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CHARACTERIZATION OF *LEISHMANIA* SPP. BY ISOZYME ELECTROPHORESIS*

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Abstract. In this study, isozyme patterns for 14 different enzymes were compared for culture strains of *Leishmania braziliensis, L. hertigi, L. mexicana, L. donovani, L. tropica,* and *L. adleri*. The isozyme separation was made by means of cellulose acetate electrophoresis. Each of the species had distinct isozyme patterns for aspartate aminotransferase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and fructokinase. For other enzymes, two or more species had identically migrating bands; however, by using combinations of the other 10 enzymes it was possible to separate any one of the six species. In addition to these interspecific differences the Panama strains of *L. braziliensis* had two different malic dehydrogenase isozyme patterns; therefore, they fell into two distinct groups. These strains otherwise had identical isozyme patterns.

The World Health Organization (WHO) Scientific Working Group on Leishmaniasis, which convened in Geneva in December 1977(TDR/LEISH-SWG(1)/77.3), assigned a high priority to developing techniques such as enzyme electrophoresis for the identification of *Leishmania* strains. Other WHO publications (TDR/WP/76.14 and 76.5), point out that it is necessary to develop simple diagnostic tests which are suitable for use under field conditions and which strengthen the biomedical research capabilities in developing countries.

Chance et al.[1] have discussed the significant contributions that electrophoresis studies have made in clarifying the taxonomy of *Leishmania*. The papers by Ebert,[2,3] Kilgour et al.,[4] Gardener et al.,[5] Lumsden,[6] Al-Taqi and Evans,[7] and Chance et al.,[8] reported biochemical variation at both the inter- and intraspecific levels. In these papers the authors reported isozyme data on a total of 10 enzyme systems. They also noted the need for additional, more comprehensive biochemical studies of these intrinsic taxonomic characters which could be used in conjuction with the already available extrinsic ones.

In previous studies starch gel and polyacrylamide electrophoresis were used for enzyme separation. To perform cellulose acetate electrophoresis no special training is required, it can be run under field conditions and original zymograms can be sent through the mail.[9] Therefore, a study was undertaken to adapt the cellulose acetate electrophoresis procedure to *Leishmania*.

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MATERIALS AND METHODS

Leishmanial strains

Thirteen strains of *Leishmania braziliensis* and one strain each of *L. hertigi* and *L. mexicana* were isolated at Gorgas Memorial Laboratory, Panama, Rep. of Panama. *L. donovani, L. tropica,* and *L. adleri* strains were obtained from the WHO International Reference Center for Leishmaniasis, Jerusalem, Israel. The source of each isolate is listed in Table 1.

Cultivation

All strains were grown on modified Senekjie's diphasic medium for hemoflagellates at 22–24°C and subcultured every 8–10 days.[10] Each stock culture was maintained in a minimum of four Senekjie slants with an overlay of 1 ml of normal saline (unbuffered 0.85% (w/v) NaCl in distilled water) containing 500 units of potassium penicillin and 1 mg of streptomycin/ml. Two different preparations of each strain were tested.

Preparation of extracts for electrophoresis

To produce sufficient material for testing, promastigotes of each strain were inoculated onto 20 ml of culture medium in 300-ml screw-capped

TABLE 1

Origin of Leishmania *strains and isozyme types*

Leishmania isolates		GML Designation	Host	Place of isolation	Isozyme types				
					ACP*	ALAT	ALD	ALP	ASAT†
IA	*L. braziliensis*	LS 333	Human	Panama	I‡	I	I II V	I	I
IA	*L. braziliensis*	LC 8	Human	Panama					
IA	*L. braziliensis*	LC 27	Human	Panama					
IA	*L. braziliensis*	M 52	*Saguinus geoffroyi*	Panama					
IA	*L. braziliensis*	3026(5)	*Choloepus hoffmanni*	Panama					
IA	*L. braziliensis*	3203	*Choloepus hoffmanni*	Panama					
IB	*L. braziliensis*	2229	*Choloepus hoffmanni*	Panama	I	I	I II V	I	I
IB	*L. braziliensis*	012	*Bassarycion gabbi*	Panama					
IB	*L. braziliensis*	LC 90	Human	Panama					
IB	*L. braziliensis*	LC 69	Human	Panama					
IB	*L. braziliensis*	LC 71	Human	Panama					
IB	*L. braziliensis*	LC 2	Human	Panama					
IB	*L. braziliensis*	LC 43	Human	Panama					
II	*L. hertigi*	C 120	*Coendou rothschildi*	Panama	II	II IV	II I V	–	II
III	*L. mexicana* (LV41)§	1746	*Oryzomys capito*	Panama	III	III	III	–	III
IV	*L. donovani* ‖ (LV136)§ Brazil8**	F 54	Human	Brazil	IV V	IV II	IV	–	IV
V	*L. tropica* ‖ BER8**	F 57	Sandfly	Israel	V IV	V	V I II	–	V
VI	*L. adleri* ‖ (LV34)§ 146**	F 53	*Latastia longicauda*	Kenya	VI	VI	VI	–	VI

* See Table 2 for enzymes.

† ASAT and GOT represent two different substrate/buffer systems to identify enzyme 2.6.1.1

‡ Roman numerals refer to isozyme types of the species shown diagrammatically in Figures 1–15. More than one number for an enzyme indicates which of the two or more species have identical enzyme bands.

§ Designation of Liverpool School of Tropical Medicine, Liverpool, England.

‖ Strains received from WHO International Reference Center for Leishmaniasis, Jerusalem, Israel.

** Designation of WHO International Reference Center of Leishmaniasis.

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 centrifuged for 30 min at 1,000 × *g* (22–24°C). The clear supernatant was decanted and the process was repeated through two additional washings. Half a milliliter of membrane buffer (14 parts distilled H_2O: 1 part 0.1 M Tris/Maleic Acid/EDTA/$MgCl_2$, pH adjusted to 7.4 with 40% NaOH) was added to the pelletized promastigotes of each strain, and the material was quick-frozen in Dry Ice and alcohol and thawed rapidly in a 37°C waterbath. This process was repeated three times and the material was examined microscopically to confirm the disruption of promastigote membranes. After a final quick-freezing, the material was stored in a Revco freezer at −70°C until being tested electrophoretically.

Enzyme activities

The enzymes studied are listed in Table 2. The alanine amino-transferase (ALAT), aspartate amino-transferase (ASAT), and malic dehydrogenase (MDH) electrophoresis procedures were similar to those reported by Kilgour and Godfrey,[11] Kilgour et al.,[4,12] Godfrey and Kilgour,[13] Miles et al.,[14] and Al-Taqi and Evans.[7] Isozymes of these three enzymes were observed under ul-

TABLE 1
Continued

	Leishmania isolates	Isozyme types									
		G6PDH	GOT†	HK	LDH	ME	6PGDH	PGI	MDH	PGM	FK
IA	*L. braziliensis*	I	I	I	I	I III	I	I V	IA	I III	I
IA	*L. braziliensis*										
IA	*L. braziliensis*										
IA	*L. braziliensis*										
IA	*L. braziliensis*										
IA	*L. braziliensis*										
IB	*L. braziliensis*	I	I	I	I	I III	I	I V	IB	I III	I
IB	*L. braziliensis*										
IB	*L. braziliensis*										
IB	*L. braziliensis*										
IB	*L. braziliensis*										
IB	*L. braziliensis*										
IB	*L. braziliensis*										
II	*L. hertigi*	II	II V	II	II III	II	II	II	II III	II V	II
III	*L. mexicana* (LV41)§	III	III	III VI	III II	III I	III	III	III II	III I	III
IV	*L. donovani* ‖ (LV136)§ Brazil8**	IV	IV VI	IV	IV	IV	IV	IV	IV	IV	IV
V	*L. tropica* ‖ BER8**	V	V II	V	V	V	V	V I	V	V II	V
VI	*L. adleri* ‖ (LV34)§ 146**	VI	VI IV	VI III	VI	VI	VI	VI	VI	VI	VI

TABLE 2
Enzymes tested in this study

Enzyme	Enzyme commission number[9]	Enzyme abbreviation
Acid phosphatase	3.1.3.2	ACP
Alanine amino-transferase	2.6.1.2	ALAT
Aldolase	4.1.2.13	ALD
Alkaline phosphatase	3.1.3.1	ALP
Aspartate amino-transferase*	2.6.1.1	ASAT
Glucose 6-phosphate dehydrogenase	1.1.1.49	G6PDH
Glutamate oxaloacetate transaminase*	2.6.1.1	GOT
Hexokinase	2.7.1.1	HK
Lactic dehydrogenase	1.1.1.2	LDH
Malic enzyme	1.1.1.40	ME
6-Phosphogluconic dehydrogenase	1.1.1.44	6PGDH
Phosphoglucoisomerase	5.3.1.9	PGI
Malic dehydrogenase	1.1.1.37	MDH
Phosphoglucomutase	2.7.5.1	PGM
Fructokinase	2.7.1.11	FK

* Two different substrate/buffer systems to identify the same enzyme.

TABLE 3

*Conditions for electrophoresis and components for developing zymograms**

Enzyme	Cell buffer	Voltage	Run time (minutes)	Reaction buffer	Developing components†
ACP‡	1	160	10	A	75 mg Naphthol as phosphoric acid; 50 mg Fast Garnet GBC
ALAT‡	2	150	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-Nicotinamide Adenine Dinucleotide, Reduced form (NADH), Na_2 salt.
ALD‡	2	160	15	C	300 mg Fructose-1,6-diphosphate (Na_4 salt); 15 mg MTT tetrazolium (MTT); 10 mg Phenazine methosulphate (PMS); 300 units Glyceraldehyde-3-phosphate dehydrogenase (Rabbit muscle $(NH_4)_2SO_4$ solution, pH 7.5); 15 mg β-Nicotinamide Adenine Dinucleotide (DPN).
ALP‡	1	180	10	D	100 mg α-Naphthyl acid phosphate (Na salt); 75 mg Fast blue RR; 2 mg $MgCl_2$; 4 mg $MnCl_2$.
ASAT‡§	1	120	20	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 (Na_2 salt).
G6PDH‡	2	150	15	C	75 mg D-Glucose-6-phosphate; 15 mg MTT; 15 mg β-Nicotinamide Adenine Dinucleotide Phosphate (TPN), Na_2 salt, 10 mg PMS.
GOT§	3	180	15	C	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.
HK	3	130	10	C	50 mg α-D(+)-Glucose; 20 units Glucose-6-phosphate dehydrogenase (Bakers yeast); 20 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 40 mg ATP (Na^2 salt from equine muscle).
LDH	2	160	15	E	2.5 ml DL-Lactic acid (Na salt); 15 mg β-DPN; 15 mg MTT; 10 mg PMS.
ME	3	180	15	F	270 mg DL-Malic acid (readjust pH to 7.5). Then add 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 mg $MnCl_2$
6PGDH	2	180	15	C	15 mg 6-Phosphogluconic acid (Na_3 salt); 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 60 mg EDTA (Na_2 salt).
PGI	3	180	15	C	20 mg Fructose-6-phosphate; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 units Glucose 6-phosphate dehydrogenase (Bakers yeast); 60 1 mg EDTA (Na_2 salt).

* *Cell buffers*:

1. 0.15 M Citric acid (Na_3)/0.24 M NaH_2PO_4, adjust to pH 6.3 with 40% NaOH.
2. Add 0.2 M NaH_2PO_4 to 0.2 M Na_2HPO_4 to reach pH 7.0.
3. 0.1 M Tris/0.1 M Maleic acid/0.1 M EDTA (Na_2)/0.1 M $MgCl_2$, adjust to pH 7.4 with 40% NaOH.
4. 0.25 M Tris/0.05 M Citric acid monohydrate; adjust to pH 7.0 with Tris.

Membrane buffers:

ALAT, ALD, MDH—1 part cell buffer and 9 parts distilled water.
All other enzymes—1 part cell buffer and 24 parts distilled water.

Reaction buffers:

A. 1 part 0.1 M Citric acid (anhydrous) and 4 parts 0.1 M Sodium Citrate.
B. 0.025 M NaH_2PO_4/0.08 M Na_2HPO_4.
C. 0.05 M Tris, adjust to pH 8.0 with 50% HCl.
D. 0.076 M Tris/0.005 M Citric Acid (monohydrate), adjust to pH 7.0 with Citric Acid.
E. 0.1 M Tris; adjust to pH 8.0 with 50% HCl.
F. 0.06 M Tris, adjust to pH 7.5 with 50% HCl.

† To make 60 ml of stain (about 50 samples). All chemicals Sigma.
‡ These systems require cooling during electrophoresis.
§ Two different substrate/buffer systems to identify the same enzyme.

TABLE 3
Continued

Enzyme	Cell buffer	Voltage	Run time (minutes)	Reaction buffer	Developing components†
MDH	4	200	15	B	15 mg oxaloacetic acid; 15 mg β-NADH (Na_2 salt).
PGM	3	200	15	C	200 mg α-D-Glucose-1-phosphate; 1.2 mg α-D-Glucose-1,6-diphosphate; 120 mg $MgCl_2$; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 units Glucose 6-phosphate dehydrogenase (Bakers yeast); 60 mg EDTA (Na_2 salt).
FK	2	150	15	C	100 mg β-D-(−)-Fructose; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 40 mg ATP; 20 mg $MgCl_2$; 50 units Phosphoglucose Isomerase.

traviolet (UV) light. Many of the other enzyme procedures were similar to those described by Shaw and Prasad,[15] and by Ayala.[16] The phosphoglucomutase (PGM) cellulose acetate procedure was described by Kreutzer et al.[9] The electrophoresis procedures for the enzymes described by these authors were modified for *Leishmania* and for cellulose acetate electrophoresis. The conditions for electrophoresis and the components of the stains are recorded in Table 3.

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

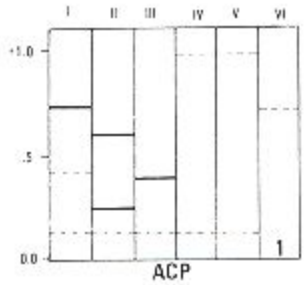
Electrophoresis

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; however, under certain conditions more than one enzyme can be assayed on a single plate. 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. Most of the enzymes in this study required a triple application of sample. 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 all 15 enzymes were made from each *Leishmania* isolate. The membranes were placed in the Zip Zone chamber. Up to three membranes (or 24 samples) can be 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 (most enzyme systems do not require cooling), 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 (10–15 min for most of the enzymes in this study). 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 100 × 15-mm petri dishes, and these were stored at 3°C until needed.

RESULTS

The results of multiple runs of separately isolated extracts of six species of *Leishmania* are shown diagrammatically in Figures 1–15. In these figures the Roman numerals at the top refer to the species. There are two MDH isozyme types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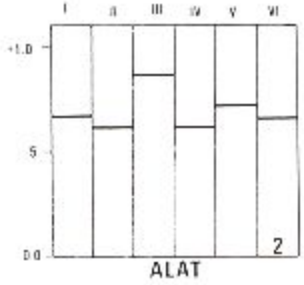
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.5
0.0
2
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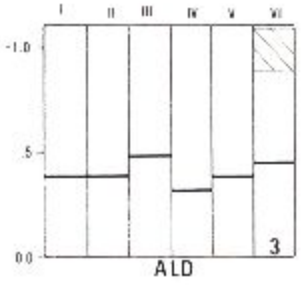
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0.0
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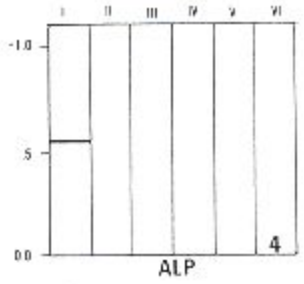
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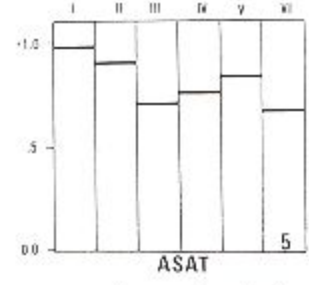
ALP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5
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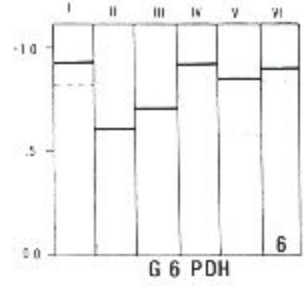
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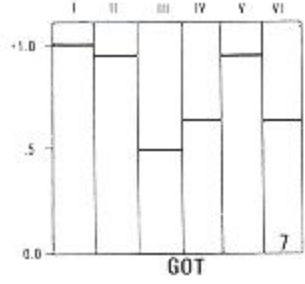
G 6 PDH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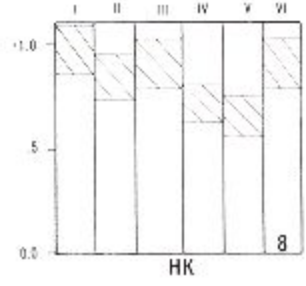
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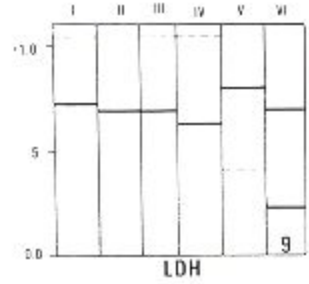
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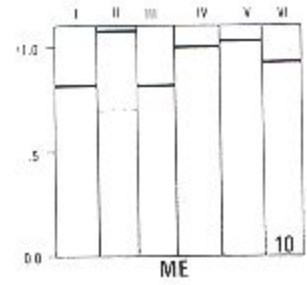
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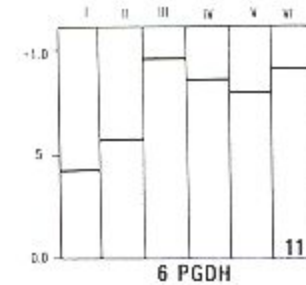
0.0
10
ME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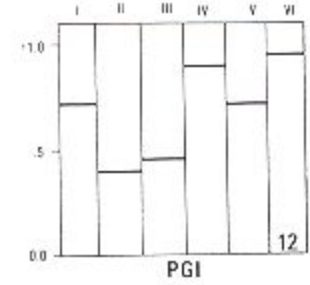
6 PGDH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0.0
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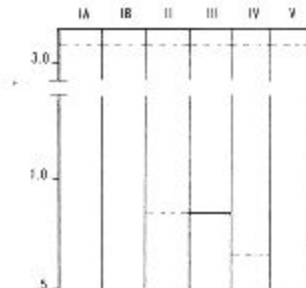
PGI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1.0
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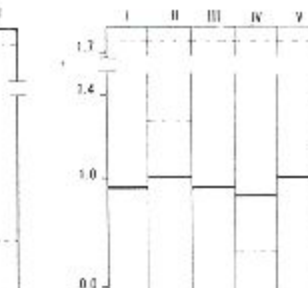
0.0
13
MDH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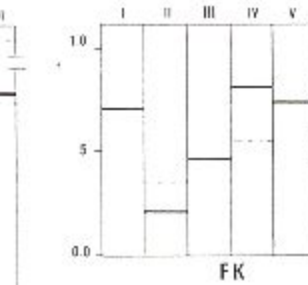
0.0
-1.0
14
PGM


I II III IV V VI
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0.0
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FK

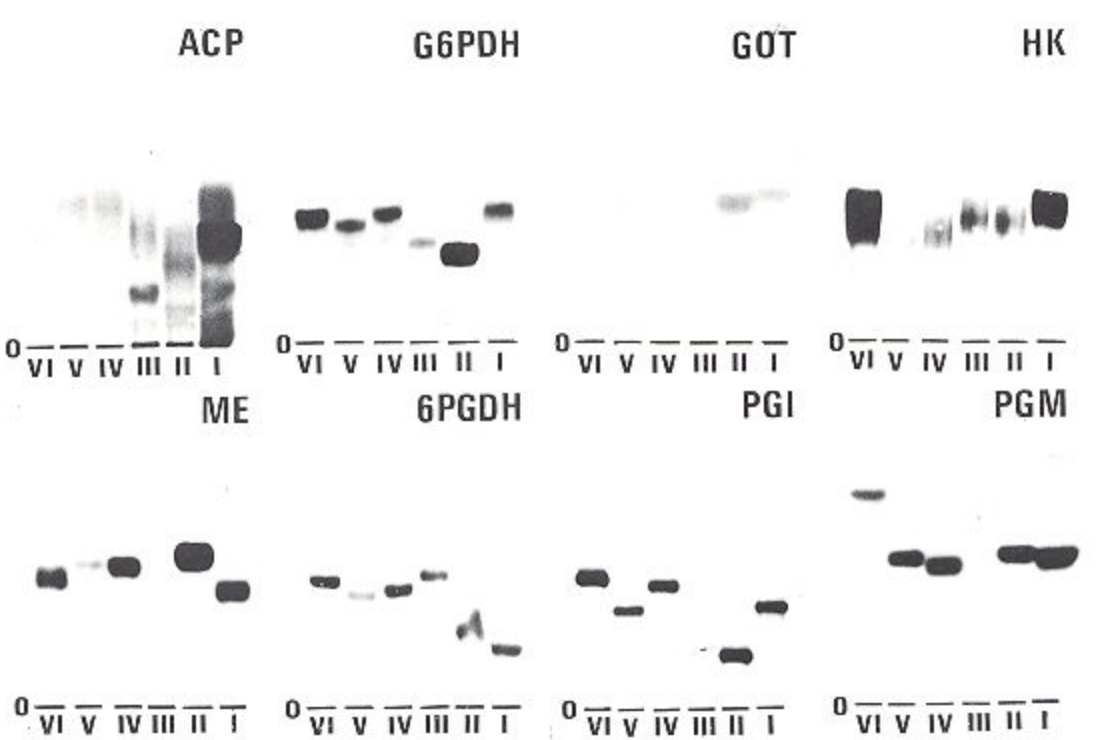

FIGURE 16. Photograph of zymograms of certain enzymes. See Table 2 for the complete names of the enzymes. I—*L. braziliensis*; II—*L. hertigi*; III—*L. mexicana*; IV—*L. donovani*; V—*L. tropica*; VI—*L. adleri*. O = origin. Anode is at the top of each zymogram.

among the *L. braziliensis* strains. They have been designated IA and IB. The isozyme types have been combined and tabulated in Table 1. More than one number indicates which of the species have an identically migrating isozyme for a specific enzyme. A photograph of eight zymograms is presented in Figure 16.

The acid phosphatase (ACP) isozyme patterns were identical in *L. donovani* and *L. tropica*, but different in the other species. The bands were not distinct and there was a certain amount of "bleeding" or running-together of bands; therefore, ACP was of limited value in separating the species studied (Figs. 1 and 16, Table 1). All *L. braziliensis* strains had identical bands.

The ALAT isozymes, which must be observed with UV, were identical in *L. hertigi* and *L. donovani*. The *L. braziliensis* strains had an identically migrating band. These bands were distinct and only one band was observed in each species (Fig. 2, Table 1).

Three of the species, *L. braziliensis* (all strains), *L. hertigi*, and *L. tropica*, had an identical single band of aldolase (ALD) activity. Two of the three other species had a single differently migrating band, and *L. adleri* had one thin distinct band and a second diffuse, more anodal, band of activity. The bands produced by this enzyme stained very lightly (Fig. 3, Table 1).

All strains of *L. braziliensis* had an identical

←

FIGURES 1–15. Diagrammatic representation of the electrophoretic patterns of 14 enzymes obtained from six species of *Leishmania*. See Table 2 for the complete names of the enzymes. Numbers at left represent relative mobilities of the isozymes. 1.0 is one inch from the origin (0.0) on the cellulose acetate plate. Anode is at the top of each diagram. IA and IB—*L. braziliensis*; II—*L. hertigi*; III—*L. mexicana*; IV—*L. donovani*; V—*L. tropica*; VI—*L. adleri*. Broken lines represent lightly staining bands.

alkaline phosphatase (ALP) band. In the other species no activity was observed in the cellulose acetate procedure here described (Fig. 4, Table 1).

ASAT was one of the four enzymes with differently migrating isozymes in all six species, but all strains of *L. braziliensis* had an identical band. These bands must be observed with UV. The bands produced by this enzyme can be used to separate the strains of the *Leishmania* species used in this study (Fig. 5, Table 1).

Another enzyme which had differently migrating isozymes in the strains of each species in this study was glucose 6-phosphate dehydrogenase (G6PDH); however, the *L. braziliensis* strains all produced the same band migration. Certain of the species had "epigenetic"[17] or "sub-bands"[18] (Figs. 6 and 16, Table 1). However, each species had one major G6PDH band (a band which indicates a high level of enzyme activity).

Each species produced a single band of glutamate oxaloacetate transaminase (GOT) activity. GOT and ASAT are different systems for identifying isozymes of enzyme 2.6.1.1 (Table 2). Although most of the relative migrations for the isozymes produced by the different species were the same in both systems, the *L. hertigi* and *L. tropica* bands had an identical migration in the GOT system. It appears then that the ASAT buffer/stain system is the preferred one for the study of isozymes of this enzyme in *Leishmania*. Each strain of *L. braziliensis* produced an identical GOT band (Figs. 7 and 16, Table 1).

The bands produced by hexokinase (HK) were thick and showed "bleeding." *L. mexicana* and *L. adleri* had a similarly migrating band, but the intensity of the staining was always greater in *L. adleri* (Figs. 8 and 16, Table 1). The bands in the *L. braziliensis* strains were identical.

The lactic dehydrogenase (LDH) bands stained lightly in each species. In *L. hertigi*, *L. mexicana*, and *L. adleri* there was an identically migrating dark band, but in *L. mexicana* there was also another, more anodal, "epigenetic" band, and in *L. adleri* there was a second, more cathodal, dark staining band (Fig. 9, Table 1). All *L. braziliensis* strains produced an identically migrating band.

The malic enzyme (ME) pattern was identical in *L. braziliensis*, all strains, and *L. mexicana*. The other species each had a differently migrating band (Figs. 10 and 16, Table 1).

The third enzyme with differently migrating bands in all six species, but identical migration among all strains of *L. braziliensis*, was 6-phosphogluconic dehydrogenase (6PGDH). This enzyme can be used to separate the strains of the six species (Figs. 11 and 16, Table 1).

The phosphoglucoisomerase (PGI) band migrations of *L. braziliensis*, all strains, and *L. tropica* were identical. The other four species produced differently migrating bands (Figs. 12 and 16, Table 1).

Each species produced at least two bands of MDH activity, one of which was a rapidly migrating, lightly staining, "epigenetic" band which moved more than three times faster than any other band. This band had an identical migration in each *Leishmania* isolate. There were two distinctly migrating major isozymes bands recovered from the *L. braziliensis* strains. One type, IA, had a more rapid anodal movement than the other, IB (Fig. 13). No single strain had both major isozyme bands; however, zymograms produced from mixed isolates, LS 333 and LC 90, had, in addition to light staining bands, two major MDH bands. Each major MDH band in each *L. braziliensis* strain had a secondary light staining lead band. The major bands in *L. braziliensis*, IA, and *L. adleri* were identically migrating; however, the major isozyme bands in the other species were distinctly migrating (Fig. 13, Table 1). The MDH bands were observed with UV.

The PGM bands had identical migrations in *L. braziliensis*, all strains, and *L. mexicana*, also *L. hertigi* and *L. tropica* produced an identically migrating band. As in MDH each species produced a rapidly migrating anodal, "epigenetic" band (Figs. 14 and 15, Table 1).

The fructokinase (FK) bands were distinctly migrating in all six species. Both *L. mexicana* and *L. donovani* had two bands of activity, one lighter staining than the other (Fig. 15, Table 1). The identically migrating FK bands from all strains of *L. braziliensis* were considerably lighter than the bands produced by the other species.

DISCUSSION

The relative migrations of major MDH bands separated by disc polyacrylamide electrophoresis[5] for *L. mexicana* strain 1746, LV41, *L. donovani* strain F54, LV 136, Brazil 8, and *L. adleri* strain F54, LV 34, 146 were the same as those reported in this study. That is, the *L. donovani* band was most anodal, the *L. adleri* isozyme migrates slightly more slowly, and the *L. mexicana* band

had the slowest anodal migration. Each of these species' strains had a second, more lightly staining, MDH band. In *L. mexicana* this band had a faster anodal migration than the light band in *L. adleri*, and the light band in *L. donovani* had the slowest migration. All light bands had a more rapid anodal migration than any major MDH band (Fig. 13). Al-Taqi and Evans,[7] who used thin-layer starch-gel electrophoresis, reported four MDH, six PGI, and four G6PDH isozymes in *L. tropica* major (LV 39), but Chance et al.,[8] using disc polyacrylamide electrophoresis, recovered a single isozyme for each of these enzymes from this strain. These observations point out the necessity of confirming relative isozyme migrations produced by all electrophoresis systems. All the enzyme systems reported here have produced identical migrations on cellulose acetate in two runs from two separate preparations of each species' strain.

In many of the systems there were, in addition to the prominent major bands, other secondary lightly staining bands. These were especially evident in G6PDH, LDH, MDH, and PGM (Figs. 6, 9, 13, 14, and 16). These "epigenetic" bands[17] have been observed in many different types of organisms. One possible explanation as to their origin in mosquitoes was proposed by Bullini and Coluzzi.[19] These authors proposed that the light staining band might be derived from the primary, more intensely staining band by secondary modifications of the enzyme. It is also possible that certain of these bands are allozymes in a diallelic strain.

All of the strains of the six leishmanial species used in this study can be separated by their ASAT G6PDH, 6PGDH, or FK isozymes. Each species had a distinctly migrating band of activity for each of these enzymes (Figs. 5, 6, 11, and 15). In addition, most of the species' strains can be separated by their distinct isozyme patterns for almost any one of the other enzymes. If electrophoresis is to be used as a taxonomic tool for species identification in *Leishmania*, multiple enzymes should be used. Many of these biochemical variants were not, as emphasized in Table 1, species exclusive; however, with up to 15 different enzyme systems, more comprehensive characterization of both intra- and interspecific lines should be facilitated by cellulose acetate electrophoresis. It is clear, however, that the species' strains of this study can be taxonomically separated by certain of their biochemical characters.

Although the isozyme patterns in most of the *L. braziliensis* strains were identical, there were two distinct MDH isozyme types. In one group, 1A, the band of activity had a fast anodal migration while in the other group, 1B, the band had a slower anodal migration (Fig. 13 and Table 1). These Panama strains then fell into two biochemically separable groups depending on which MDH allozyme they had. Intraspecific variation has been reported in most of the *Leishmania* isozyme studies cited in this paper, and the intraspecific MDH variability reported here showed the existence of isozyme polymorphism in the Panama isolates of *L. braziliensis*. Neither geographic location nor host-source of the two enzymatically distinct groups of *L. braziliensis*, delineated by MDH (Fig. 13, Table 1), appear to influence their phenotypic expression, since both groups were comprised of isolates from man and natural reservoirs from identical localities.

The six *Leishmania* species, each one compared with any one of the other five, exhibited extensive isozyme variability; however, the strains of the Panama *L. braziliensis* showed variability only in MDH isozymes. Similar high levels of interspecific isozyme variability and comparatively low levels of intraspecific variability were reported in *Leishmania* from the Aethiopian zoogeographical region of Africa[8] and from Kuwait,[7] as well as from studies on *Drosophila*[16] and mosquitoes.[19] Additional studies on *L. braziliensis* strains from other sites within the species range are contemplated. It is possible that more intraspecific isozyme variation will be discovered, and that specific isozymes might turn out to be associated with a specific host, geographical location, or other parameter.

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