours at 35°C with 5% CO_2. The cells were then washed three times using the 10% fetal bovine serum in RMPI (1640) media. The infected cells were then counted and placed in 10% buffered formalin phosphate at pH 7.2.

Electrophoresis

The procedures for separating cells from the growth medium, set up of certain subtrate/ buffer systems, and cellulose acetate electrophoresis have been reported elsewhere (Kreutzer and Christensen, 1980). All equipment was manufactured by Helena Laboratories, Beaumont, Texas.

Isolates were identified by cellulose acetate electrophoresis (Tables 3 and 4). A list of the enzymes examined is presented in Table 5.

TABLE 3: *Leishmania* ISOLATES

*Isolates tested by cytophotometric analysis and also by isoenzyme electrophoresis in results.

SPECIES	NUMBER	DESIGNATION (WHO)	HOST	SOURCE
Promastigotes				
Leishmania panamensis	N111	MHOM/HN/90/ANGLE	Human	Honduras
Leishmania panamensis	CL064	MHOM/CO/82/CL064	Human	Columbia
Leishmania braziliensis	N103	MHOM/HN/90/HN-190	Human	Honduras
Leishmania braziliensis	CL063	MHOM/CO/82/CL063	Human	Columbia
(*Leishmania panamensis*/ *Leishmania braziliensis*)				
**Leishmania* sp.	N112	MHOM/HN/90/HANDLEY	Human	Honduras
**Leishmania* sp.	N109	MHOM/HN/90/HN-178	Human	Honduras
**Leishmania* sp.	CL090	MHOM/CO/85/CL090	Human	Columbia
Leishmania major	N136	MHOM/MA/91/N136	Human	Mediterranean
Leishmania donovani	N135	MHOM/ET/90/N135	Human	Ethiopia
Amastigotes				
Leishmania panamensis	CL064	MHOM/CO/82/CL064	Human	Columbia
Leishmania braziliensis	CL063	MHOM/CO/82/CL063	Human	Columbia
Leishmania tropica	WR1063	MHOM/SA/91/WR1063	Human	Saudi Arabia
Leishmania major	WR1075	MHOM/IQ/91/WR1075	Human	Iraq

TABLE 4 : LEISHMANIA ISOLATES (CAE)*

L. panamensis/ L. braziliensis "hybrids" (WHO Designations)

MHOM/HN/84/H-10
MHOM/HN/84/H-12
MHOM/HN/90/HANDLEY
MHOM/HN/90/HN-171
MHOM/HN/90/HN-178
MHOM/PA/88/CA292
MHOM/CO/85/CLO90 (PLUS 11 CLONES)
MHOM/CO/85/CL122 (PLUS 8 CLONES)
MHOM/CO/86/CL228
MHOM/CO/88/CL452

*Identification by cellulose acetate electrophoresis

TABLE 5: ENZYMES TESTED IN THIS STUDY *

ENZYME	ABBREVIATION
Oxidoreductases	
Lactate dehydrogenase	LDH
Malate dehydrogenase	MDH
Malic enzyme	ME
Isocitrate dehydrogenase	ICD
Phospogluconate dehydrogenase	6PGDH
Glucose-6-phosphate dehydrogenase	G6PDH
Glutathione reductase	GSR
Transferases	
Glutamine-oxaloacetate transaminase	GOT & ASAT
Glutamate-pyruvate transaminase	ALAT
Hexokinase	HK
6-Phosphofructokinase	PFK
Phosphoglucomutase	PGM
Pyruvate kinase	PK
Hydrolases	
Acid phosphatase	ACP
Peptidase D	LP
Lyases	
Fumarate hydratase	FUM
Isomerases	
Mannose phosphate isomerase	MPI
Glucose phosphate isomerase	GPI

* Adapted from (Kreutzer et al., 1983)

Slide Preparation

Parasites contained in the growth medium were fixed with 10% buffered formalin phosphate at pH 7.2 obtained from Fisher Scientific, Fair Lawn, New Jersey. The formalin was added to the medium to acheive a ten percent fixative solution.

The samples were placed in 50 ml tubes and pelleted via the use of ans IEC HN-SII centrifuge at 1000xg for 10 minutes. The supernatant was then removed, and the remaining pellet was washed with the 10% formalin phosphate and incubated for 24 hours. The following day, the samples were once again centrifuged at 1000xg for 10 minutes. The supernatant was removed and the pellet was washed with 70% ethanol. The parasites were again pelleted at 1000xg for 10 minutes. The supernatant was removed, and the remaining pellet was resuspended in 70% ethanol.

The ending mixture containing 70% ethanol and a concentration of parasites was separated to form a homogeneous mixture using a Branson Sonifier 250 obtained from VWR Scientific, Philadelphia, Pennsylvania. Output control remained at level 6, and the mixture was sonicated for 2 seconds for three repititions (for promastigotes). This was a crucial step in the procedure for prolonged exposure could lyse the cells and nuclei. The amastigote pellets were vortexed in order to not break the macrophage cells while effecting separation. The parasite solution was then transferred by pipet to previously albuminized slides. The slides were allowed to air dry in a ventilated hood system and were then ready for staining. The same procedure was used for samples of promastigotes and amastigotes.

Feulgen Staining and Cytochemical Methods

Parasites prepared for microspectrophotometry were stained using the Feulgen nuclear reaction (Feulgen and Rosenbach, 1924). The Feulgen nuclear reaction is based upon the observation that the stain is stoichiometric for DNA. The Schiffs reagent forms a stable bond with the exposed aldehyde groups of hydrolized DNA. The bonding occurs as a 1:1 ratio with the DNA, and the result is the production of a magenta color localized in the nuclei of the cells. The stability of the reaction provides an accurate way of determining the relative DNA content of individual nuclei (Kasten, 1967).

Variations of the reaction can occur as a result of differences in hydrolysis time, pH, manufacturer of stain, and exposure time to Schiffs reagent (Olkowski, 1976). The parasites in this study were stained simultaneously to minimize these variations.

The two key steps involved in the Feulgen reaction are 1) hydrolysis of DNA and 2) stoichiometric staining of the DNA. An indepth study varying hydrolysis times with acid concentration performed by Yemma and Therrien in 1972 concluded that an optimal hydrolysis time for the Feulgen reaction was 45 minutes using 5N HCl. Stoichiometric staining of the DNA was achieved using Schiffs reagent. The staining reagents are presented in Table 6. The staining procedure was as follows:

1). The prepared parasite smears were washed in distilled water for 5 minutes.

2). The smears were than transferred to 5N HCl for 45 minutes to hydrolyze the DNA (critical step).

3). Following hydrolysis, the slides were transferred to fortified Schiffs reagent for a two hour staining period.

4). After staining, the slides were transferred to a 10% potassium meta-bisulfite solution and washed for 10 minutes. This procedure was repeated a second time.

5). The smears were placed in distilled water for 5 minutes.

6). The smears were then run through a graded ethanol series (70%, 90%, absolute) starting with lowest concentration to highest concentration for dehydration. The slides remained in each concentration for 5 minutes.

7). The slides were cleared in xylene for 5 minutes.

8). The smears were mounted in permount, and coversliped.

Microspectrophotometric Determinations

All determinations were made using a Zeiss Type 01 microspectrophotometer with use of a 100x Planachromat oil immersion objective. Measurements of DNA were taken using the two wavelength method employed by Patau in 1952. This method provides an accurate way to measure DNA of materials too irregular in distribution to give valid results with conventional methods. In other words, the material does not conform to the Beer-Lambert Law.

To choose the wavelengths, an absorption curve was created measuring a single homogeneously stained nuclei from the population to be measured. Several readings were taken for wavelengths of 450 nm- 600 nm, with intervals of 5-10 nm. For each wavelength, a measurement was taken for nuclear material and for background material. Extinction coefficients ($E = \log I_0 / I_S$; where, I_0 = reading of background light, and I_S = light passing through the specimen) were plotted against the corresponding wavelengths. The two wavelengths were chosen so that $2E_1 = E_2$ where $E_1 = \log I_0 / I_S$ at $_1$, and $E_2 = \log I_0 / I_S$ at $_2$.

TABLE 6: STAINING REAGENTS

Schiffs Reagent: Dissolve 5.0g of basic fushin in 1000ml of boiled distilled water. Cool to 50°C and filter. Add 100ml of 1N HCl and 20g of potassium meta-bisulfite ($K_2S_2O_5$) to the filtrate. Stir thouroughly and store in dark for 24 hours. Add 2.5g of neutral activated charcoal and filter.
*Right before the staining procedure the stain must be fortified with 10% potassium meta-bisulfite solution, 1:4 mixture.

10% Potassium meta-bisulfite: Dissolve 10g potassium meta-bisulfite in 100ml water.

Hydrochloric Acid Rinse: 5N HCl

Ethyl Alcohol Rinse: Variations on concentrations of ethyl alcohol rinses were prepared by using a stock of absolute alcohol and by making the appropriate dilutions using distilled water.

After the two wavelengths were selected, the nuclear content of the parasite samples were measured. For each cell, four readings were taken:

Io_1 = background light at $_1$
Is_1 = light through specimen at $_1$
Io_2 = background light at $_2$
Is_2 = light through specimen at $_2$

The amount of dye (DNA) in the measured area is given as:

$$M = KAL_1Q$$

where

K = constant (1/e) where e is the extinction coefficient at .
$L_1 = (1-T_1)$ = absorption, where $T_1 = I_s/I_o$ at $_1$.
$L_2 = (1-T_2)$ = absorption, where $T_2 = I_s/I_o$ at $_2$.
A = area of photometer aperature in nm (r^2).
$Q = L_2/L_1$.
C = 1/(2-Q) ln 1/(Q-1)

With the four separate readings for each nuclei, relative DNA calculations and statistical analysis were made on the main computer at Youngstown State University with use of a program created by Dr. John Yemma.

Nine isolates of promastigotes were examined in this study. Fifty nuclei of each isolate were analyzed for their nuclear DNA content. Two isolates of amastigotes with infection for 24 hours and two isolates with infection for 48 hours were examined with measurements of fifty nuclei each. All the DNA readings are presented as histograms in order to analyze the data. Changes or shifts in the histograms will detect differences in ploidy level and mitotic activity.

RESULTS

The results of this investigation are presented in the form of histograms, tables, graphs, and zymograms representing CAE plates. Histograms are presented in order to show changes in measured nuclear DNA content and the DNA content within a population of cells. All values of DNA content are expressed in arbitrary units. The relative amount of DNA for a given population is derived from the stoichiometric DNA binding relative to its concentration in each nucleus. The graphs of zymograms represent a clear and accurate duplicate of the allomorphs present on the actual CAE plates.

ELECTROPHORESIS

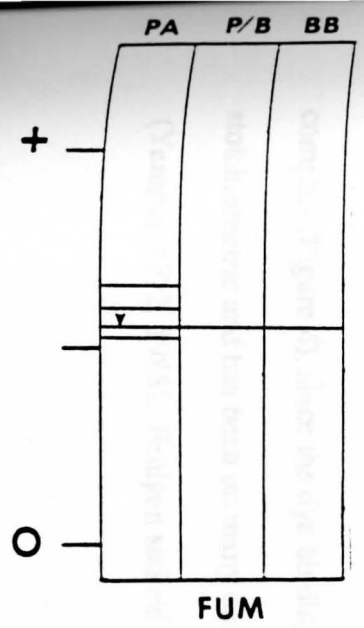
Each isolate noted in Table 4 was characterized for 20 enzyme systems (Table 5). Each of the 20 enzyme systems were extensively studied, and species of *Leishmania* can be properly identified according to the method used here (Kreutzer et al., 1983). Approximately 1000 runs of isolates for *L. panamensis* and 400 for *L. braziliensis* provided the information for the allomorphs presented. The results here are adapted from unpublished data collected by Dr. Richard Kreutzer.

Figure 1 presents zymogram plates for enzymes of the isolates which produced a single dark band identical to the most common allomorph in *Leishmania panamensis* and *Leishmania braziliensis*. The most common allomorph is the band(s) with the imprinted arrow. Either *L. panamensis* or *L. braziliensis* alone, *L. panamensis* and *L. braziliensis* in mixed culture , or a hybrid species would produce the same single band.

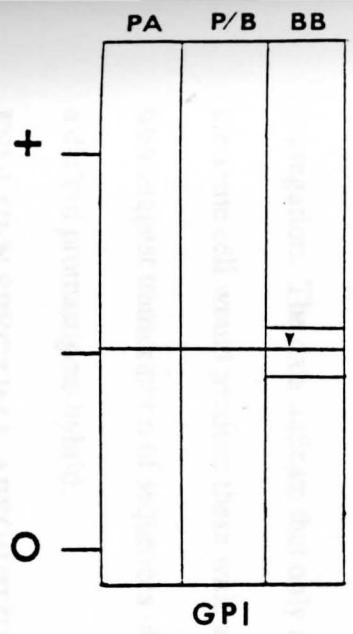
FIGURE 1

Zymograms of Enzymes

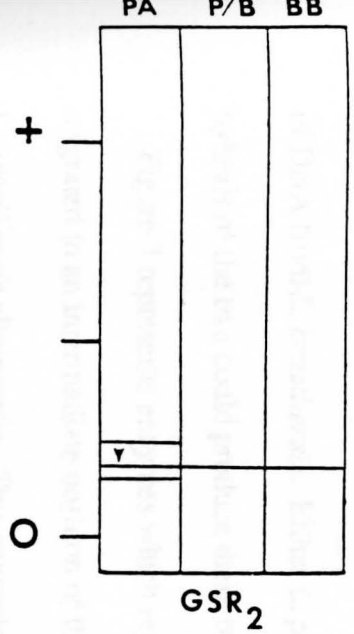
(Producing a Single Dark Band)



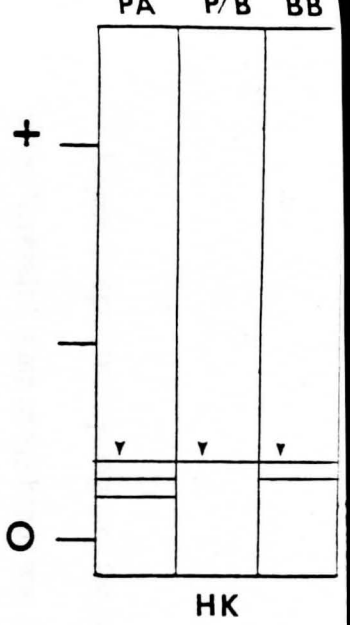
FUM



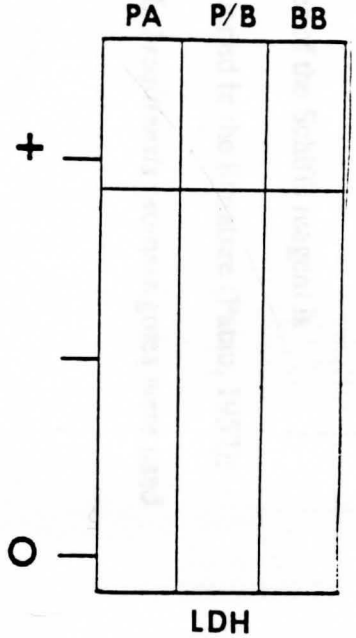
GPI



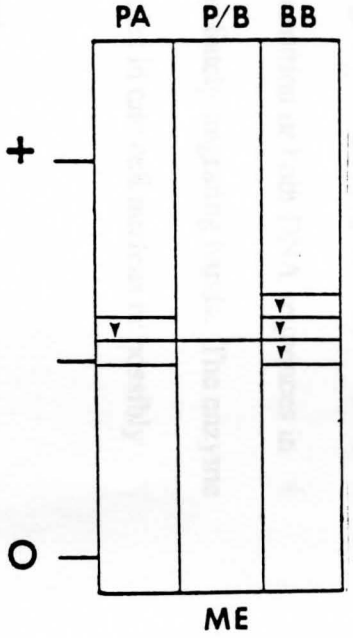
GSR_2



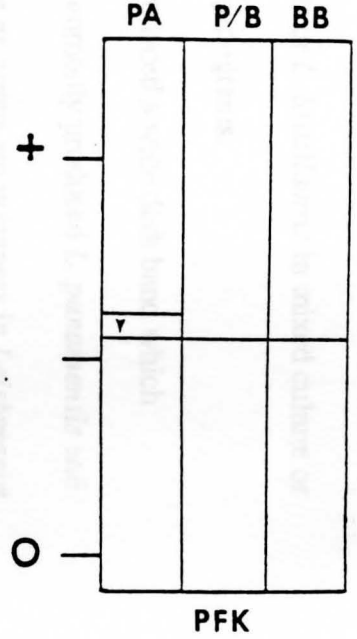
HK



LDH



ME



PFK

Figure 2 represents zymograms for enzymes which regularly produced two dark bands in the "hybrids". One of the bands was a common allomorph in *L. panamensis*, and the other was a common allomorph in *L braziliensis*. The two separate bands suggest that the enzymes could be monomers with transcription of a sequence from *L. panamensis* to produce one polypeptide sequence with the other sequence coming from transcription of DNA from *L. braziliensis*. Either *L. panamensis* or *L. braziliensis* in mixed culture or hybrids of the two could produce these two band zymograms.

Figure 3 represents enzymes which regularly produced a wide dark band which migrated to an intermediate position of the most commonly produced *L. panamensis* and *L. braziliensis* allomorphs. This suggests that these enzymes are polymers in *Leishmania*, with some of the strands being produced by *L. panamensis* and others being produced by *L. braziliensis*. The wide band suggests that the genes for the enzyme follow Mendelian segregation. The data indicate that only a hybrid condition or both DNA sequences in the same cell would produce these wide and intermediately migrating bands. The enzyme data suggest transcription of sequences of both species in one cell nucleus or possibly a diploid promastigote hybrid.

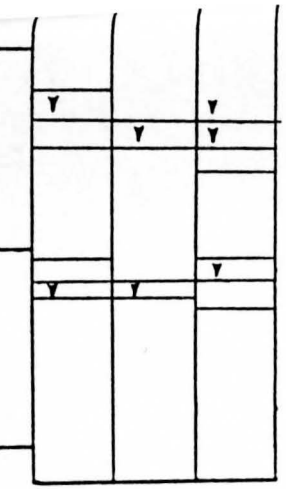
FEULGEN SPECTRAL ABSORPTION CURVE

When using the two wavelength method of microspectrophotometry, an absorption curve must be established in order to find the maximum absorption for the Feulgen-DNA complex (Figure 4), since the dye binding properties of the Schiffs reagent is stoichiometric and has been on many occasions reported in the literature (Patau, 1952); (Yemma, 1972; 1985). Feulgen stained nuclei of *L. braziliensis* promastigotes were used

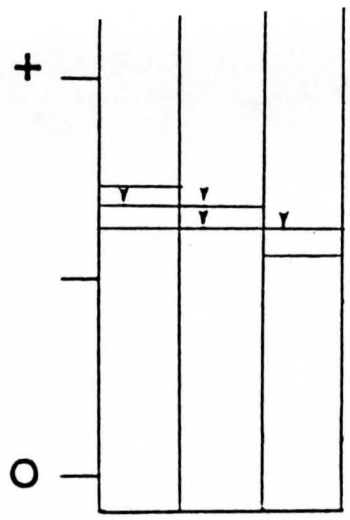
FIGURE 2

Zymograms of Enzymes

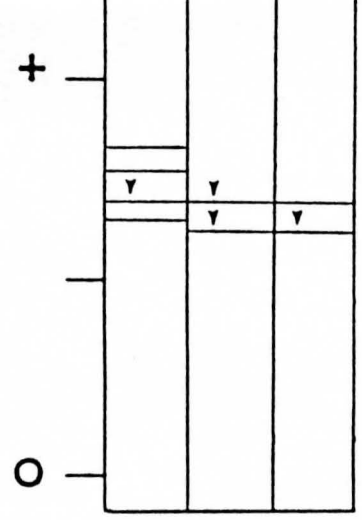
(Producing Two Dark Bands in "Hybrids")



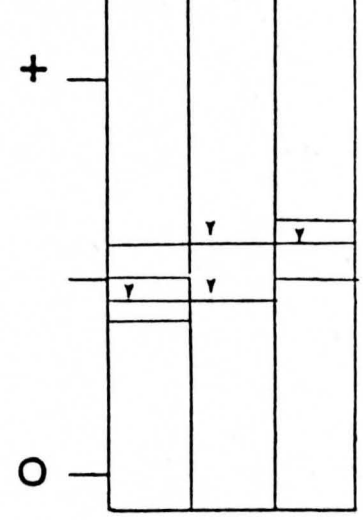
ACP



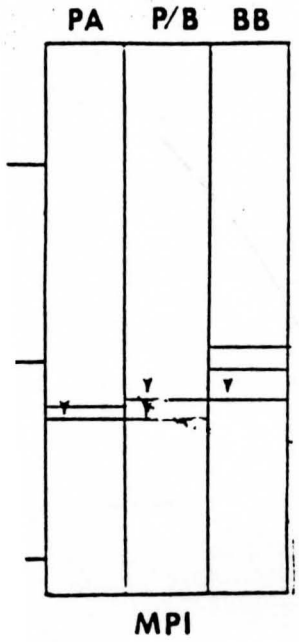
ASAT



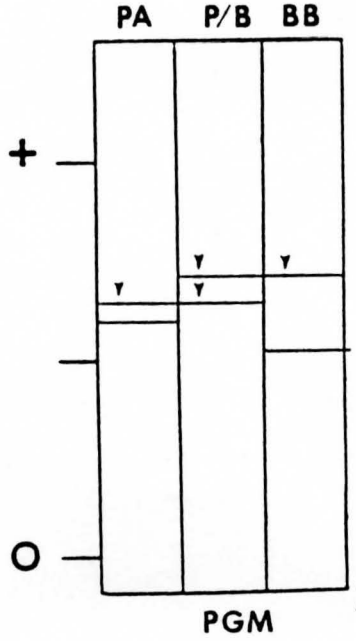
GOT



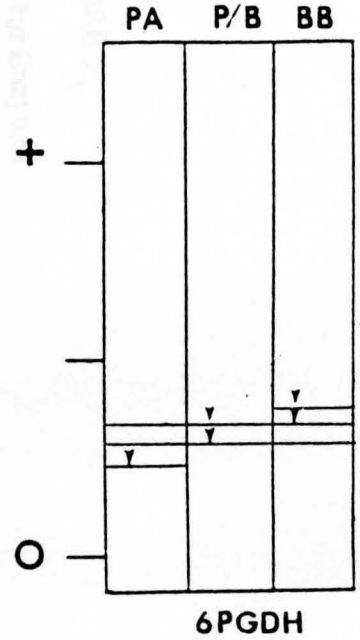
LP



MPI



PGM

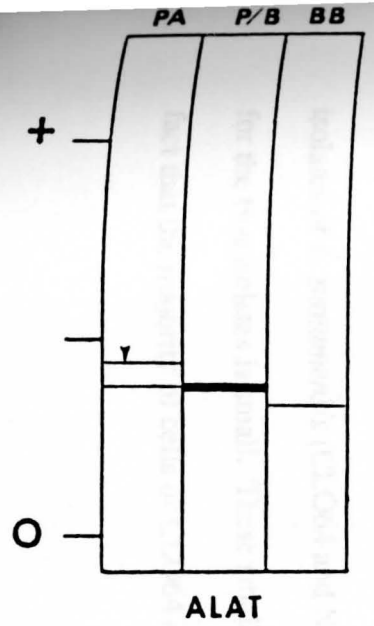


6PGDH

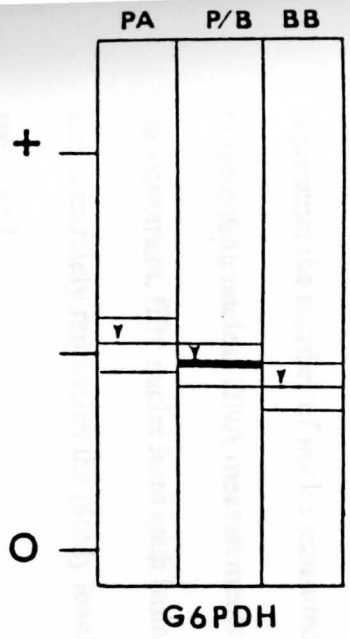
FIGURE 3

Zymograms for Enzymes

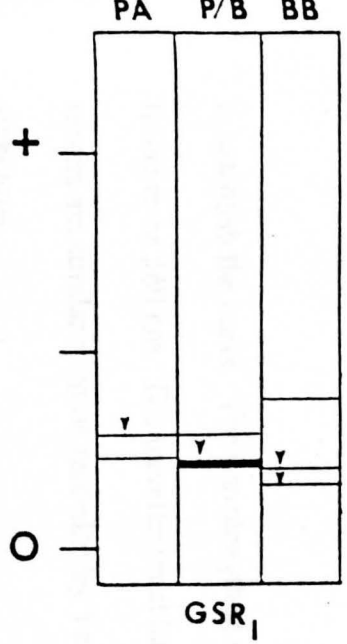
(Producing Wide Intermediate Bands for "Hybrids")



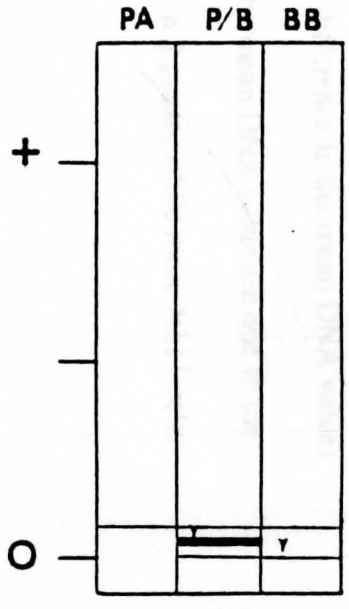
ALAT



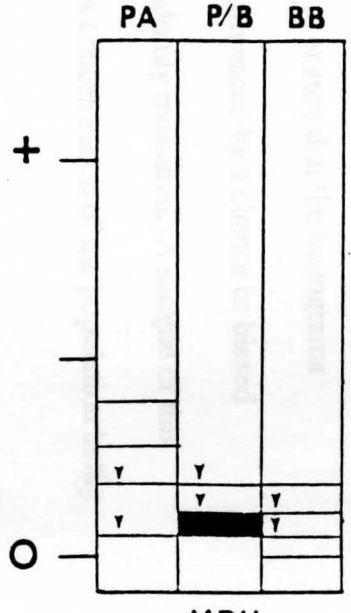
G6PDH



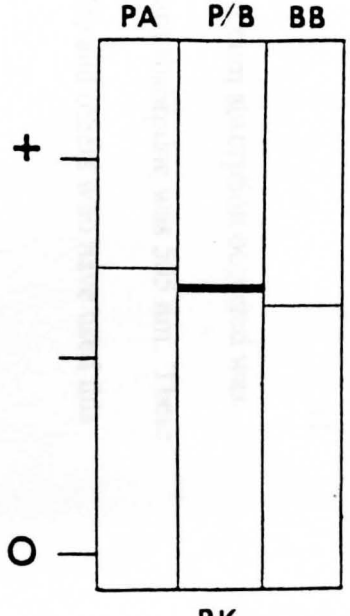
GSR_1



ICD



MDH



PK

to establish the curve. The wavelength at which maximum absorption occurred was found to be 560 nm. The wavelength at half-maximum absorption was 505 nm. These results are similar to those recorded by Yemma (1971) and others who have used this technique.

CYTOPHOTOMETRIC ANALYSIS OF NUCLEAR DNA

Promastigotes

The measured nuclear DNA content of promastigote cells for the isolates given in Table 3 are presented as frequency histograms (Figures 5-9). The abscissa represents the dye concentration of nuclei within a population, given in arbitrary units, and the ordinate represents the number of nuclei measured. In order to ensure that the histograms representing nuclear DNA measurements were not influenced by a chance or biased measurement, fifty nuclei from each isolate were randomly measured. A sample of this size accurately represents the ploidy level(s) or DNA concentration of the population being measured.

To describe the conditions of haploid, diploid, or tetraploid, the designations of 1C, 2C, and 4C are used respectively (Swift, 1950). The terminology of G_1 (presynthetic gap), S (DNA synthesis), and G_2 (post-synthetic gap) used by Howard and Pelc (1953) will be used to indicate synthetic activity.

Figures 5a and 5b show the relative nuclear DNA content for cells of two separate isolates of *L. panamensis* (CLO64 and N111). The difference in the mean DNA values for the two isolates is small. These small shifts in the mean DNA value are due to the fact that the majority of cells of CLO64 are present in S phase, while more N111 cells

FIGURE 4

Feulgen Spectral Absorption Curve

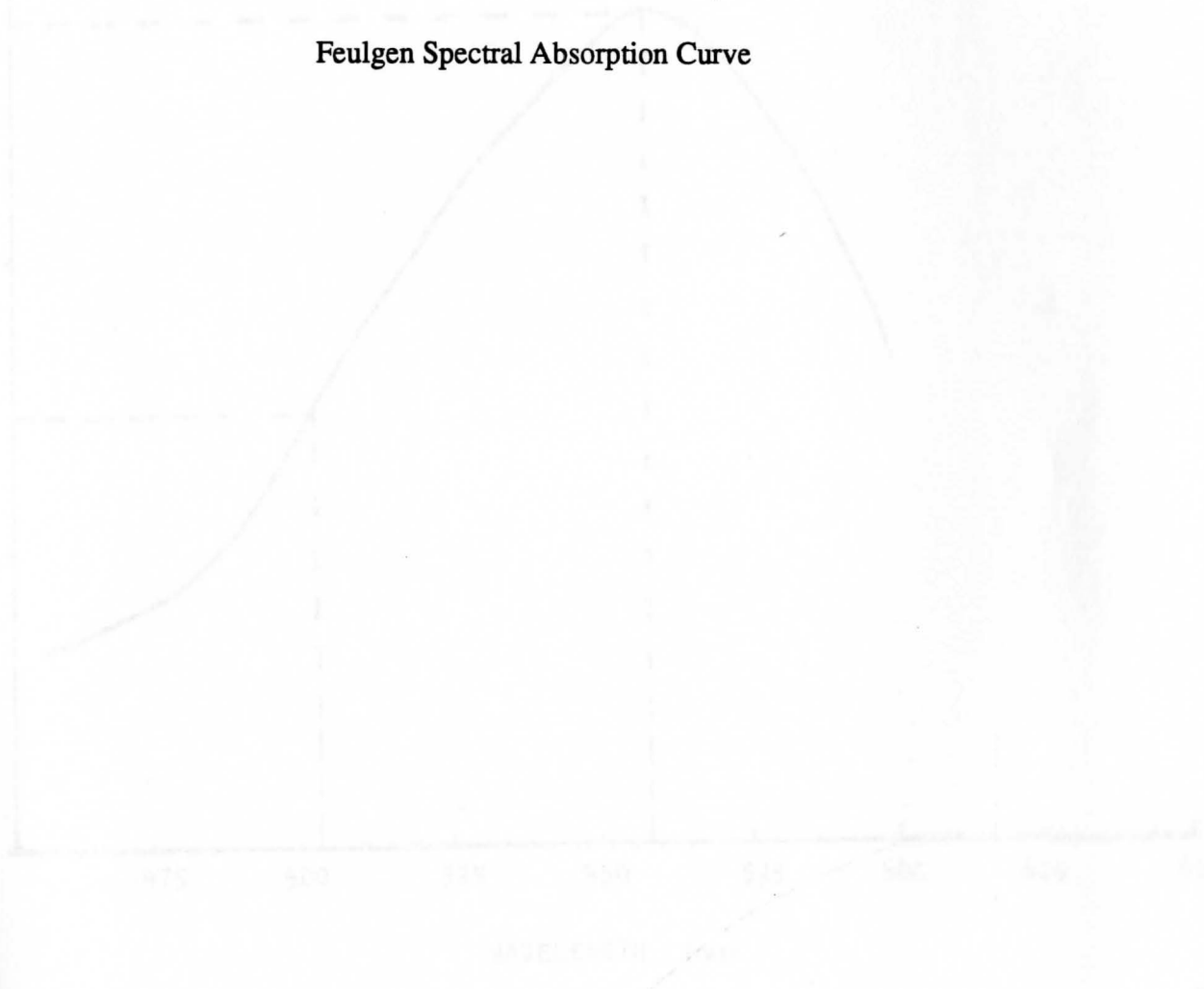


FIGURE 4 : FEULGEN SPECTRAL ABSORPTION CURVE

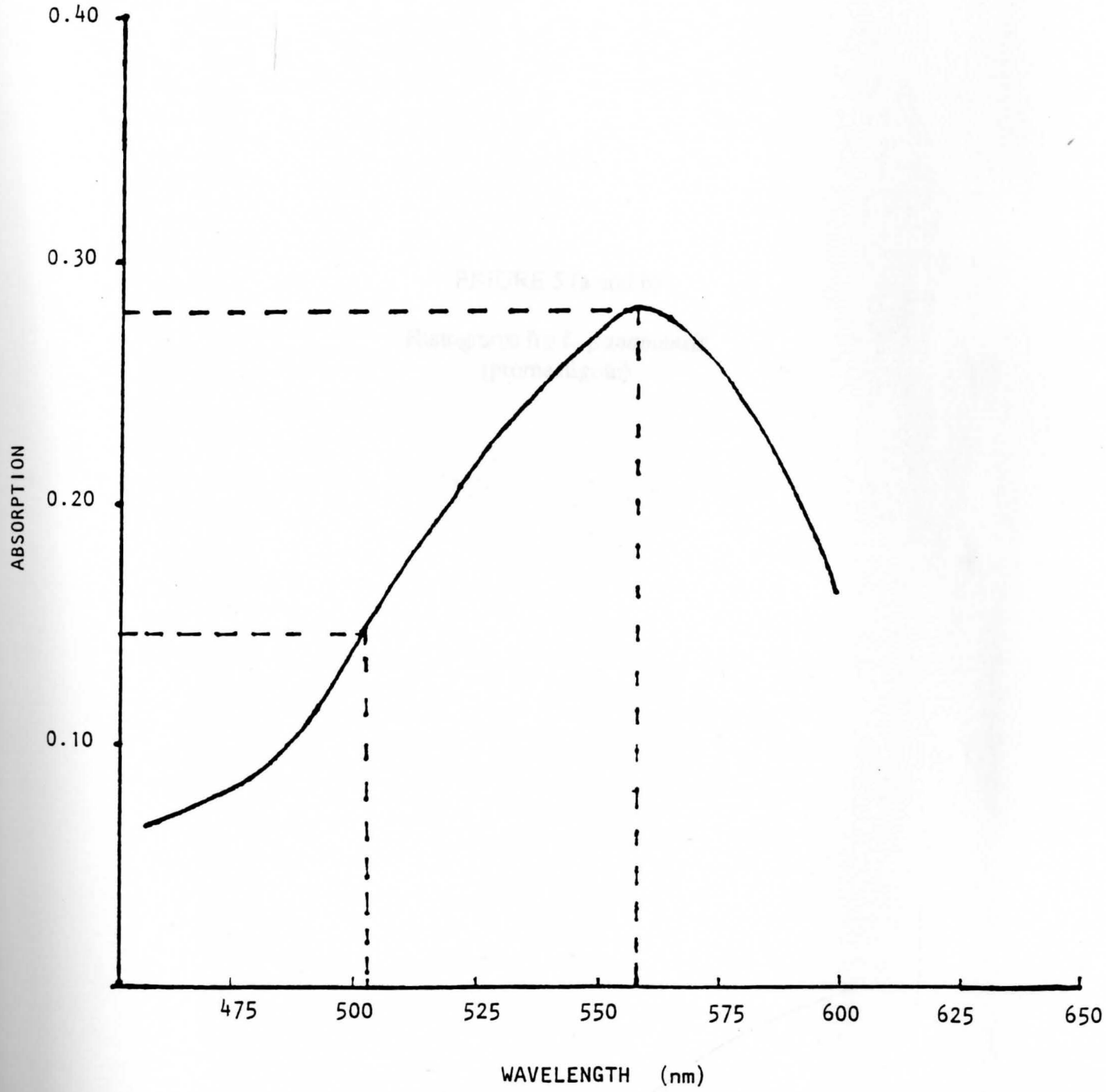
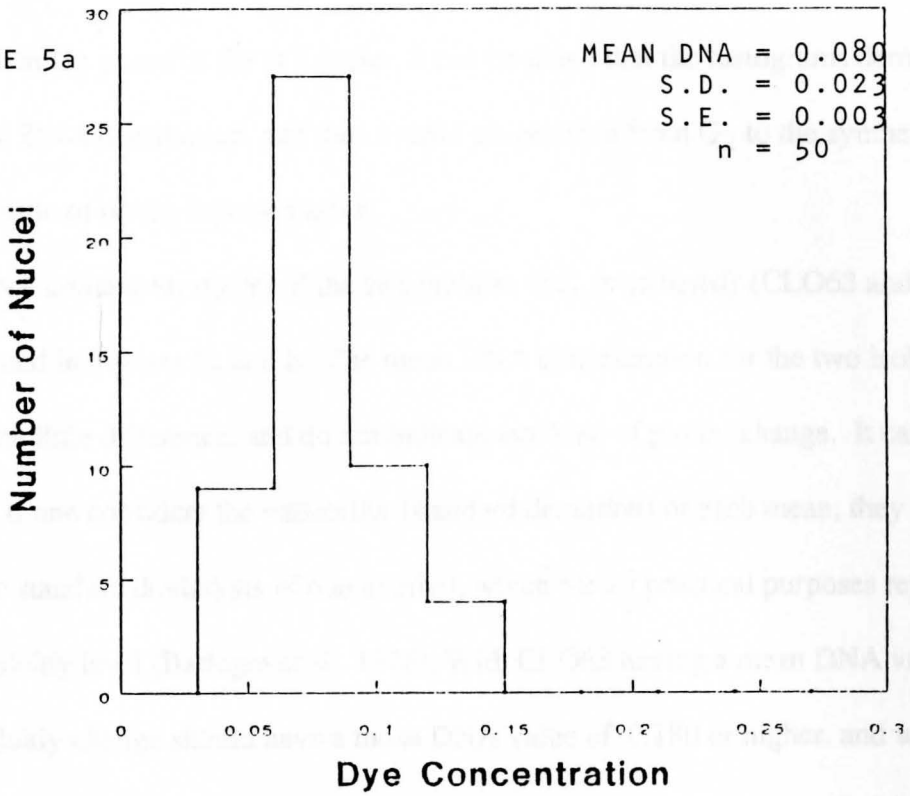


FIGURE 5 (a and b)

Histograms for *L. panamensis*
(promastigote)

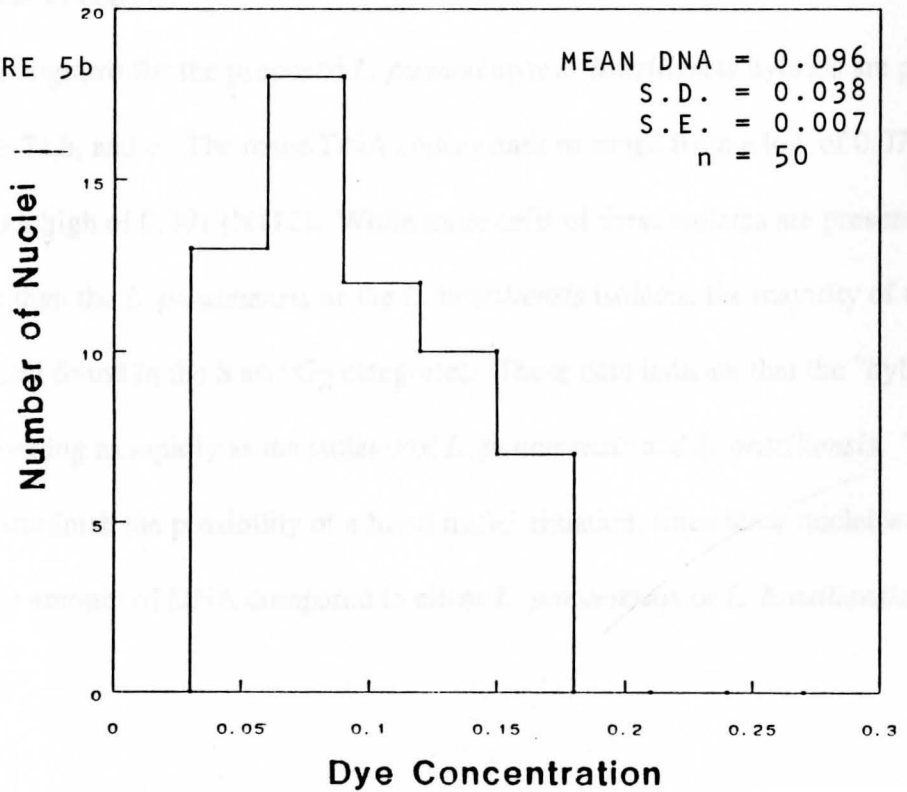
Leishmania panamensis
CLO64

FIGURE 5a



Leishmania panamensis
N111

FIGURE 5b



are present in G_2 phase of the cell cycle. It can be noted that the histograms demonstrate a unimodal DNA distribution, and thus a rapid progression from G_2 to the synthetic phase, with very little or no G_1 representation.

The DNA content for nuclei of the two isolates of *L. braziliensis* (CLO63 and N103) is represented in Figures 6a and b. The mean DNA concentration for the two isolates demonstrate little difference, and do not indicate any kind of ploidy change. It can be noted that if one considers the variability (standard deviation) of each mean, they are well within two standard deviations of one another, which for all practical purposes represents the same ploidy level (Barlogie et al., 1978). With CLO63 having a mean DNA value of 0.093, a ploidy change should have a mean DNA value of 0.180 or higher, and we see the value of 0.127 in N103. Once again the condition is caused by capturing cells in different stages of synthetic activity. The histogram of CLO63 is positively skewed with more cells falling in S phase and a few in G_1; while N103 is negatively skewed, having a large peak of cells in G_2.

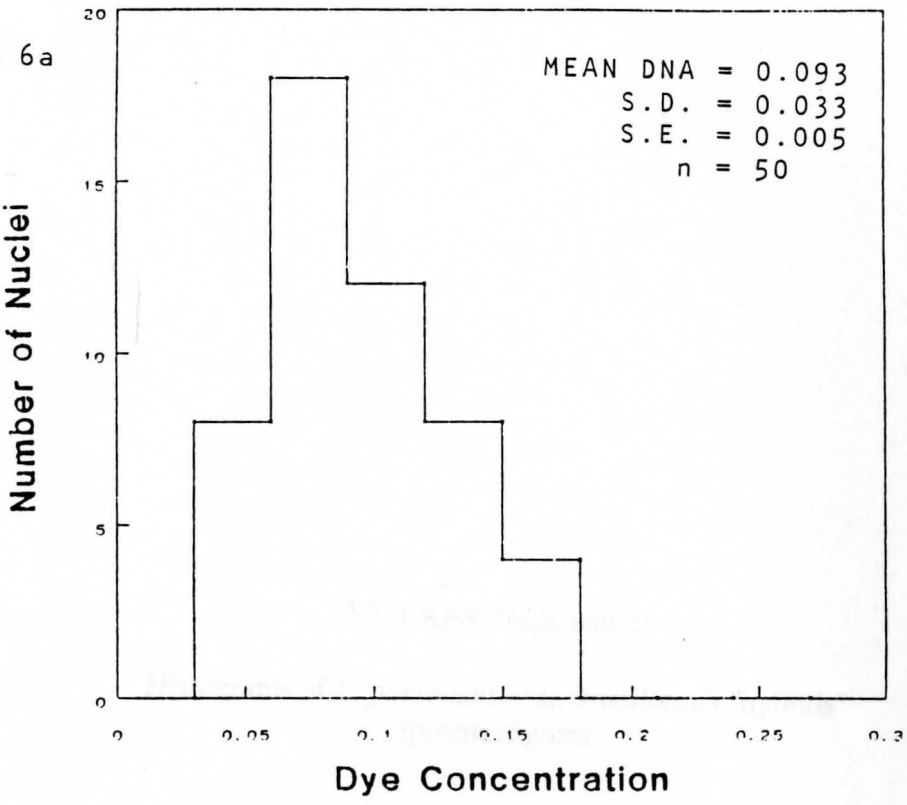
The histograms for the proposed *L. panamensis/ L. braziliensis* hybrids are presented in Figures 7a,b, and c. The mean DNA concentrations range from a low of 0.074 (N109) to a high of 0.091 (N112). While more cells of these isolates are present in G_1 phase than the *L. panamensis* or the *L. braziliensis* isolates, the majority of the cells are still found in the S and G_2 categories. These data indicate that the "hybrids" are not dividing as rapidly as the isolates of *L. panamensis* and *L. braziliensis*. The data also diminish the possibility of a fused nuclei situation, since these nuclei would have double the amount of DNA compared to either *L. panamensis* or *L. braziliensis*.

FIGURES 6(a and b)

Histograms of *L. braziliensis*
(promastigote)

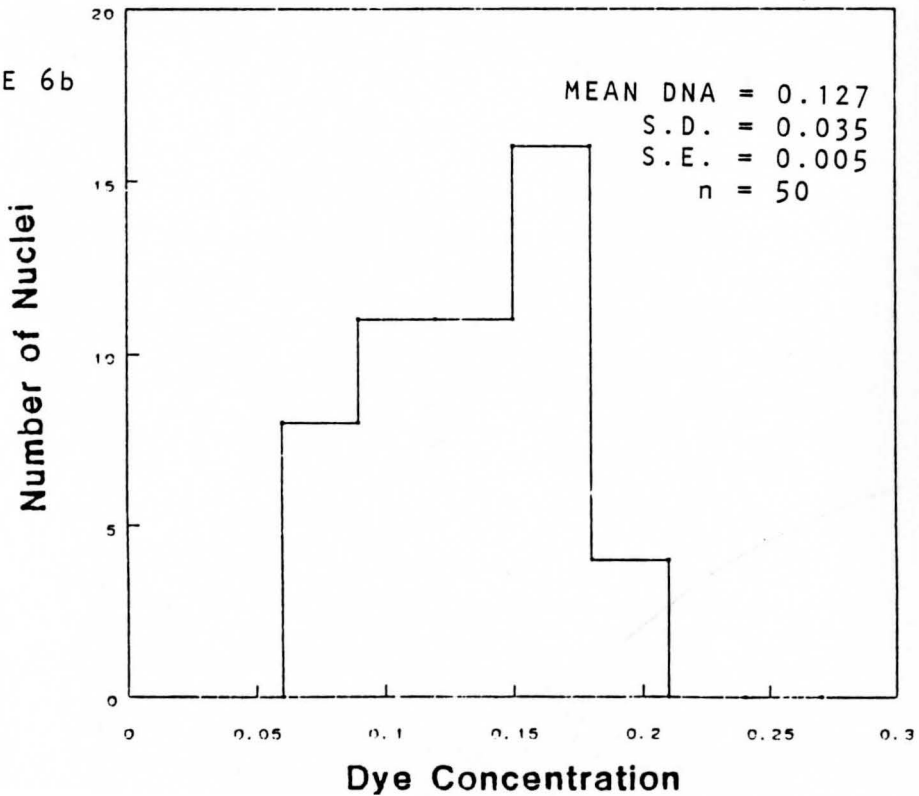
Leishmania braziliensis
CL063

FIGURE 6a



Leishmania braziliensis
N103

FIGURE 6b



FIGURES 7(a,b, and c)

Histograms of *L. panamensis/ L. braziliensis* "hybrids"
(promastigote)

FIGURE 7a

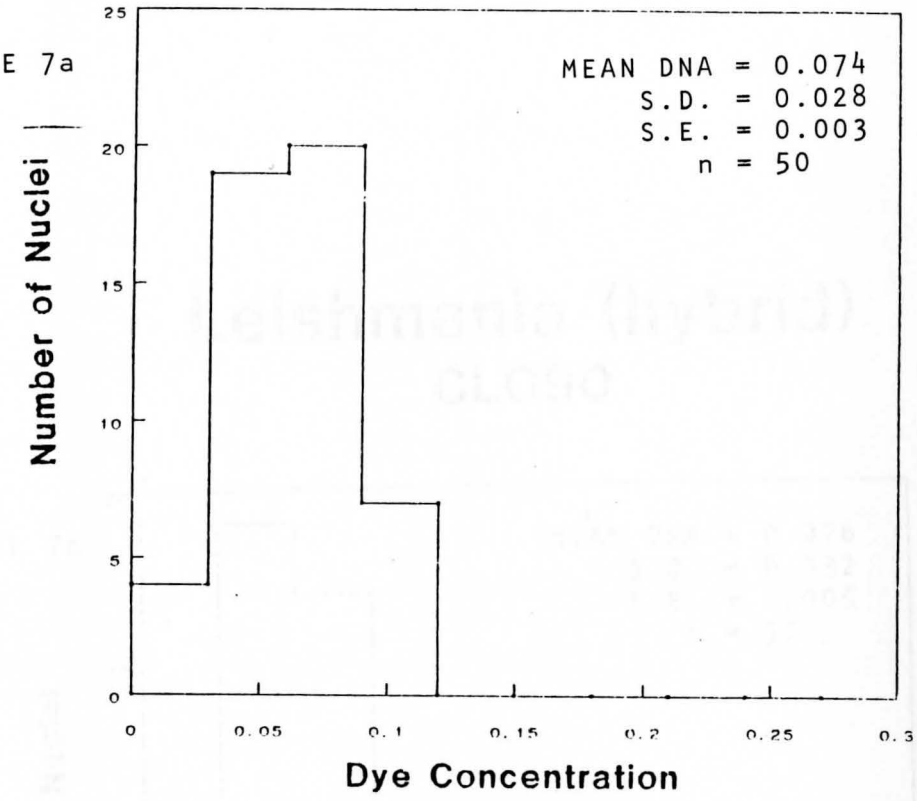
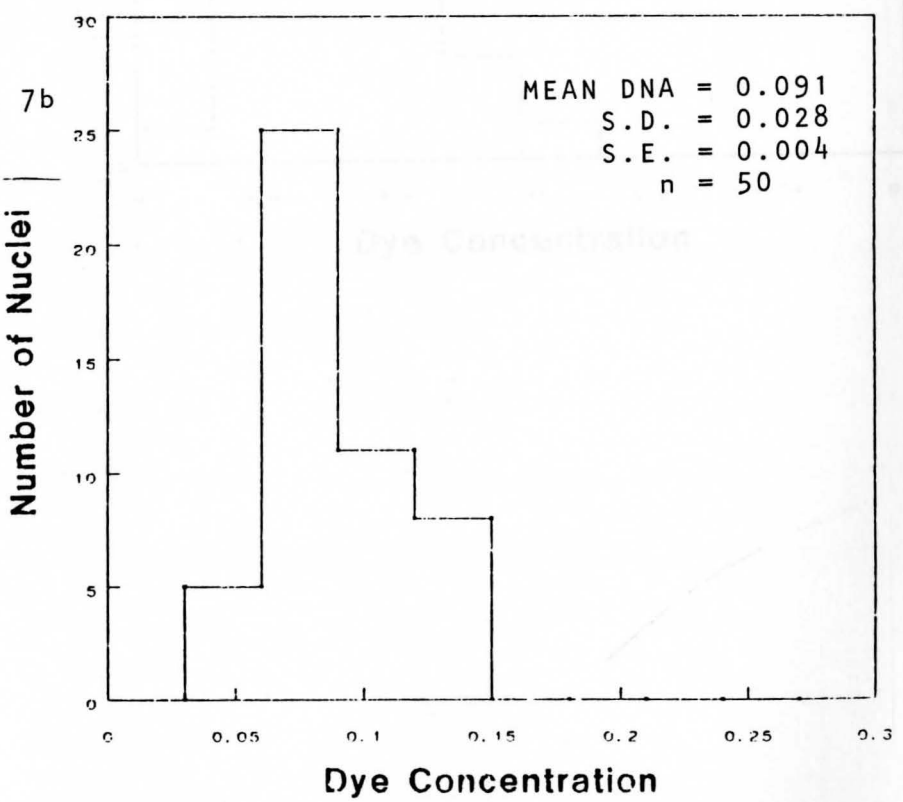


FIGURE 7b



Leishmania (hybrid)
CLO90

FIGURE 7c

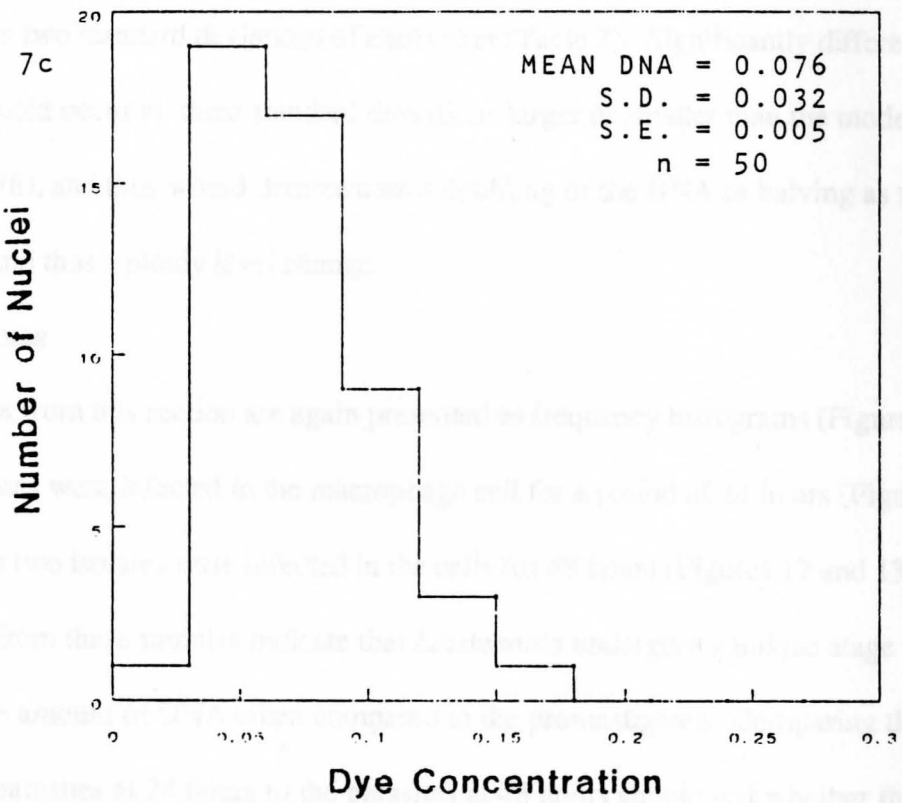


Figure 8 presents a frequency histogram for an isolate of *L. major* (N136). The histogram of *L. donovani* (N135) is depicted in Figure 9. The mean DNA content of the two isolates do not differ to a large extent with any of the other isolates studied, and both samples demonstrate the majority of their cells in S phase and G_2 phase.

The promastigote cells of the isolates examined can be called the same population. When analyzing the mean DNA values of the given isolates, it is found the values all fall within two standard deviations of each other (Table 7). Significantly different DNA values would occur at three standard deviations larger or smaller than the mode (Barlogie et al., 1978), and thus would demonstrate a doubling of the DNA or halving as the case may be, and thus a ploidy level change.

Amastigotes

Results from this section are again presented as frequency histograms (Figures 10-13). Two isolates were infected in the macrophage cell for a period of 24 hours (Figure 10 and 11), while two isolates were infected in the cells for 48 hours (Figures 12 and 13). The data from these samples indicate that *Leishmania* undergoes a unique stage which has double the amount of DNA when compared to the promastigotes. Comparing the data from the parasites at 24 hours to the parasites at 48 hours should test whether the appearance of this stage is time-dependent.

Figure 10 presents the data of amastigote cells of *Leishmania major*. Two populations of cells are present in this figure as is demonstrated by the bimodal DNA distribution. The group of cells with lower DNA amounts represent a population similar to that of

FIGURE 8

Histogram of *Leishmania major*
(promastigote)

FIGURE 8

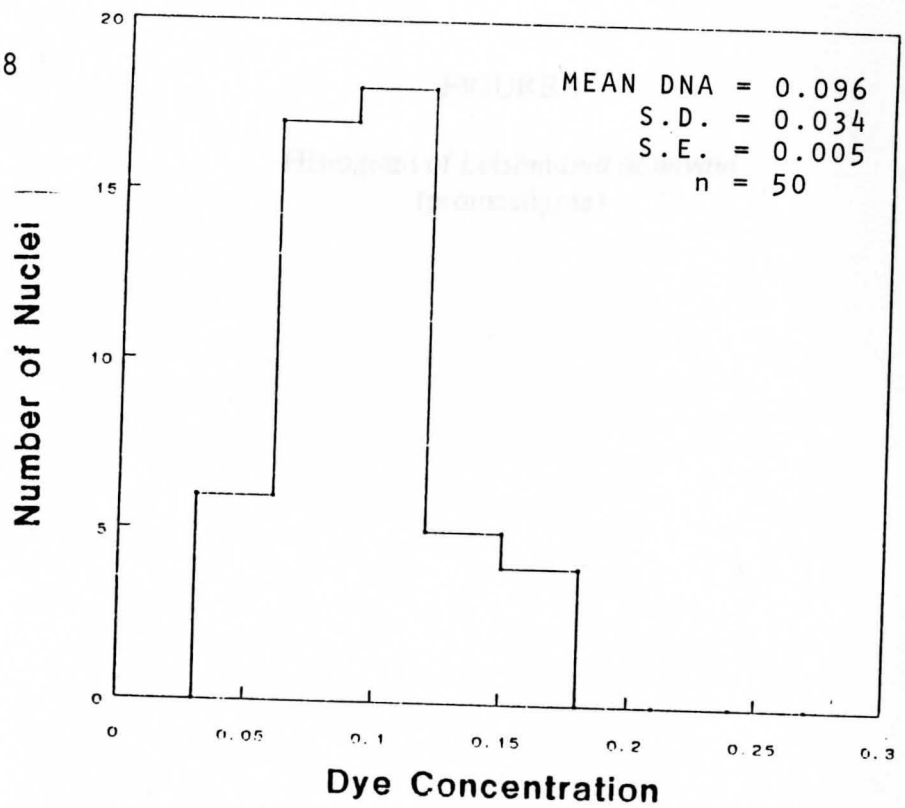

Leishmania major
N136
MEAN DNA = 0.096
S.D. = 0.034
S.E. = 0.005
n = 50
Number of Nuclei
20
15
10
5
0
0
0.05
0.1
0.15
0.2
0.25
0.3
Dye Concentration

FIGURE 9

Histogram of *Leishmania donovani*
(promastigote)

Leishmania donovani
N135

FIGURE 9

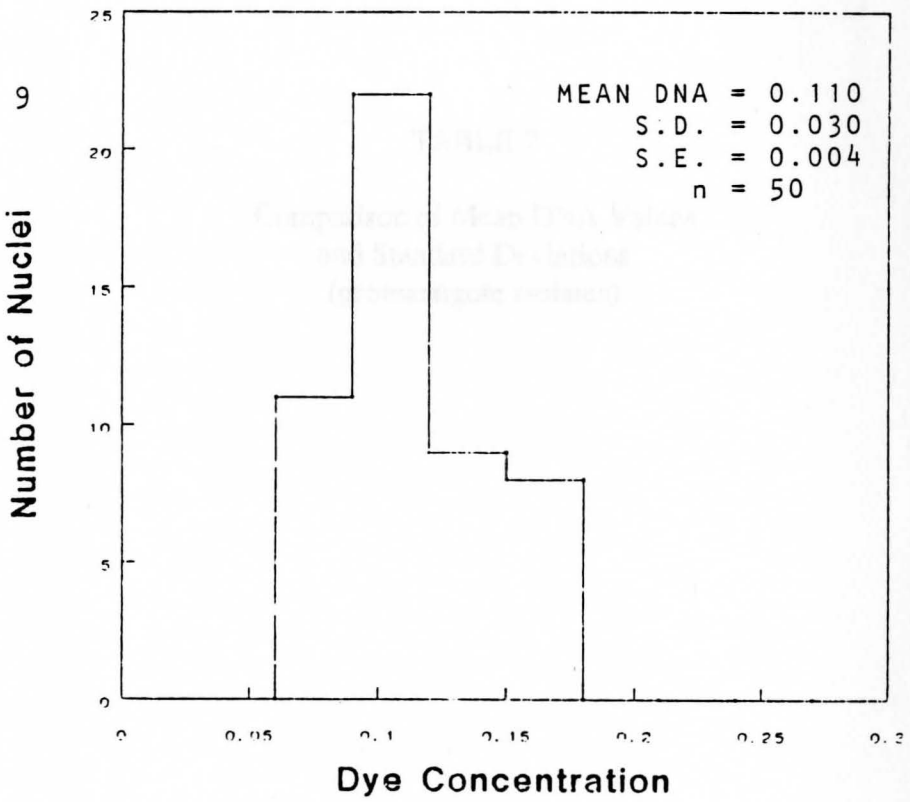


TABLE 7

Comparison of Mean DNA Values
and Standard Deviations
(promastigote isolates)

TABLE 7: COMPARISON OF MEAN DNA AND STANDARD DEVIATION
(PROMASTIGOTE ISOLATES)

Largest mean DNA value: 0.127
+/- 2 S.D.: 0.057-0.197

Smallest mean DNA value: 0.074
+/- 2 S.D.: 0.018-0.130

Acceptable range: 0.057-0.130

Isolate	Mean DNA Value
N103	0.127
N135	0.110
N136	0.095
N109	0.074
N112	0.091
N111	0.096
CLO90	0.076
CLO63	0.093
CLO64	0.080

the promastigote cells. *L. major* (WR1075) has more cells in G_1 than the promastigotes and less in G_2, but still represent a population of actively dividing cells. There is a noticeable break in the histogram and a second population of cells appears with higher amounts of DNA. Twelve percent of the nuclei examined were found in this second population.

There are a slightly larger number of cells found in the second group in *Leishmania tropica* (WR1063) (Figure 11). Once again we see a bimodal DNA distribution; the typical promastigote population with lower dye concentrations followed by a break and another population of cells with higher amounts of DNA. Eighteen percent of the cells examined were found in the second population. The amastigote data so far presented have been of the 24 hour infection stage. The next two isolates studied represent cells infected at 48 hours.

Figure 12 presents the 48 hour infection of cells of *L. panamensis* (CLO64). The cells typical of the promastigote population increase in number from G_1 through S to early G_2. There is a break, followed by a second dividing population, containing 34 percent of the nuclei measured. Forty-six percent of the fifty nuclei examined of *L. braziliensis* (CLO63) (Figure 13) were found in the second population. Nuclei falling within the second population have roughly double the amount of DNA of those nuclei found in the first population. These cells also fall out of the range of two standard deviations from the mean of the first population; therefore, they can be considered a different ploidy level.

The mean DNA values of the amastigote cells examined range from 0.110 (WR1075) to 0.145 (CLO64). The mean DNA values increase proportionally

as the amount of nuclei falling into the second population increases. The value of *L. major* (WR1075) does not differ from the promastigote cells examined because 44 of the 50 cells examined were found to be of the same population as the promastigotes, as indicated by the mean DNA. *L. braziliensis* (CLO63) with a mean DNA value of 0.133 is about 1.5 times the amount of the majority of the promastigotes. This should be expected, since roughly one-half of the cells are found as the promastigote population, while the other half are found in the second population.

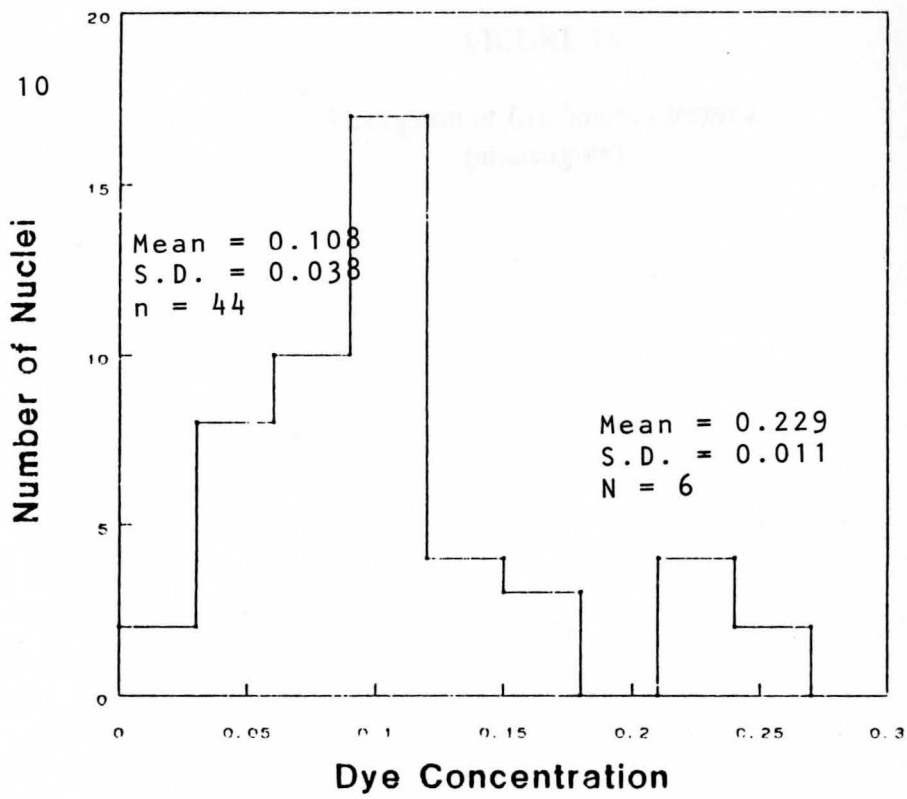
A better examination can be made by comparing the mean DNA value of the first population to the mean DNA value of the second population (Table 8). The mean DNA values for the second population found in the amastigotes is roughly double the mean DNA values of the first group for all of the isolates. Also the mean DNA values of the first group fall into the acceptable range to be the same population as the promastigotes examined (Table 7). The number of nuclei present in the second group increased with period of infection. About one-fifth of the cells were present in the second group at 24 hours; however, at 48 hours there were two-fifths of the cells present in this group.

FIGURE 10

Histogram of *Leishmania major*
(amastigote)

Leishmania major
WR1075

FIGURE 10



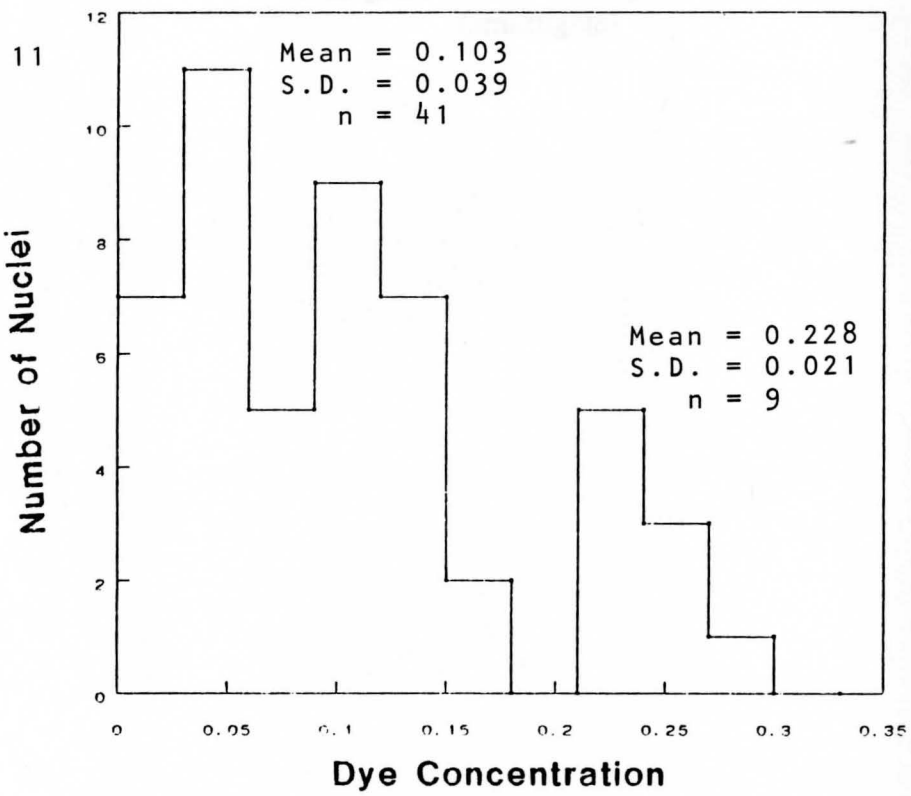
TOTAL
MEAN DNA = 0.110
S.D. = 0.051
S.E. = 0.008

FIGURE 11

Histogram of *Leishmania tropica*
(amastigote)

Leishmania tropica
WR1063

FIGURE 11



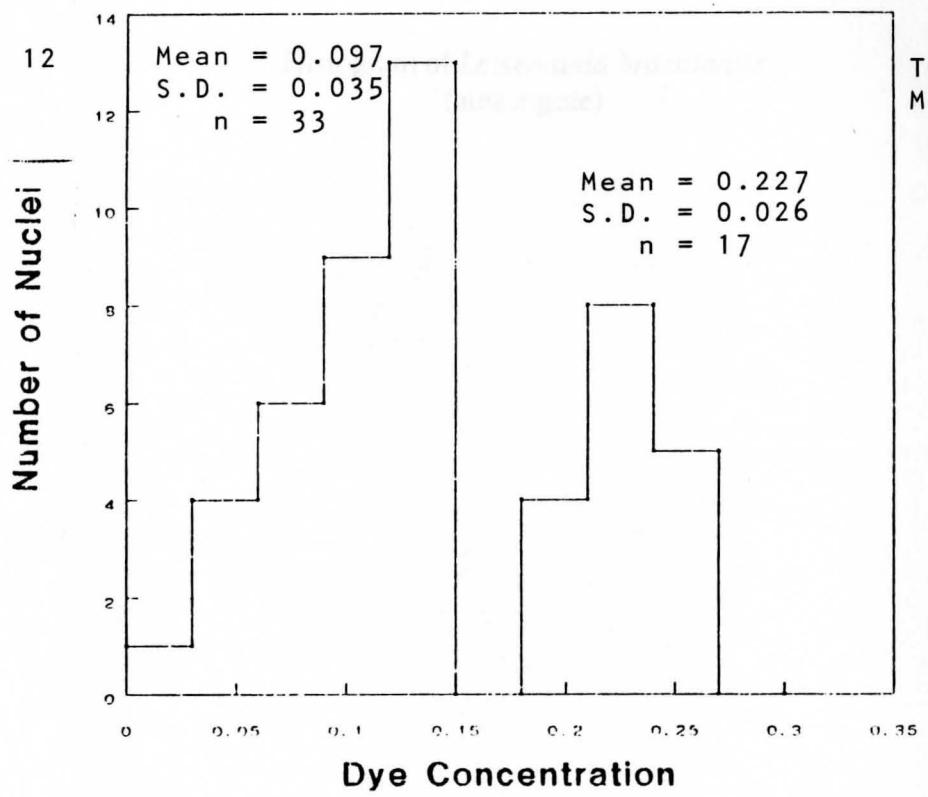
TOTAL
MEAN DNA = 0.116
S.D. = 0.056
S.E. = 0.009
n = 50

FIGURE 12

Histogram of *Leishmania panamensis*
(amastigote)

Leishmania panamensis
CL064

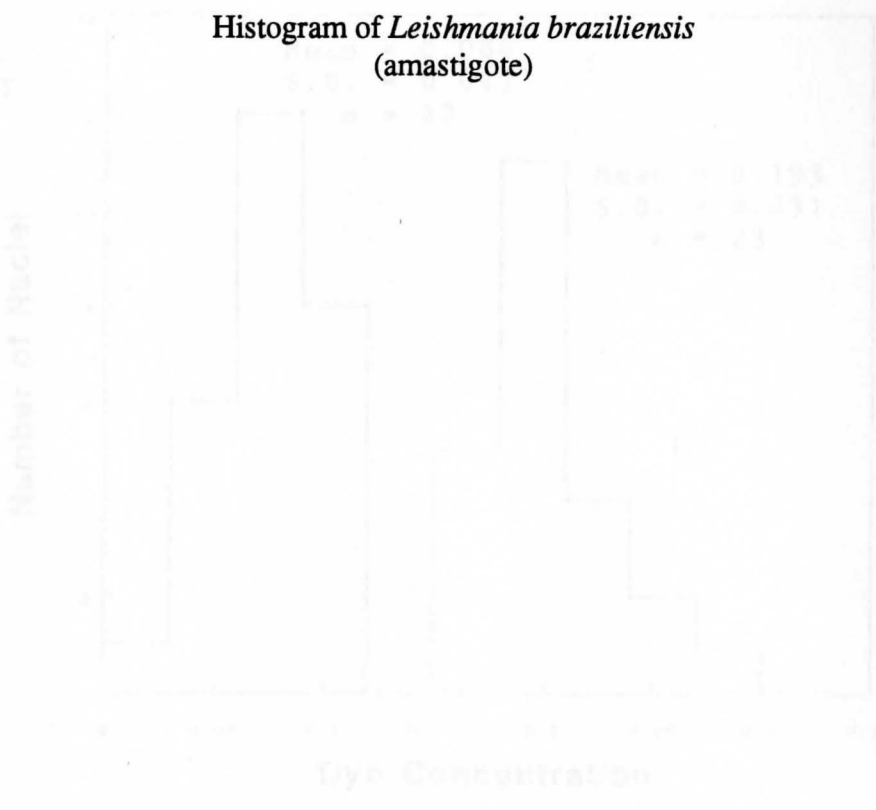
FIGURE 12



TOTAL
MEAN DNA = 0.145
S.D. = 0.069
S.E. = 0.010
n = 50

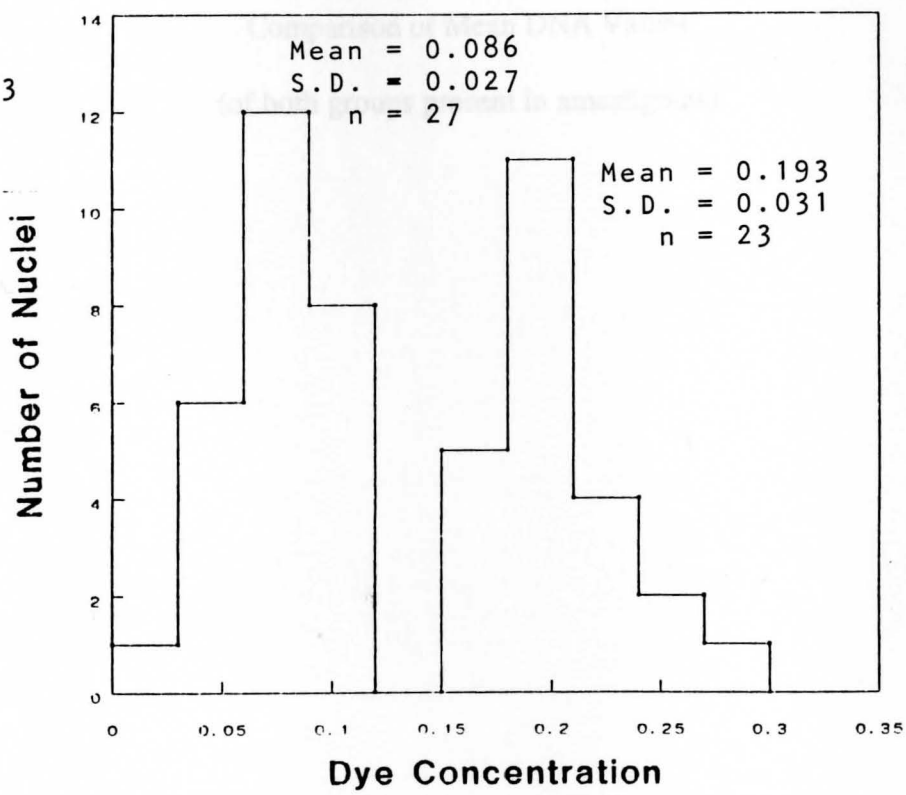
FIGURE 13

Histogram of *Leishmania braziliensis*
(amastigote)



Leishmania braziliensis
CLO63

FIGURE 13



TOTAL
MEAN DNA = 0.133
S.D. = 0.072
S.E. = 0.010
n = 50

TABLE 8

Comparison of Mean DNA Values

(of both groups present in amastigotes)

TABLE 8 : COMPARISON OF MEAN DNA VALUES IN AMASTIGOTES

Species	Group 1		Group 2	
	Mean	**S.D.**	**Mean**	**S.D.**
Leishmania major	0.108	0.038	0.229	0.011
Leishmania tropica	0.103	0.039	0.228	0.021
Leishmania panamensis	0.097	0.035	0.227	0.026
Leishmania braziliensis	0.086	0.027	0.193	0.031

DISCUSSION

The electrophoretic and cytophotometric data presented in this study support the contention that cell fusion occurs in the amastigote stage of the life cycle in Leishmania to produce nuclei with double the amount of DNA found in the promastigotes. The cytophotometric data clearly indicate cell fusion as is evidenced by a dramatic increase in nuclear DNA content in the amastigote stage. The electrophoretic data support these data through the production of what appears to be heterozygous isoenzyme allomorphs. Together these data suggest a scheme whereby genetic exchange may occur in *Leishmania.*

Isolates from ten human patients classified as having simple cutaneous leishmaniasis were analyzed by cellulose acetate electrophoresis for 20 enzymes. These isolates were identified as *L. panamensis/ L. braziliensis* "hybrids" by the bands they produced. Microscopic examination revealed that each cell had a single nucleus; thus, they are not heterocaryons.

The results of zymograms for enzymes which produced a single band identical to the most common allomorph in *L. panamensis* and *L. braziliensis* conclude that either *L. panamensis* or *L. braziliensis* alone, *L. panamensis* and *L. braziliensis* in a mixed culture of the two, or a hybrid would produce these bands. The zymograms for enzymes which regularly produced two dark bands for the isolates show that one band was the most common allomorph in *L. panamensis*, the other the most common in *L. braziliensis*. The two bands suggest that transcription of one genetic sequence from *L. panamensis* and one from *L. braziliensis* occurs to produce the polypeptide components of a monomeric

enzyme. Either *L. panamensis* and *L. braziliensis* in a mixed culture of the two, or a hybrid of the two species could produce these two bands. The zymograms for enzymes which produced a wide band intermediate of the most common allomorphs found in *L. panamensis* and *L. braziliensis* suggest that the enzymes are polymers in *Leishmania*, with some strands being produced by *L. panamensis* and some by *L. braziliensis*. Mixed cultures of L. panamensis and L. braziliensis produced two separately migrating bands. The data indicate that only a hybrid condition or both DNA sequences in the same cell would produce these bands. The enzyme data suggest transcription of sequences of both species in one cell nucleus or probably a diploid promastigote hybrid.

The data presented in this study by quantitative cytophotometric analysis provide insight regarding the measured relative amount of DNA present in the nuclei of promastigote and amastigote cells of different species of *Leishmania* and fully support the electrophoretic data present. Of special interest is the presence of nuclei having double the amount of DNA within the amastigote population, as well as the observation that the number of these nuclei appear to rise in proportion to the period of infection within the macrophage. Data representing the promastigote stage indicate that these cells have one-half the nuclear DNA content of the fused cells found in the amastigotes.

The nuclei of the promastigote isolates follow a unimodal distribution. Increases in nuclear DNA content occur as the cells move from G_1 into DNA synthesis, and finally into G_2, but never exhibit the nuclear DNA value observed in the fused cells of the amastigotes. As is characteristic of actively mitotic cells, these cells are in various stages of the cell cycle with DNA synthesis predominating. With fast growing

cultures as is exhibited in *Leishmania*, the cells have no appreciable G_1 phase of the cell cycle , or it is very short in duration. These cells very quickly enter the DNA synthesis phase of the cell cycle with growth becoming very rapid. This was observed in the culture itself, when clear cultures became turbid in a matter of hours. The average DNA level in the host macrophage nucleus was 37.5.

The histograms for the promastigote isolates follow the same general pattern. The G_2 stage of the cell cycle represents a fully replicated nuclear DNA cell ready to undergo division, and therefore commonly has replicated DNA levels present during growth phase or 2C DNA content. Since very few G_1 or 1C cells are noted in the histograms representing the relative DNA concentration in the promastigotes, it can be assumed that at stationary phase the cells are in G_2 arrest. For the majority of the promastigote histograms, the highest DNA concentration occurs at approximately 0.15, representing the completely replicated DNA G_2 stage. Half of that value typically represents cells found at G_1, with intermediary cells in S phase.

When examining the relative DNA content of nuclei of the amastigote isolates, an entirely new population of cells not found in the promastigotes appears. It is interesting to note that when comparing the mean DNA value of these cells to the mean DNA value of the promastigote cells, the amount is approximately double that found in the promastigotes. Here it is evident that two different ploidy levels occur in *Leishmania*. It can also be noted that apparently not all cells are at this time capable of fusion, or have not fused, indicating that fusion may depend on relative maturation of cells, or is time-dependent.

One special characteristic present in all of the histograms for the amastigote isolates is the break seen dividing the two separate cellular populations. This break is typical for cells that undergo rapid fusion or zygote formation (Yemma and Perry, 1985). If the cells doubled their DNA, going through a smooth transition from one ploidy level to a second, there should not be a break in the histogram because the cells would be continually synthesizing DNA, and would represent cells of intermediate DNA concentration, just as the transition from G_1 to G_2 is made. The histograms of the amastigotes show that the fused cell population as stated are approximately double in DNA concentration when compared with the promastigotes, and also with the first population of cells present in the amastigotes. For an average of the fused cell population, a mean DNA concentration of approximately 0.21 is observed, i.e., not quite double the promastigote 2C levels (G_2 phase), indicating that fusion of cells can occur while DNA synthesis is in progress. Once the cells have fused, synthesis continues and subsequently this population undergoes mitotic division. This can best be seen in the amastigotes that were infected at 48 hours; *Leishmania panamensis* (CLO64) and *Leishmania braziliensis* (CLO63). In these two isolates, it is evident from finding nuclei with higher amounts of DNA than after the initial fusion occurs, there is continued synthesis followed by a G_2 phase.

It is evident from the data representing the amastigotes that a distinct cell type arises that was not found in the promastigotes. Even more interesting is the fact that there is an increase in the amount of cells falling into this category at a period of 48 hours post infection when compared to those of 24 hours. It is likely that the cell fusion that occurs in *Leishmania* is time-dependent, and longer periods of infection could lead to the absence of

the promastigote population.

When combining the cytophotometric data with the electrophoretic data (Kreutzer), it is possible to designate ploidy levels to the two different populations. By examining the zymograms, it can be said that the promastigotes that were examined are diploid. The production of two bands for the hybrids and the intermediary migrating bands for these isolates show conditions that could not occur in a haploid organism.

Using these data to provide insight the life cycle of *Leishmania* becomes clearer. The promastigotes are 2C cells, which after DNA synthesis becomes 4C followed by a mitotic division. The amastigotes can exist either as 2C cells or as fused (4C) cells, with each group independently dividing.

These data also explain the appearance of *Leishmania* "hybrids" occurring in nature. It is possible for different species to develop simultaneously within the gut of the sandfly (Panton et al., 1991), or to have multiple infection from two different sandflies. When the sandfly infects the host, parasites from the two separate species can enter the macrophage cell. There is then the opportunity for sexual reproduction followed by genetic exchange to occur by amphimixis (Bell, 1988). The two different species undergo cell fusion to from the 4C cell. The cell exists then as having genetic material from both parents in one cell nucleus.

It is noted that not all of the amastigotes examined were present as 4C cells. Although a larger number of cells were present as 4C cells at the 48 hour infection period compared to the 24 hour infection time, it cannot be assumed that all amastigote cells fuse. In fact, it is just as likely they do not. The 4C population seems to be

independent of the 2C cells. If cell fusion occurred just prior to meiotic division, a large group of cells falling within the G_1 category of the promastigotes would be expected, since this is the condition in which cells are found in their restored state.

It is likely that one stage may be necessary for the spread of the infection in the host, and the other necessary for transmission of infectivity to the sandfly. According to the quantitative cytophotmetric data, meiosis does not occur in the macrophage cell, for haploid G_1 cells were not found. When the sandfly ingests cells of the 4C type, the new environment could stimulate meiotic division and genetic recombination could occur. It is also possible that as cyclic macrophage reinfection occurs, the 4C cells are released, meiosis is initiated, and these now 2C "promastigotes" infect a new macrophage, and additional rounds of amphimixis occur.

The data suggest that two ploidy levels exist simultaneously within in the same macrophage cell. This indicates a possible receptor mechanism which would trigger potential cells into fusing. It is probable that receptor sites are a prerequisite for mating, and their production requires cellular maturation. Production of these receptors may be accomplished by chemical inducers and affector molecules produced by compatible mating types (Yemma et al., 1974; 1991).

Evidence of cell fusion in some *Trypanosoma* has been recorded. Trypomastigotes of *Trypanosoma cruzi* (Chagas) appear to fuse, and the genetic exchange observed in *Trypanosoma conorhiri* (Donovan) may also occur by cell fusion. Ellis and others (1982) recorded the presence of multinucleate trypanosomes, which appeared to have resulted from the fusion of one or more organisms. Cell fusion of

Leishmania promastigotes has been recorded by videomicroscopy (Lanotte and Rioux, 1990).

Many other experiments performed in the study of *Leishmania* suggest that genetic recombination occurs. Until the present study there was no proof of the mechanism by which this occurred. It is now evident that *Leishmania* can exchange genetic information by cell fusion to achieve a 4C state in the amastigote, followed by reduction to the genetic level (2C) found in the promastigotes.

Further investigations of cell fusion in this organism are necessary in order to state the complete mechanism by which fusion occurs. Another important area that needs to be closely examined is the location where reduction back to the 2C ploidy level takes place. A continued experiment involving specific timed infection periods should also be performed in order to see if there is a threshold level at which cell fusion ceases.

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