GENETIC CHARACTERIZATION AND IDENTIFICATION OF *LEISHMANIA* SPP. BY ISOZYME ELECTROPHORESIS

by

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ABSTRACT

GENETIC CHARACTERIZATION AND IDENTIFICATION OF
LEISHMANIA SPP. BY ISOZYME ELECTROPHORESIS

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Master of Science
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Biochemical or genetic data as enzyme profiles which were obtained by cellulose acetate electrophoresis (CAE) were reported on 44 Leishmania isolates. These enzyme profiles contain data from 25 enzyme systems (possibly 29 gene loci).

Calculations from the CAE data on average polymorphism indicated that Leishmania species/types or groups can be expected to be about 21% polymorphic, which suggests isolate pairs which have profiles about 75% or more identical should be considered samples from the same species/type, and isolates that are significantly less than 75% identical are therefore samples from different species/types.

There were five major groupings of isolates according to enzyme profiles which were for the most part consistent with groupings of the genus based on other criteria: braziliensis, mexicana, donovani, tropica and hertigi profiles. Within these groups there were natural subgroups of isolates among which there was 75% or more allozyme or allomorph (genetic) identity. The braziliensis profile group had two subgroups: panamensis and braziliensis or guayanensis, and the mexicana profile group had three subgroups: mexicana, amazonensis, and peruviana. There was an indication that an L. donovani infantum isolate might be

different from the other L. donovani isolates, and that the L. tropica isolates could be samples from more than one group.

The data reported here were consistent with previously reported CAE data. As more known samples are characterized specific enzyme profiles including additional enzyme polymorphism will become available facilitating and enhancing biochemical identification and characterization of Leishmania isolates.

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CHAPTER I

INTRODUCTION

The subdivision of the genus Leishmania into species is complicated by the fact that all species are morphologically very similar in both the promastigote and the amastigote stages (Hommel, 1978), although some size differences have been noted in the amastigote stage among certain leishmanias (Shaw and Lainson, 1976; Gardener et al., 1977). Methods which rely on differences other than mainly morphological features, therefore, have been used to characterize the different leishmanias. One of the methods recently being used is isozyme electrophoresis (Gardener and Howells, 1972; Gardener et al., 1974; Ebert, 1973, 1974; Kilgour et al., 1974).

Studies have shown that enzymes and other proteins are the translational products of genes, and that variation in enzymes and proteins substantiates allelic variation in the genes coding for them (Avise, 1974; Ayala, 1975; Myers, 1978). Since cell proteins are gradually modified by genetic mutation, it is possible to estimate the relatedness of organisms by comparing the structures of their homologous proteins. Enzymes are chosen for study since they are easily identified by their substrate specificity. If the differences in molecular structures of enzymes are associated with charged amino acids, they may be separated in an electrical field by electrophoresis. When comparisons are made between closely related species, electrophoresis provides more information than most other techniques for a given amount of work

and cost (Ayala, 1975). According to Ayala (1975), if the tissues from two individuals are subjected to electrophoresis, and if the assayed enzyme of one individual migrates faster than that of the other, one can assume that the two enzymes are different at least by one amino acid substitution. Further they are therefore coded for by different genes, i.e. by different alleles (allozymes or allomorphs) if the enzyme is coded by the same gene locus in both individuals. It is assumed, on the other hand, that they are coded for by identical genes if the enzymes of the two individuals migrate equally.

It is estimated that only a third of possible amino acid substitutions would result in alteration of electrophoretic mobility since amino acid substitutions can occur that do not change the net electrical charge of a protein or substantially modify its configuration (Ayala, 1975, 1982; Harris and Hopkinson, 1976; Shaw and Prasad, 1970). However, as Gibson et al. (1980) point out it is reasonable to assume that the more enzymes that are electrophoretically identical between two samples, the greater is the likelihood that they are related; furthermore the greater the number of identities, the closer is the relationship. Avise (1974) in describing the systematic value of electrophoretic data notes that many multilocus studies have revealed very high levels of genetic similarity between conspecific populations with nearly identical allelic (allozyme) content in 85 percent or more of their loci, and further that genetic similarities even among very closely related species are usually much lower. Ayala (1975), Ayala et al. (1974) and Myers (1978) have reported similar conclusions.

The EPILEISH sub group of the Scientific Working Group of Leishmaniasis in 1979 reported that there is still a priority for typing leishmanial isolates by such procedures as isozyme electrophoresis (TDR/LEISH-SWG(2)/79.3). There have been numerous applications of the technique to identify and classify other parasitic protozoa including Plasmodium (Carter, 1978), Trypanosoma (Godfrey and Kilgour, 1976; Kreutzer and Sousa, 1981), Eimeria (Rollinson, 1975; Shirley, 1975) and amoebae (Sargeaunt and Williams, 1978). A recent World Health Organization workshop (1980) on biochemical characterization of Leishmania recommended that cellulose acetate electrophoresis (CAE) be used for enzyme studies on leishmanial isolates. In addition it was noted that electrophoresis studies should include multiple enzyme systems; the results of such studies could establish "enzyme profiles" specific for the various leishmanial types.

Al-Taqi and Evans (1978), Brazil (1978), Chance (1979), Change et al. (1977, 1978), Ebert (1973, 1974), Gardener et al. (1974), Kilgour et al. (1974), Lumsden (1974), and Rassam et al. (1979) described intra- and interspecific biochemical variation of up to ten enzymes detected by gel enzyme electrophoresis in Leishmania isolates, and certain of the authors discussed the necessity to continue and expand such studies to further clarify the taxonomy of Leishmania. Kreutzer and Christensen (1980) recently reported electrophoresis techniques on cellulose acetate for fourteen enzyme systems which have been used to identify certain Leishmania species and species' strains. The study reported extensive biochemical interspecific differences and minor intraspecific differences in strains of Leishmania braziliensis (panamensis). In addition to these CAE enzyme systems more substrate/buffer systems have

been adapted for Leishmania so that isolates can now be characterized for at least 25 systems. Certain Walter Reed Army Institute of Research (WRAIR) and National Institutes of Health (NIH) isolates were characterized for these systems, and the results of the study are reported here.

CHAPTER II

MATERIALS AND METHODS

Leishmanial strains

Forty-four strains of Leishmania spp. were obtained from the WRAIR and NIH isolate banks. Some of these had been identified by the classical procedures based on their clinical aspects in the host along with their epidemiology and geographical distribution; these are specifically designated in Table 1. Other isolates had been identified only to the genus level and are noted in Table 1 as Leishmania sp.

Cultivation

WRAIR strains were cultured in Schneider's Drosophila medium (GIBCO) plus 30% (V/V) fetal calf serum following the procedures of Hendricks et al. (1978) and Childs et al. (1978) to produce about 10^8 promastigote cells. Stock cell culture medium was prepared according to Schneider (1974). Cultures of organisms were established in 25 cm^2 plastic-culture flasks using 5 ml of medium. The standard incubation temperature was 25.5 ± 1.5^{o} C.

The NIH strains were grown in Medium 199 with 20% fetal calf serum and in the presence of Penicillin, Streptomycin and Gentamycin. All strains had been passaged no more than 10 to 12 times after incubation, which was always on NNN medium originally.

Preparation of extracts for electrophoresis

The proceeding steps for preparation of extracts for electrophoresis are those described by Kreutzer and Christensen (1980). To

TABLE 1

LEISHMANIA ISOLATES

Species[a]	WRAIR No.	Other Designation	Host	Source/Exposure
L. braziliensis (panamensis)	209	Murray	Human	Panama
L. braziliensis (panamensis)	148	Jimenez	Human	Hawaii
Leishmania sp.	-	NIH/BR, Broos	Human	Costa Rica
L. braziliensis (braziliensis)	063	Terborgh	Human	Peru
L. b. braziliensis or L. m. amazonensis	345	Marsden's Irene	Human	Brazil
L. braziliensis quayanensis	075	de Sousa M 2061	Human	Brazil
Leishmania sp.	442	Bennett	Human	New World
L. mexicana mexicana	127	ATCC 30883	Human	Texas
Leishmania sp.	404	OK#11	Dog	Oklahoma
Leishmania sp.	220	Hendricks	Human	Panama
Leishmania sp.	222	Sluss	Human	Panama
Leishmania sp.	223	Kaletta	Human	Panama
Leishmania sp.	224	Locke	Human	Panama
Leishmania sp.	225	Huggins	Human	Panama
Leishmania sp.	226	Hernandez	Human	Panama
Leishmania sp.	227	Jones	Human	Panama
Leishmania sp.	228	Farrell	Human	Panama
L. mexicana amazonensis	303	LV72 H6 (Liverpool)	Human	Brazil
Leishmania sp.	-	NIH/MA, Maria	Human	Brazil
Leishmania sp.	-	NIH/DM, Dina-Maria Laboratory infection of NIH/MA	Human	Brazil
L. mexicana mexicana	183	Farrell 1-VLM	Human	British Honduras
L. braziliensis peruviana	140	L-677 (Burstain) UTA, p-Ls286 (Zeludon) ATCC 30880	Human	Peru
Leishmania sp.	-	NIH/IS, Isabel same as WR338	Human	Dominican Republic

TABLE 1 (CONTINUED)

LEISHMANIA ISOLATES

Species[a]	WRAIR No.	Other Designation	Host	Source/Exposure
Leishmania sp.	338	Isabella, same as NIH/IS	Human	Dominican Republic
Leishmania sp.	-	NIH/EM, Emilio	Human	Dominican Republic
L. donovani	130	Khartoum	Human	Sudan
L. donovani	425	NLB008, Mutiso	Human	Kenya
L. donovani	426	NLB023, Kasina	Human	Kenya
L. donovani	427	NLB021, Muindi	Human	Kenya
Leishmania sp.	435	Old world visceral	Human	Kenya
L. donovani	269	Stauber's 3s Strain	Human	North Africa
L. donovani chagasi	116	ATCC 30881 (Herrera)	Human	Honduras
L. donovani infantum	351	LRC-L47	Man	France
L. donovani	352	W.H.O. Strain	Human	India
L. donovani	378	Old world visceral	Human	Khartoum
L. tropica	308	LRC-L207, Old Israeli vaccine strain	Human	Israel
L. tropica	309	LRC-L251, New Israeli vaccine strain	Human	Israel
Leishmania sp.	-	NIH/FR, Friedlin	Human	Israel
L. tropica major[b]	-	NIH/SE, Seidman	Human	Senegal
Leishmania sp.	-	NIH/AC, Ackerman, same as WR261	Human	Asia Minor
L. tropica	261	Ackerman, same as NIH/AC	Human	Unknown
Leishmania sp.	259	Jennifer, McGillivary	Human	Kenya
L. donovani	047	L117RC (Bray's) LRC160	Human	Bengal, India
L. hertigi	151	New world animal cutaneous	Coendou rothschildi	Panama

[a]Hommel, 1978.

[b]Neva, et al., 1979.

Bjorvatn and Neva, 1979.

produce sufficient material for testing, promastigotes of each strain were inoculated onto 20 ml of culture medium in 300-ml screw-capped flasks and overlayed with 10 ml of saline/antibiotic solution. After 8-10 days, 50 ml of normal saline (as above without antibiotics) was added and the flasks were agitated for 1 min (22-24° C). The supernatant was then filtered through gauze pledgets into 80-ml centrifuge tubes. All tubes were centrifuges for 30 min at 1,000 x g (22-24° C). The clear supernatant was decanted and the process was repeated through two additional washings. A quantity of buffer (14 parts distilled H_2O : 1 part 0.1 M Tris/0.1 M Maleic acid/0.01 M EDTA (Na_2)/0.01 M $MgCl_2$, pH adjusted to 7.4 with 40% NaOH) which depends on the size of the pellet of promastigotes was added to each isolate (.25 ml buffer : a button of cells 4-5 mm in diameter; more or less buffer for a larger or smaller pellet). The cells were resuspended in the buffer and the cell membranes were disrupted by three freeze-thaw cycles. The material was examined microscopically to confirm disruption and the lysate was stored in a cryobank at -195° C until being characterized by electrophoresis.

Enzyme activities

The enzymes studied are listed in Table 2. The conditions for electrophoresis and the components of the stains are recorded in Table 3. Certain of these electrophoresis procedures were adapted for Leishmania and for cellulose acetate electrophoresis from those reported by Al-Taqi and Evans (1978); Ayala (1975); Godfrey and Kilgour (1976); Harris and Hopkinson (1976); Kilgour and Godfrey (1973); Kilgour et al. (1974, 1975); Kreutzer et al. (1977) Miles et al. (1977); and Shaw and Prasad (1970).

TABLE 2

ENZYMES TESTED IN THIS STUDY[a]

Enzyme	Enzyme Commission Number	Enzyme Abbreviation	Rating[b]	Number of Loci[c]
Oxidoreductases				
Lactate dehydrogenases	1.1.1.27	LDH	2-3	1-2
Malate dehydrogenase	1.1.1.37	MDH	1	1-2
Malic enzyme	1.1.1.40	ME	1-2	1
Isocitrate dehydrogenase	1.1.1.42	ICD	2	1-2
Phosphogluconate dehydrogenase	1.1.1.44	6PGDH	1	1
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	1	1-2
Glyceraldehyde-phosphate dehydrogenase	1.2.1.12	$GAPDH_1$, $GAPDH_2$	2	2 or more
Glutathione reductase	1.6.4.2	GSR	2-3	1-2
Transferases				
Glutamate-oxaloacetate transaminase	2.6.1.1	GOT & ASAT	1-2	1
Glutamate-pyruvate transaminase	2.6.1.2	ALAT	1	1
Hexokinase	2.7.1.1	HK	2	1-3
6-Phosphofructokinase	2.7.1.11	FK	2-3	1-2
Adenylate kinase	2.7.4.3	AK	1-2	1-2
Guanylate kinase	2.7.4.8	GUK	4	1-2
Phosphoglucomutase	2.7.5.1	PGM	1-2	1-2
Hydrolases				
Esterases	3.1.1.1	EST	2	1-2
Acid phosphatase	3.1.3.2	ACP	1	1-3
Peptidases	3.4.11-13	PEP	1	1
Peptidase D	3.4.13.9	PEPD	1	1
Lyases				
Aldolase	4.1.2.13	ALD	4	1-2
Fumerate hydratase	4.2.1.2	FUM	2	1-2
Isomerases				
Mannose phosphate isomerase	5.3.1.8	MPI	1	1
Glucose phosphate isomerase	5.3.1.9	GPI	1	1

[a] Commission on Biochemical Nomenclature, 1973.

[b] The ratings indicate facility of operation. Enzyme systems which produce distinct, rapidly appearing bands from a single application of the isolate are rated 1.

[c] Enzymes which regularly produce more than one band for most isolates are possible multilocus systems; however, no substantiating genetic data are available. In this study if two isolates had identically migrating bands for one band of a multiband system, they were considered identical for that system.

TABLE 3

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	Developing Components[a]
LDH[b]	1	160	15	A	3.0 ml DL-Lactic acid, Na salt; 15 mg β-Nicotinamide Adenine Dinucleotide (β-NAD); 15 mg MTT tetrazolium (MTT); 10 mg Phenazine Methosulfate (PMS).
MDH[c]	2	180	15	B	15 mg cis-Oxalacetic acid; 15 mg β-Nicotinamide Adenine Dinucleotide, Reduced Form (β-NADH), Na_2 salt.
ME	3	180	15	C	270 mg DL-Malic acid (readjust pH to 7.5). Then add 15 mg MTT; 15 mg β-Nicotinamide Adenine Dinucleotide Phosphate (β-NADP), Na salt; 10 mg PMS; 25 mg $MnCl_2$.
ICD[b]	1	180	15	A	100 mg DL-Isocitric acid, Na_3 salt (readjust pH to 8.0). Then add 15 mg MTT; 15 mg β-NADP; 10 mg PMS.
6PGDH[b]	1	180	15	D	15 mg 6-Phosphogluconic acid, Na_3 salt; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-NADP; 10 mg PMS; 60 mg EDTA, Na_2 salt.
G6PDH[b]	1	160	15	D	75 mg D-Glucose-6-phosphate; 15 mg MTT; 15 mg β-NADP; 10 mg PMS.
$GAPDH_1$[b]	3	300	15	E	50 mg Fructose-1,6-diphosphate, Na_4 salt (dissolve in 2 ml reaction buffer). Add 2.7 units Aldolase and incubate at 37°C for 1 hour. Then add 5 mg MTT; 30 mg β-NAD; 2.5 mg PMS; 50 mg Arsenic acid, Na_2 salt; 50 mg Sodium pyruvate.

TABLE 3 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	Developing Components[a]
$GAPDH_2$[b]	3	220	10	E	80 mg Fructose-1,6-diphosphate, Na_4 salt (dissolve in 2 ml reaction buffer). Add 90 units Aldolase and incubate at 37°C for 1 hour. Then add 15 mg MTT; 20 mg β-NAD; 10 mg PMS; 100 mg Arsenic acid, Na_2 salt; 50 mg Sodium pyruvate.
GSR[b]	1	180	12	F	30 mg Glutathione (oxidized form); 5 mg β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β-NADPH); 1 mg 2,6-Dichlorophenol-indophenol, Na salt; 15 mg MTT.
GOT	3	180	15	A	100 mg L-Aspartic acid; 75 mg α-Ketoglutaric acid (readjust pH to 8.0). Then add 10 mg Pyridoxal-5-phosphate. 75 mg Fast Blue BB salt (separate plate).
ASAT[b,c]	4	180	15	B	30 mg α-Ketoglutaric acid, Na salt; 70 mg L-Aspartic acid (readjust pH to 7.4). Then add 100 units Malic dehydrogenase (Porcine heart in phosphate buffer pH 7.5); 15 mg β-NADH.
ALAT[b,c]	1	160	10	B	30 mg α-Ketoglutaric acid, Na salt; 50 mg L-alanine; 30 units Lactic dehydrogenase (Beef heart in phosphate buffer pH 7.5); 15 mg β-NADH.
HK	5	160	15	D	50 mg α-D(+)-Glucose; 20 units Glucose-6-phosphate dehydrogenase (Baker's yeast); 20 mg $MgCl_2$; 15 mg MTT; 15 β-NADP; 10 mg PMS; 40 mg Adenosine 5'-Triphosphate (ATP), Na_2 salt.

TABLE 3 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	Developing Components[a]
FK	3	160	15	A	200 mg β-D-(-)-Fructose; 15 mg MTT; 15 mg β-NADP; 10 mg PMS; 40 mg ATP; 20 mg $MgCl_2$; 50 units Phosphoglucose isomerase.
AK	3	150	15	G	30 mg Adenosine 5'-Diphosphate (ADP), Na_2 salt; 120 mg $MgCl_2$; 120 mg ∝-D(+) Glucose; 15 mg β-NADP; 15 mg MTT; 10 mg PMS; 15 units Glucose-6-Phosphate dehydrogenase; 30 units Hexokinase.
GUK[c]	6	250	15	H	30 mg ATP; 50 mg Guanosine 5'-Monophosphoric acid (GMP), Na salt; 15 mg Phosphoenolpyruvate, K salt; 15 mg β-NADH; 60 mg $MgCl_2$; 150 mg KCl; 30 mg $CaCl_2$; 280 units Lactate dehydrogenase; 20 units Pyruvate kinase.
PGM	7	180	15	D	200 mg ∝-D-Glucose-1-phosphate; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-NADP; 10 mg PMS; 25 units Glucose-6-phosphate dehydrogenase (Baker's yeast); 60 mg EDTA, Na_2 salt; 1.2 mg ∝-D-Glucose-1,6-diphosphate.
EST[c]	8	180	15	I	25 mg 4-Methylumbelliferyl acetate (dissolve in 10 ml 50% acetone).
ACP[b]	4	160	15	J	75 mg Naphthol as phosphoric acid; 50 mg Fast Garnet GBC salt.

TABLE 3 (CONTINUED)

CONDITIONS FOR ELECTROPHORESIS AND COMPONENTS FOR DEVELOPING ZYMOGRAMS

Enzyme	Cell Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	Developing Components[a]
PEP[c]	9	160	10	K	20 mg L-Leucyl-L-Leucyl-L-Leucine; 1.5 mg L-Amino Acid Oxidase (Crotalus adamanteus venom); 250 units Peroxidase (from horse radish); 20 mg $MnCl_2$; 15 mg 3-Amino-9-ethylcarbazole.
PEPD[c]	9	160	10	K	20 mg L-Leucyl-L-Proline (hydrochloride); 1.5 mg L-Amino Acid Oxidase; 250 units Peroxidase (from horse radish); 20 mg $MnCl_2$; 15 mg 3-Amino-9-ethylcarbazole.
ALD[b]	1	160	15	D	250 mg Fructose-1,6-diphosphate, Na_4 salt; 15 mg MTT; 15 mg β-NAD; 10 mg PMS; 300 units Glyceraldehyde-3-phosphate dehydrogenase (Rabbit muscle $(NH_4)_2SO_4$ solution, pH 7.5); 60 mg Sodium Arsenate.
FUM	3	180	15	L	500 mg Fumaric acid, K salt (readjust pH to 7.0). Then add 15 mg β-NAD; 15 mg MTT; 10 mg PMS; 200 units Malic dehydrogenase.
MPI	10	160	15	M	20 mg Mannose-6-phosphate, Ba salt; 40 mg $MgCl_2$; 15 mg β-NADP; 15 mg MTT; 10 mg PMS; 40 units Phosphoglucose isomerase (Rabbit muscle); 15 units Glucose-6-phosphate dehydrogenase (Baker's yeast).
GPI	3	180	15	D	20 mg Fructose-6-phosphate; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-NADP; 10 mg PMS; 25 units Glucose-6-phosphate dehydrogenase (Baker's yeast); 60 mg EDTA, Na_2 salt.

Cell buffers:

1. 0.2 M Phosphate, pH 7.0.
2. 0.25 M Tris/0.09 M Citric acid monohydrate; adjust to pH 7.0 with Tris.
3. 0.1 M Tris/0.1 M Maleic acid/0.01 M EDTA (Na_2 salt)/0.01 M $MgCl_2$; adjust to pH 7.4 with 40% NaOH.
4. 0.15 M Citric acid (Na_3)/0.24 M NaH_2PO_4; adjust to pH 6.3 with 40% NaOH.
5. 0.233 M Tris/.065 M Citric acid (monohydrate); adjust to pH 7.0 with 50% HCl.
6. 0.1 M Tris/0.1 M Maleic anhydride /0.01 M $MgCl_2$; adjust to pH 7.4 with 40% NaOH.
7. 0.1 M Tris/0.1 M Maleic acid/0.01 M EDTA/0.01 M $MgCl_2$; adjust to pH 8.0 with 40% NaOH.
8. 0.1 M Tris/0.01 M EDTA; adjust to pH 7.2 with boric acid crystals.
9. 0.1 M Tris/0.1 M NaH_2PO_4; adjust to pH 7.4 with 40% NaOH.
10. 0.05 M Tris/0.05 M NaH_2PO_4; adjust to pH 8.3 with 40% NaOH.

Membrane buffers:

HK;MPI - 1 part cell buffer and 5 parts distilled water.
MDH;GSR;ALAT;ALD - 1 part cell buffer and 9 parts distilled water.
AK;GUK - 1 part cell buffer and 10 parts distilled water.
All other enzymes - 1 part cell buffer and 14 parts distilled water.

Reaction buffers:

A. 0.1 M Tris; adjust to pH 8.0 with 50% HCl.
B. 0.025 M NaH_2PO_4/0.08 M Na_2HPO_4; pH 7.4.
C. 0.06 M Tris; adjust to pH 7.5 with 50% HCl.
D. 0.06 M Tris; adjust to pH 8.0 with 50% HCl.
E. 0.05 M Tris; adjust to pH 7.5 with 50% HCl.
F. 0.25 M Tris; adjust to pH 8.4 with 50% HCl.
G. 0.5 M Tris; adjust to pH 8.0 with 50% HCl.
H. 0.5 M Tris; adjust to pH 7.5 with 50% HCl.
I. 0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, pH 6.3.
J. 1 part 0.1 M Citric acid (anhydrous) and 4 parts 0.1 M Sodium Citrate.
K. 0.02 M Na_2HPO_4; adjust to pH 7.5 with 50% HCl.
L. 0.05 M NaH_2PO_4/0.05 M Na_2HPO_4, pH 7.0.
M. 0.2 M Tris; adjust to pH 7.5 with 50% HCl.

[a] To make 60 ml of stain (about 50 samples). All chemicals Sigma.
[b] These systems require cooling during electrophoresis.
[c] These isozymes observed under ultraviolet light.

Control membranes were run for each enzyme system. The membranes were run under conditions identical to those of the experimental, but the specific enzyme substrate was omitted from the developing components. Bands were not observed on the control membranes.

Electrophoresis

An aliquot of 5 µl was taken from the specimen sample with a microdispenser and transferred to a well of a Zip Zone Well Plate (all electrophoresis equipment is manufactured by Helena Laboratories, Beaumont, Texas). A maximum of eight samples was run on each cellulose acetate membrane. Presoaked (20 min) Titan III cellulose acetate membranes were removed from the membrane buffer, blotted, and aligned on the Super Z Aligning Base. About 0.5 µl of the aliquot was transferred from the well plate to the membrane with a Super Z applicator. Additional applications were made to other membranes from the original aliquot, and three or four enzyme systems were studied from one 5-µl aliquot. Two assays of the 25 enzyme/substrate systems were made for each of the Leishmania isolates. The membranes were placed in the Zip Zone chamber. Two membranes (or 16 samples) were run in a single chamber. To prepare the cell, 100 ml of cell buffer was added to both buffer wells, and filter paper wicks were placed over the cell/membrane contact areas. The Titan Power Supply was set for the appropriate voltage and time, and if cooling was necessary during electrophoresis, ice cubes were placed in the center wells prior to turning on the power supply. After electrophoresis the membranes were removed from the cell, trimmed with a scissors, and placed, enzyme side down, on a previously prepared substrate/stain. Zymograms were incubated at 37° C until the

bands which indicate enzyme activity were dark enough to be observed (5-15 min for most of the enzymes in this study). Isozymes of MDH, ASAT, ALAT, GUK, EST, PEP, and PEPD were viewed under ultraviolet light. Each band was marked as soon as it appeared on the membrane. The membranes were then removed from the reaction mixture, placed in 5% acetic acid to stop the reaction, washed in tap water, blotted, and allowed to air dry.

To prepare the staining dishes, the specific components (Table 3) were dissolved in 30 ml of reaction buffer, combined with 30 ml of 2% Noble Agar (50° C), 10-ml portions were placed in 100X 15-mm petri dishes, and these were stored at 3° C until needed.

CHAPTER III

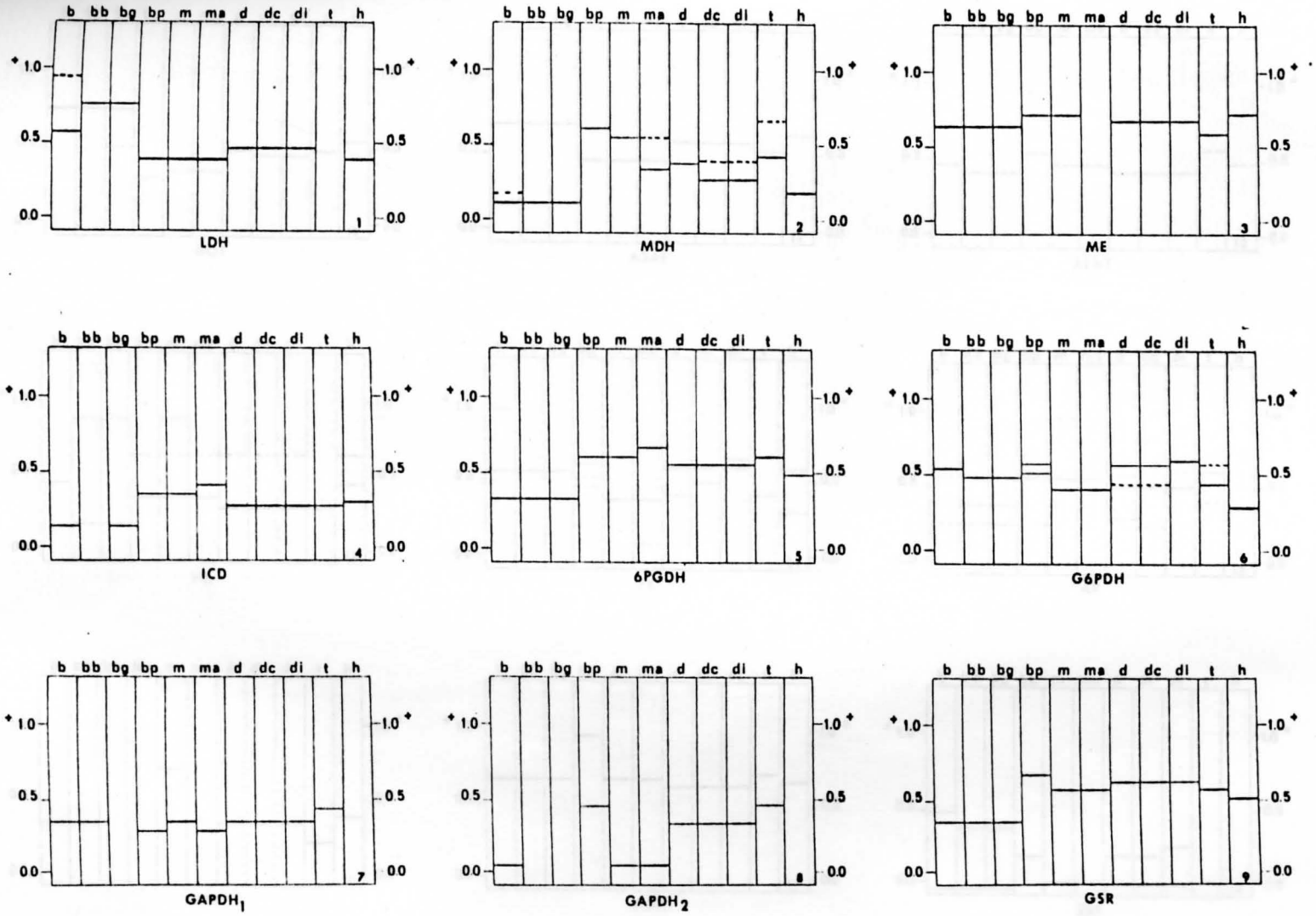
RESULTS

Each isolate listed in Table 1 was characterized for the 25 enzyme systems in Table 2. The isozyme patterns of eleven isolates are shown diagrammatically in Figs. 1-25. Pair comparisons were made of the resulting enzyme profiles and these were converted to percent similarity, Table 4A, B, C. Systems rated 1, 1-2, or 2, Table 2, are ones which produce thin, rapidly appearing bands (enzyme activity) with the smallest number of cells. Those rated 2-3 or 4 require fresh, highly concentrated isolate homogenates to produce bands. Also reported in Table 2 are estimates of the number of loci which might be actively producing (coding) enzymes in the parasite; however, no genetic data are available to sustain these estimates. Many isolates produced more than one band of enzyme activity, Figs. 2, 6, 13, 14, 15, 18, 19, 23. Multiple locus enzymes (isozymes) have been reported in other organisms (Avise, 1974; Ayala, 1975; Ayala et al., 1974; Harris and Hopkinson, 1976; Myers, 1978) and it is possible that a similar situation exists in these protozoan parasites.

In certain of the Leishmania isolates which produced multiple bands one of these bands was dark and the second was light, Figs. 2, 6, 13, 14, 15, 19. Preliminary studies suggest that the isozyme which produces the dark band might be associated with the kinetoplast and the light band isozyme is cytoplasmic (Krassner, 1968); however, further study is indicated. Any differences in the light bands were not considered in this study. Preliminary studies also suggest that GPI is a dimer in Leishmania as it is in many other organisms (Harris and

FIGURES 1-25

Diagrammatic representations of the electrophoretic patterns of 25 enzyme/substrate systems from eleven isolates of Leishmania. See Table 2 for complete names of enzymes. Numbers at the sides of each figure represent the mobilities of the enzymes. 0.0 is the origin on the cellulose acetate plate. Anode is at the top of each figure. Broken lines indicate lightly staining bands. b-WR209; bb-WR063; bg-WR075; bp-WR140; m-WR127; ma-WR303; d-WR130; dc-WR116; di-WR351; t-WR308; h-WR151.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0.5
0.0
1
LDH
2
MDH
3
ME
4
ICD
5
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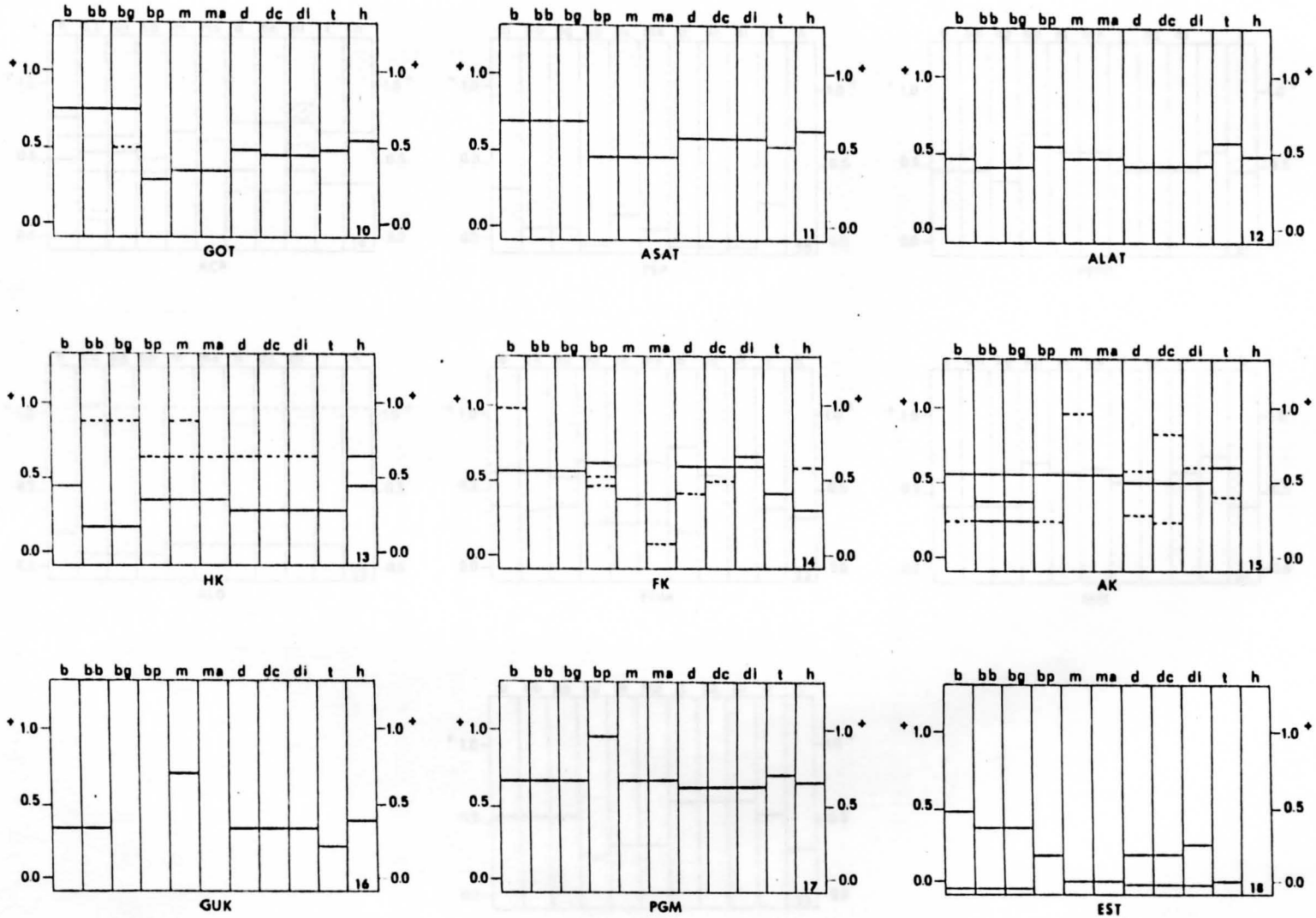
G6PDH
7
GAPDH1
8
GAPDH2
9
GSR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1.0
0.5
0.0
10
GOT
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ASAT
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ALAT
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HK
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FK
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AK
16
GUK
17
PGM
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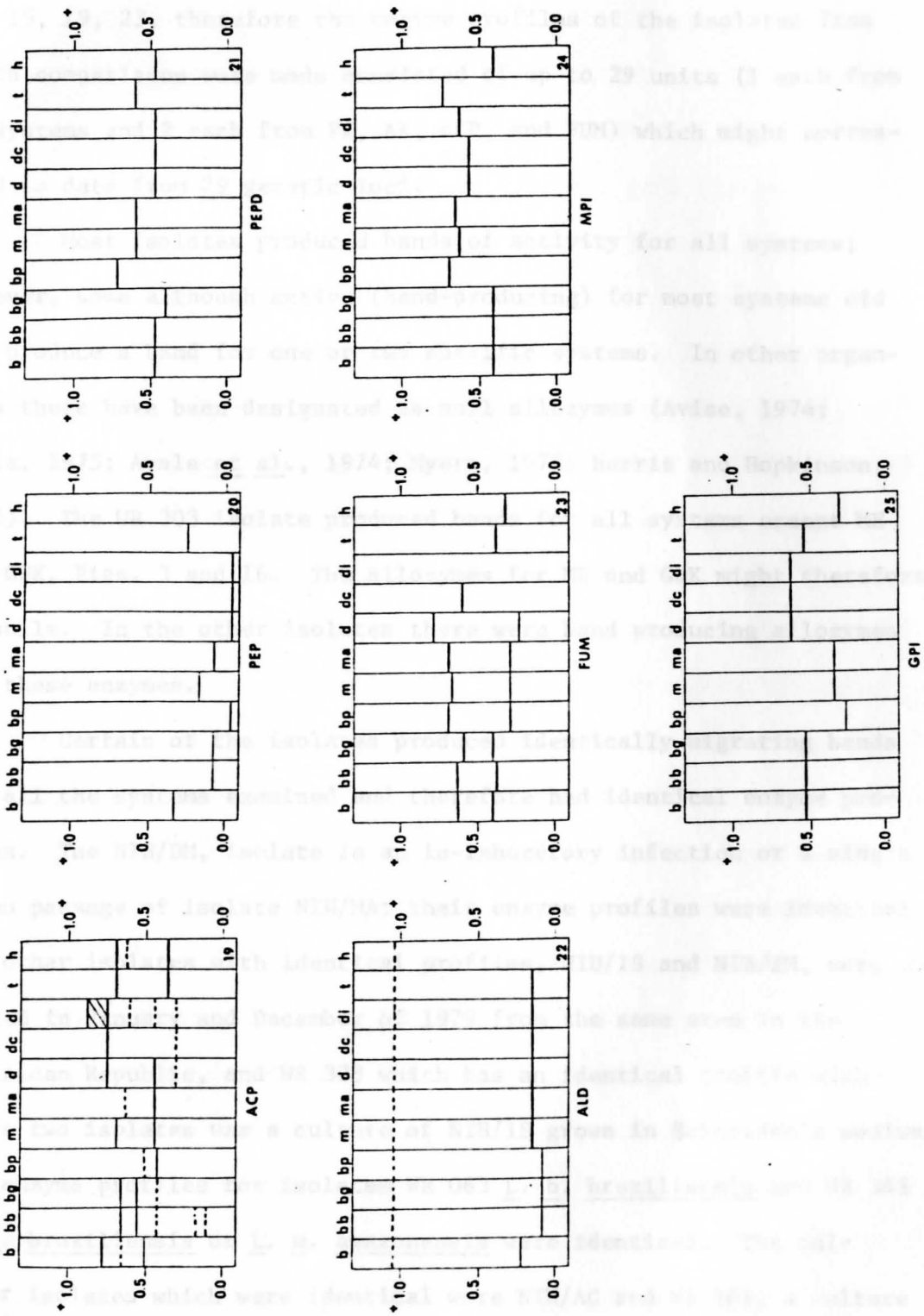
EST


PEPD
MPI
PEP
FUM
GPI
ACP
ALD
b bb bg bp m ma d dc dl t h
1.0
0.5
0.0
21
24
20
23
25
19
22

Hopkinson, 1976). Four of the enzyme systems, FK, AK, ACP, and FUM, regularly produced at least two dark bands for most isolates, Figs. 14, 15, 19, 23, therefore the enzyme profiles of the isolates from which comparisons were made consisted of up to 29 units (1 each from 21 systems and 2 each from FK, AK, ACP, and FUM) which might correspond to data from 29 genetic loci.

Most isolates produced bands of activity for all systems; however, some although active (band-producing) for most systems did not produce a band for one or two specific systems. In other organisms these have been designated as null allozymes (Avise, 1974; Ayala, 1975; Ayala et al., 1974; Myers, 1978; Harris and Hopkinson, 1976). The WR 303 isolate produced bands for all systems except ME and GUK, Figs. 3 and 16. The allozymes for ME and GUK might therefore be nulls. In the other isolates there were band producing allozymes for these enzymes.

Certain of the isolates produced identically migrating bands for all the systems examined and therefore had identical enzyme profiles. The NIH/DM, isolate is an in-laboratory infection or a single human passage of isolate NIH/MA; their enzyme profiles were identical. Two other isolates with identical profiles, NIH/IS and NIH/EM, were obtained in January and December of 1979 from the same area in the Dominican Republic, and WR 338 which has an identical profile with these two isolates was a culture of NIH/IS grown in Schneider's medium. The enzyme profiles for isolates WR 063 L. b. braziliensis and WR 345 L. b. braziliensis or L. m. amazonensis were identical. The only other isolates which were identical were NIH/AC and WR 261, a culture of NIH/AC. It is not surprising but rather expected that isolate pairs,

one of which is a culture of the other, should be so similar; however most isolate profiles differed one from another.

Among the isolates certain pairs were more than 80% identical. The two L. b. (panamensis) isolate profiles, WR 209 and 148, were 82% identical, and the profiles of isolates WR 063, WR 345, WR 075, and WR 442 were at least 79% identical. These isolates plus NIH/BR which are referred to as the braziliensis group in Fig. 26 were at least 40% identical to each other, Tables 1 and 4A.

A group of unidentified isolates, WR 220, WR 222, WR 223, WR 224, WR 225, WR 226, WR 227, and WR 228, were identical for over 90% of the enzyme systems, and have been designated group 1 (GR1). These isolates were more than 80% identical with an L. m. mexicana isolate, WR 127, and another unknown, WR 404; WR 183, identified as L. m. mexicana, was less than 50% identical with any of these isolates. The GR1 and WR 404 isolates were more than 75% identical to a pair of unknown isolates, NIH/MA and NIH/DM, but WR 127 was less than 70% identical with them. These two NIH isolates were more than 75% identical with WR 303, L. m. amazonensis, which was less than 70% identical with WR 127, GR1 isolates and WR 404. The origin and histories of these two isolates when correlated with the data indicate that they are probably L. m. amazonensis and not L. m. mexicana; however is should be noted that although these isolates, WR 127, GR1, WR 404, of the mexicana subgroup and WR 303, NIH/MA, NIH/DM of the amazonensis subgroup, are placed in separate subgroups in the mexicana profile, they are all quite similar, Fig. 26. Greater than 80% similarity was noted among isolates WR 183 identified as L. m. mexicana, WR 140 L. b. peruviana, NIH/IS, WR 338 (a clone of NIH/IS), and NIH/EM, therefore these data identify WR 183

TABLE 4A

PERCENT ENZYME PROFILE IDENTITY BETWEEN PAIRS OF ISOLATES IN THE BRAZILIENSIS PROFILE. THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS.

Lpb[d] 209[a]	Lbp 148	Lsp BR[b]	Lbb 063	Lbb 345	Lbg 075	Lsp 442	
	82[c]	74	60	60	56	52	209
		81	48	48	52	44	148
			58	58	54	42	BR
				100	79	79	063
					79	79	345
						79	075
							442

Footnotes:

a WRAIR isolates

b NIH isolates

c

$$\text{Percent Enzyme Profile Identical} = \frac{\text{Total isozyme systems in which identically migrating bands were produced}}{\text{Total isozyme systems which produced a band for both isolates}} \times 100$$

d L.bp (L. b. panamensis), L.sp. (Leishmania sp.)
L.bb (L. b. braziliensis), L.bg (L. b. guayanensis)

as L. b. peruviana. All isolates noted as the mexicana group in Fig. 26 were at least 30% identical to one another, Tables 1 and 4B.

The isolates referred to as the donovani group in Fig. 26 were more than 70% identical to each other, and some of these isolate pairs were over 80% identical. The three isolates WR 425-7 were over 90% identical and were designated as GR2, Tables 1 and 4C.

Two L. tropica isolates, WR 308, and WR 309, were 79% identical and these were over 60% identical with two NIH isolates, NIH/SE L. t. major and an unknown, NIH/FR, Tables 1 and 4C.

The profile of an L. hertigi isolate, WR 151, was less than 15% similar to any of the others.

Although WR 047 when initially isolated was identified as L. donovani, the later history of the isolate is not clear and possible mishandling is indicated (Bray - personal communication). It does have over 20% similarity with the other visceral isolates. Another isolate WR 378, L. donovani, is about 30% identical with the donovani group. Three isolates, WR 261, NIH/AC, and WR 259, all unknowns, are 61% identical. These three have low levels of profile similarity with both the donovani and tropica groups, Tables 1 and 4C and Fig. 26.

TABLE 4B

PERCENT ENZYME PROFILE IDENTITY BETWEEN PAIRS OF ISOLATES IN THE "*MEXICANA* PROFILE". THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH ANY OF THE OTHERS

Lmm[d] 127[a]	Lsp 404	Lsp GR1[e]	Lma 303	Lsp MA[b]	Lsp DM	"Lmm" 183	Lbp 140	Lsp IS	Lsp 338	Lsp EM	
	88[c]	81	63	67	67	39	29	32	32	32	147
		85	67	77	77	48	39	44	44	44	404
			67	80	80	48	39	44	44	44	GR1
				77	77	38	27	32	32	32	303
					100	50	39	37	37	37	MA
						50	39	37	37	37	DM
							86	81	81	81	183
								72	72	72	140
									100	100	IS
										100	338
											EM

Footnotes:

a-c See Table 4A.

d *L.mm* (*L. m. mexicana*), *L.* sp. (*Leishmania* sp.), *L. ma* (*L. m. amazonensis*), "*L.mm*" this study identifies this isolate as *L. b. peruviana* not *L. m. mexicana*, *L.bp* (*L. b. peruviana*).

e GR1 isolates WR 220, 222, 223, 224, 225, 226, 227, 228 (>90% identical).

TABLE 4C

PERCENT ENZYME PROFILE IDENTITY BETWEEN PAIRS OF ISOLATES IN THE OLD WORLD ISOLATES (INCLUDING WR 116). THESE ISOLATES HAVE LESS THAN 15% IDENTITY WITH THE OTHERS

Ld[d] 130[a]	Ld GR2[e]	Lsp 435	Ld 269	Ldc 116	Ldi 351	Ld 352	Ld 378	Lt 308	Lt 309	Lsp FR[b]	Lsp SE	Lsp AC	Lt 261	Lsp 259	"Ld" 047	
	79[c]	75	82	81	70	68	54	-[f]	-	-	-	13	13	19	26	130
		82	68	81	73	75	50	-	-	-	-	26	26	27	30	GR2
			69	75	77	75	46	-	-	-	-	22	22	23	22	435
				78	77	71	50	-	-	-	-	22	22	19	19	269
					81	70	50	-	-	-	-	30	30	27	26	116
						77	47	-	-	-	-	26	26	27	26	351
							38	-	-	-	-	22	22	27	19	352
								-	-	-	-	14	14	13	19	378
									79	77	77	18	18	-	-	308
										67	64	33	33	-	-	309
											77	32	32	-	-	FR
												21	21	-	-	SE
													100	61	26	AC
														61	26	261
															32	259
																047

Footnotes:

a-c See Table 4A

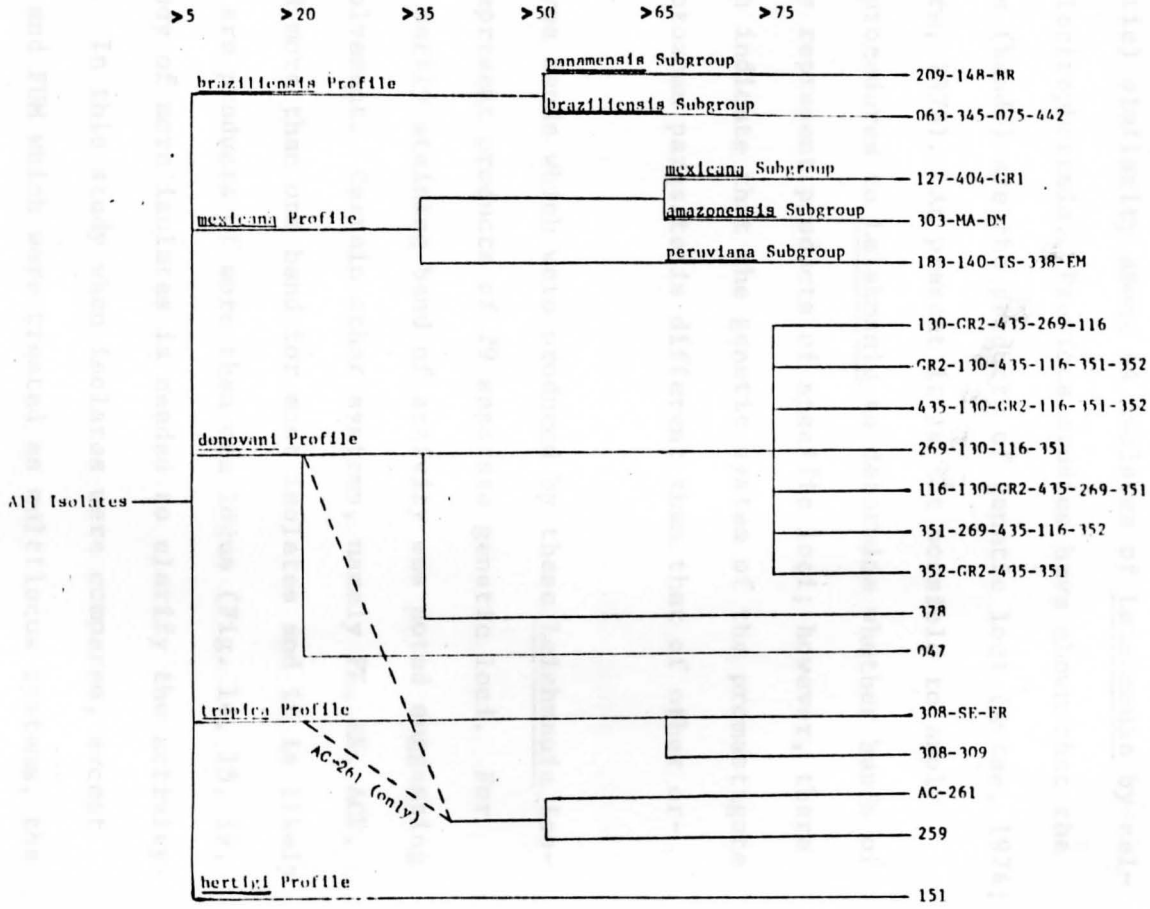
d L.d (L. donovani), L.sp (Leishmania sp.), L.dc (L. d. chagasi), L.di (L. d. infantum), L.t (L. tropica), "L.d" this isolate is possibly contaminated.

e GR2 isolates WR 425, 426, 427 (> 90% identical).

f 15% similarity

FIGURE 26

DENDROGRAM SHOWING THE GROUPING OF ISOLATES ACCORDING TO THEIR LEVELS OF ENZYME PROFILE SIMILARITIES. PROFILES OF ISOLATES GROUPED AT THE RIGHT ON THE SAME LINE ARE ABOUT 75% OR MORE IDENTICAL. SEE TABLE 1 AND 4 FOR ISOLATE INFORMATION



CHAPTER IV

DISCUSSION

A total of 25 enzyme systems were tested to measure the amount of isozyme (genetic) similarity among 44 isolates of Leishmania by cellulose acetate electrophoresis. Previous studies have shown that the enzyme activities (bands) are the products of genetic loci (Avise, 1974; Ayala, 1975; Myers, 1978). At present it is not possible to apply similar testing procedures to Leishmania to determine whether bands of activity actually represent products of specific loci; however, there are no data which indicate that the genetic system of the promastigote stage of this protozoan parasite is different than that of other organisms.

The isozyme bands which were produced by these Leishmania isolates probably represent products of 29 separate genetic loci. For most systems one darkly staining band of activity was noted suggesting single locus involvement. Certain other systems, namely FK, AK, ACP, and FUM, produced more than one band for most isolates and it is likely that these bands are products of more than one locus (Fig. 14, 15, 19, 23). Further study of more isolates is needed to clarify the activity in these systems. In this study when isolates were compared, except for FK, AK, ACP, and FUM which were treated as multilocus systems, the isolates were considered identical for a system if the darkly staining bands migrated equally, Figs. 1-25.

Different levels of isozyme identity or genetic similarity were noted among the isolates ranging from about 5% to as much as 100% or total identity, Tables 4A, B, and C. In most cases when isolates

were 100% identical, one was a culture of the other. There were two exceptions. Isolates WR 063, identified as L. b. braziliensis, and WR 345, identified as L. b. braziliensis or L. m. amazonensis, had identical profiles. The enzyme data suggest they are both isolates of L. b. braziliensis. The other exception is isolate pair NIH/IS and NIH/EM. Both of these were unknowns collected about a year apart in the same locality; the enzyme data suggest that both are L. b. peruviana.

As a measure of genetic variation, the proportion of polymorphic loci was calculated for certain isolate groups among which there were high levels of enzyme profile identity, Table 5. An estimate of the average polymorphism was then calculated to be 21.6%. It must be noted that in most of these groups polymorphism was calculated from data of only two or three isolates, and certain of these isolates were unknowns and were included in the group due to their enzyme identity with other isolates of the group. Usually these estimates are calculated from much larger numbers of identified samples. Disregarding the possible bias which might be the result of sample size the average polymorphism data suggests that isolates which have enzyme profiles which are about 21% different or about 75% identical are probably samples of the same species type or group, and that those with profiles which differ by much more than 21% should probably be grouped differently. In other words any species group of Leishmania is expected to contain on the average 21% naturally occurring polymorphism, and any two isolates with enzyme profiles about 75% or more identical are probably samples from the same group and are therefore samples from the same species. Ayala (1982) when reporting on the genic variation in natural populations

TABLE 5

AVERAGE ALLOZYME POLYMORPHISM* AMONG GROUPED ISOLATES. SEE TABLES 1 AND 4 FOR ISOLATE INFORMATION

Group	Average Polymorphism
WR209-148-BR	.210
WR063-075-442	.210
WR127-404-GRI	.140
WR303-MA	.230
WR140-183-IS	.203
WR130-GR2-435-269-116-351-352	.246
WR308-309-FR-SE	.265
All Groups	.216
Invertebrates (Diploid)	.469

$$\text{*Average Allozyme Polymorphism} = \frac{\text{Loci with allozymes not as standard} \,/\, \text{Total Loci}}{\text{Total isolates}}$$

of some major groups of animals and plants indicated that the average polymorphism in diploid invertebrates is 46.9% or about twice that found in these promastigote isolates. This suggests that the stage of Leishmania tested by CAE is haploid.

Another measure of genetic variation is the heterozygosity of the population. This is calculated by obtaining the frequency of heterozygotes at each locus and then averaging these frequencies over all loci. Of the 44 isolates characterized except for FK, AK, ACP, and FUM any one isolate produced only one dark band of activity (no heterozygotes) which also suggests that the promastigote is haploid. Kreutzer and Christensen (1980) reported similar results for 18 isolates. The group of identified, geographically separate L. donovani isolates, WR 130, GR2, WR 435, WR 269, and WR 378, had an average polymorphism of about 25% which indicates they could be expected to be heterozygous (at least two allozymes or allomorphs in one isolate) at about seven of 29 loci. It is unlikely that no heterozygosity would be present among five isolates even though the trauma of cloning on synthetic media might result in a drastic reduction in such heterozygosity. Studies in colonized diploid invertebrates have shown that laboratory colony populations do have lower levels (not zero) of polymorphism than do natural ones (Avise, 1974; Ayala, 1975). These data which suggest haploidy are not inconsistent with data reported on cell division studies on promastigotes (Bianchi et al., 1969; Christophers et al., 1926; Croft, 1976; Simpson and Braly, 1970).

The enzyme profile of each isolate has been compared pairwise with the profile of each other isolate, Tables 4A, B, C. The data from the comparisons indicate that there are at least five different

major groups among the 44 isolates and these have been designated as braziliensis, mexicana, donovani, tropica, and hertigi, Fig. 26. Each group is less than 15% similar to any other. Avise (1974), Ayala (1975) and Ayala et al. (1974), have reported similar groupings based on enzyme similarities in other organisms including vertebrates, invertebrates, and plants. The groupings according to enzyme profiles are for the most part consistent with subdivisions of Leishmania proposed by Hommel (1978). The genetic data from the enzyme profile comparisons also suggest that within certain of these major groups there are subdivisions.

The profiles of the isolates in the braziliensis profile group are all about 50% identical, but among these isolates there are two groups which themselves are about 75% or more similar. They are the L. b. (panamensis) group consisting of WR 209 and WR 148 which have been identified as L. b. (panamensis) and an unknown isolate NIH/BR which has a history indicating it could also be L. b. (panamensis), and the L. b. braziliensis (or guayanensis) group consisting of WR 063 identified as L. b. braziliensis, WR 345 either L. b. braziliensis or L. m. amazonensis but genetically the former, WR 075 identified as L. b. guayanensis and WR 442, an unknown, Table 4A.

When the enzyme profiles of isolates were over 80% identical, they were grouped and identified as samples of the same species. WR 209, WR 148, and NIH/BR are probably samples of the same type; likewise WR 063, WR 345, WR 075, and WR 442 were all about 80% identical. The L. b. guayanensis isolate, WR 075, was placed with and not separate from the L. b. braziliensis isolate, WR 063, because both enzyme

profiles were so similar. Study of additional isolates of these two types is indicated.

The isolates of the mexicana profile are all at least 35% identical, Table 4B, but two subgroups, mexicana and amazonensis, are more than 65% identical. There is over 90% enzyme profile identity among the group of unknown isolates from Panama, designated GR1. Unknowns WR 404 and GR1 are over 80% identical with WR 127 identified as L. m. mexicana and less than 70% identical with WR 303 identified as L. m. amazonensis. The isolates WR 404, GR1, NIH/MA, and NIH/DM are over 75% identical with each other. The groupings which place WR 404 and GR1 with WR 127, and therefore identify them as L. m. mexicana, and place NIH/MA and NIH/DM with 303, L. m. amazonensis, as indicated in Fig. 26 are most consistent with the genetic and isolate history data. The genetic (isozyme) data also suggest a third subgroup (peruviana) in the mexicana profile. WR 183, identified as L. m. mexicana, which is only about 50% identical with WR 127 or any isolate in the mexicana or amazonensis subgroups, is over 80% identical with WR 140 identified as L. b. peruviana and the three unknowns from the Dominican Republic, NIH/IS, WR 338 (a culture of NIH/IS), and NIH/EM. The isozyme or genetic data suggest that these five isolates are samples from one group, and furthermore they should be included in the mexicana profile group (more than 35% similarity) rather than the braziliensis profile group (less than 15% similarity). It is possible that WR 183, which this study identifies as L. b. peruviana, NIH/IS, WR 338, and NIH/EM represent samples from a Central American focus and WR 140 represents a sample from a Peruvian focus of a genetically very similar group

designated in Fig. 26 as peruviana, a subgroup in the mexicana profile group. Additional study is indicated.

Another group among which most isolates showed over 75% identity has been designated the donovani profile group, Table 4C. This group included isolates of L. donovani from India and various sites in Africa as well as an isolate of L. d. infantum, WR 351, and of L. d. chagasi, WR 116. It is unclear whether the profiles of these three donovani types are different enough from one another so isozyme analysis can be used for separation; however the data weakly suggest that WR 351 might be different from the other visceral isolates. The genetic data indicate that unknown WR 435 is L. donovani.

Another major group is the tropica profile group which consists of three identified isolates, WR 308 and WR 309, both L. tropica, and NIH/SE, identified as L. t. major, and an unknown, NIH/FR, with a history which suggests it is L. tropica, Table 4C. WR 308 is over 75% identical with the other three, and NIH/SE and NIH/FR are over 75% identical, but WR 309 has a lower level, about 65%, of identity with NIH/SE and NIH/FR. The genetic data along with each isolate's history suggest that these are four isolates of L. tropica. The average polymorphism of most isolate groups is 21.6%; the average polymorphism of these four isolates is higher, 26.5%. It is possible, as more isolates are characterized by CAE, the tropica profile group might require subdivision. The last major group is the hertigi profile group which consists of one isolate, WR 151.

The profiles of certain isolates were not significantly similar to any major profile. WR 378, L. donovani, was over 35% identical with the other visceral samples which indicates similarity to the donovani

group. Three unknown isolates, NIH/AC, WR 261 (a culture of NIH/AC), and WR 259, had profiles which were 61% identical which suggests significant genetic relationships. The profiles of these isolates had low levels, 20-30%, of similarity with both the donovani and tropica group profiles. Perhaps data from additional isolates will clarify the identification of these isolates.

The enzyme data reported here are similar to CAE data previously reported by Kreutzer and Christensen (1980). WR 209 is identical with the IB type (enzyme profile) L. braziliensis (panamensis) from Panama and WR 148 differs from type IA only in a slightly more cathodal ME isozyme for WR 148. The WR 127 isolate differs from the type III profile, both L. m. mexicana, by the ME band which is more cathodal in WR 127. WR 130 has a more anodal band for G6PDH than type IV, L. donovani (LV 136), otherwise they are identical. The L. tropica isolate, WR 308, is similar to enzyme profile type V, L. tropica, except that the 6PGDH band is more anodal, and the GOT band is identical with the band produced by another L. tropica isolate, WR 309. The profiles of WR 151 and type II, L. hertigi, are identical. The data in both studies sustain the suggestion that Leishmania species like other species have a high level of isozyme similarity, and that the species' isolates can be grouped according to their enzyme profile similarities.

Certain enzyme systems can be used to rapidly but tentatively group the isolates characterized in this study. All braziliensis profile group isolates produced a single identically migrating band for GPI which was characteristically different than the ones produced by the mexicana and donovani groups, themselves different, Fig. 25. ASAT can be used to place any of these 44 isolates in a particular major

group because each group has a distinctly migrating band of ASAT activity, Fig. 11. The groupings and identifications noted above do not, nor should any isolate identification, rely on one or two systems but rather are based on multiple system identity. The greater the number of systems included in the identification the greater the likelihood of a correct one, conversely the fewer the number of systems used the greater the likelihood of an incorrect identification.

The CAE data from identified isolates in this study suggest that the isolates which have similar epidemiologies and geographic distributions and have been identified using these criteria usually have high levels of genetic similarity as evidenced by electromorph identity. Species of other organisms have high levels of electromorph similarity (Avise, 1974; Ayala, 1975; Ayala et all, 1974; Myers, 1978). The groupings and identifications of the 44 isolates by enzyme profile similarity from the CAE data are simply grouping together genetically similar isolates and grouping apart genetically different isolates. The data have not been used to propose a new classification of the genus, but in fact are for the most part consistent with already established groupings of the genus Leishmania.

Continued studies on Leishmania will enable the establishment of enzyme profiles based on data from these and additional enzyme systems and on data from large numbers of isolates which will include more population enzyme polymorphism. Once these profiles are available, it will enhance the use of CAE for rapid and extensive biochemical (genetic) characterization of Leishmania isolates.

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