A COMPARISON OF AXENIC CULTURES OF *GIARDIA* TROPHOZOITES FROM DIFFERENT MAMMALIAN HOSTS

by

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ABBREVIATIONS

αGP	α-glycerophosphate dehydrogenase
C/P	cat-1/Portland
E_A	energy of activation
ELISA	enzyme-linked immunosorbent assay
G6P	glucose-6-phosphate dehydrogenase
G/P	guinea pig-1/Portland
H/B	human-1/Bethesda
H/E	human-1/England
H/P	human-1/Portland
ID	immunodiffusion (Ouchterlony)
IEP	immunoelectrophoresis
isozyme	isoenzyme
Km	Michaelis-Menton constant
mb-	median bodies absent
mb+	median bodies present
mb+,-	equal numbers of organisms with and without median bodies
MDH	malate dehydrogenase (NAD+)
ME	malate dehydrogenase (decarboxylating)
MW	molecular weight
6PG	6-phosphogluconate dehydrogenase
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point

SDS	sodium dodecyl sulfate
SGE	starch gel electrophoresis
Q_{10}	rate of increase of enzyme activity for each 10°C rise in temperature

I. Introduction and Statement of the Problem

Giardia are parasitic protozoa commonly found as intestinal inhabitants of a variety of animals. A clear understanding of the interrelationships and taxonomic status among *Giardia* spp. has not been attained. New *Giardia* isolates have been traditionally assigned to separate species categories based on the animal host. These species designations are made on the assumption of animal host specificity and in spite of the fact that many *Giardia* from different animals are morphologically indistinguishable.

The possibility that humans may be infected by *Giardia* from other animals has recently been raised by epidemiologic and laboratory data. Therefore, it has become more important to develop unequivocal phenotypic markers for the further study and characterization of *Giardia* isolates.

The objective of this study is to evaluate the efficacy of several methodologies used in the characterization of *Giardia* by their application to axenic cultures of *Giardia* from different hosts.

The specific aims of the research are:

i. Evaluate morphometrics as a criterion of *Giardia* speciation.

ii. Identify electrophoretic isozymes through which morphologically identical *Giardia* isolates may be distinguished.

iii. Characterize kinetic and physical properties of selected Giardia isozymes.

iv. Compare the total protein profile of Giardia isolates after SDS polyacrylamide gel electrophoresis and by Ouchterlony comparative double diffusion.

"Wherein I have sometimes also seen animacules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and the belly, which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but slow progress."

van Leeuwenhoek (19)

9 November 1681

II. Literature Review

Organisms of the genus Giardia are flagellated protozoan parasites of all classes of vertebrates and are widely distributed in nature. Dobell (18) has argued that Giardia was the motile organism which van Leeuwenhoek observed in his own feces in 1681 after experiencing an illness whose description and duration was consistent with giardiasis. The first description of the structure of Giardia was made by Lambl in 1859 (50) and the organism was named by Kunstler in 1882 (49).

Since the late 1880's, this genera has been the subject of continued disputes over nomenclature. These disputes are intermixed with concommitant taxonomic arguments which continue to this date. The details of the taxonomic dispute will be addressed later.

A. Life cycle and morphology of Giardia.

The life cycle of Giardia spp. includes a vegetative form called a trophozoite and a cyst, the form which serves to transmit the disease (Figure 1). The shape of the trophozoite (Figure 2) body resembles a longitudinally split pear. It is rounded anteriorly, pointed posteriorly, and convex dorsally. Trophozoites are bilaterally symmetrical, 9 to 21 m long, 5 to 15 m wide and 2 to 4 m thick.

The ventral surface of the trophozoite is modified to form a large adhesive disk (AD) by which the organism attaches

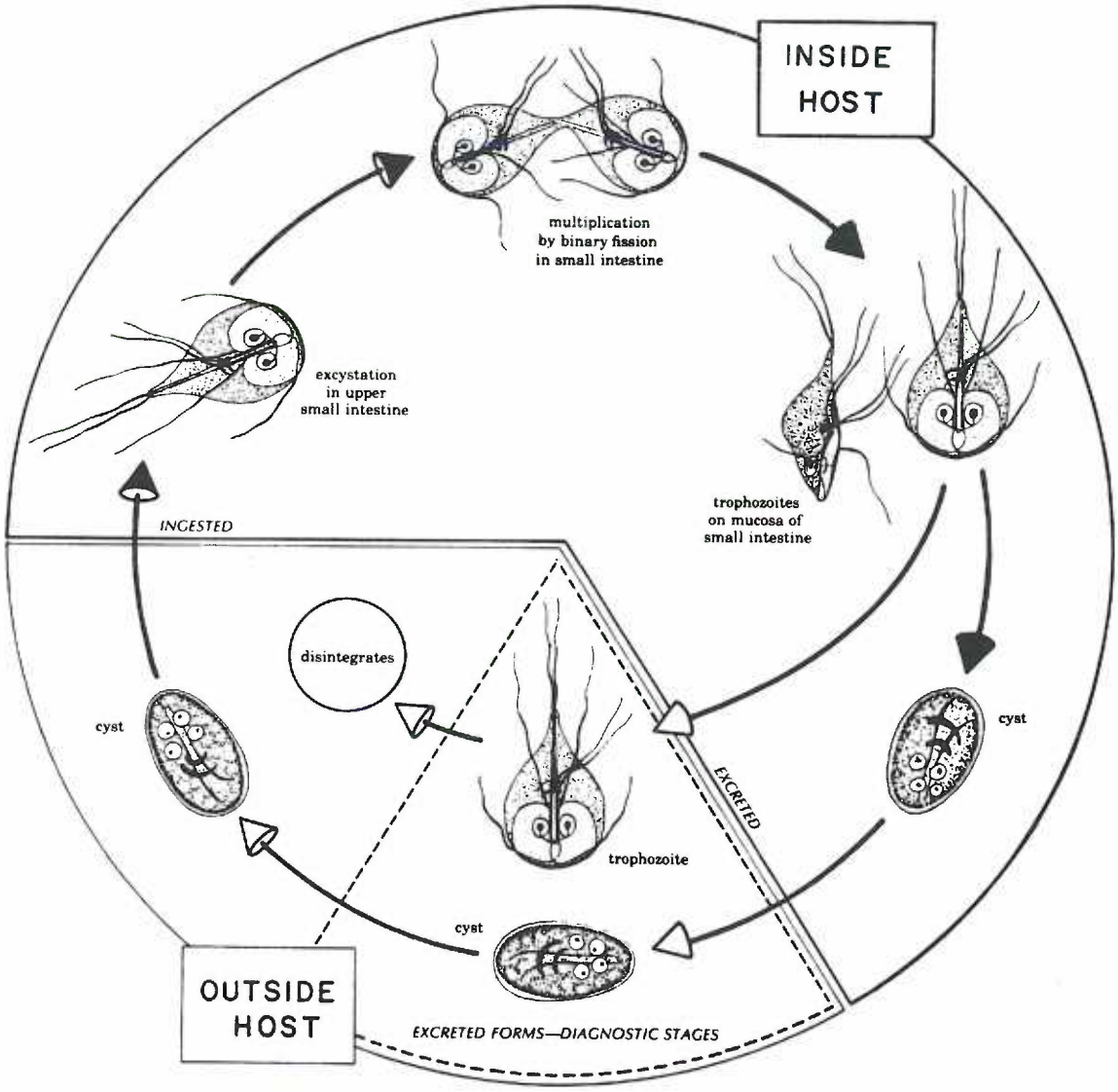
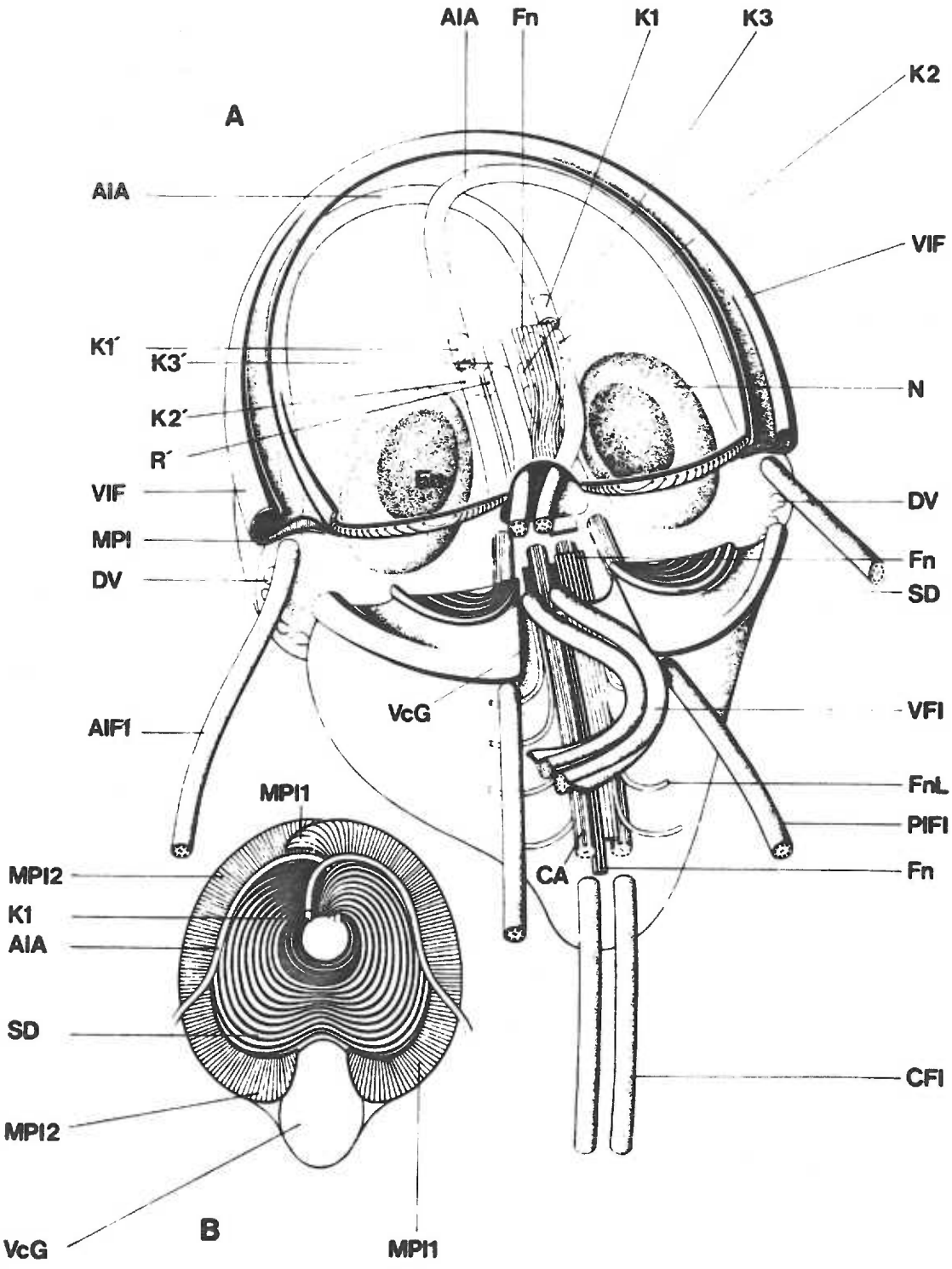

INSIDE HOST
multiplication by binary fission in small intestine
excystation in upper small intestine
trophozoites on mucosa of small intestine
INGESTED
disintegrates
cyst
cyst
EXCRETED
trophozoite
cyst
OUTSIDE HOST
EXCRETED FORMS—DIAGNOSTIC STAGES

Figure 2. Ultrastructure of Giardia. Schematic diagrams of a trophozoite of Giardia muris. (A) Ventral view. Kinetosomes of both mastigonts form a single group of parallel cylinders in the midline between the anterior ends of nuclei (N). The kinetosomes K1 and K1' are directed anteriorly; other kinetosomes are directed backward. The anterolateral flagella (A1F1) originate in kinetosomes K1 and K1'. Their axonemes (A1A) proceed anteriorly, cross, and turn backward, following dorsally the lateral margins of the adhesive disk. The ventral flagella (VF1) confined to the ventrocaudal groove (VcG) possess lateral extensions enlarging their surface. Their axonemes originate in kinetosomes K2 and K3'. Axonemes of the posterolateral flagella (P1F1) originate in kinetosomes K2' and K3 and run along the sides of the ventrocaudal groove (VcG). Caudal flagella (CF1) emerging at the posterior tip of the body originate in kinetosomes R and R'. Their axonemes (CA) run dorsally to the striated disk (SD) and ventrocaudal groove following the longitudinal axis of the body. Funis (Fn) forms two microtubular ribbons accompanying the caudal axonemes (CA), one in the ventral, and one in the dorsal position. Posteriorly, individual microtubules separate subsequently from each ribbon, radiate laterally (FnL), and few of them only reach together with the caudal axonemes to the posterior end. DV, digestive vacuoles; MP1, marginal plate; En, endosome; V1F, ventrolateral flange. (B) Dorsal view showing marginal plates (MP1) and striated disk (SD). From Kulda (48).


A
AIA
Fn
K1
K3
K2
AIA
VIF
K1´
K3´
K2´
R´
N
VIF
DV
MPI
Fn
DV
SD
VcG
AIFI
VFI
MPI1
FnL
PIFI
MPI2
CA
Fn
K1
AIA
SD
CFI
MPI2
VcG
B
MPI1

to the surface of the small intestine (in vivo) or a culture vessel (in vitro). Two oval nuclei are located dorsally to this AD. Eight flagella originate in the kinetosomal complex situated on the midline between the nuclei. There are one or two median bodies situated dorsally to the posterior end of the AD. The shape of the median bodies helps to define the main morphological types of the genus. The median bodies are composed of bundles of microtubules. These bodies, whose function is unknown, may disappear during cell division (24, 86). The dimensions (morphometrics) and morphology of the trophozoite have been the principle criteria used for taxonomic comparison.

Giardia cysts are oval bodies 8 to 12 µm by 10 to 20 µm. Two or four nuclei (mature form), axonemes, ribosomes, fragments of the microtubular skeleton of the AD and kinetosomes are found internally.

Trophozoites attach to the epithelial brush border of the duodenal and jejunal region of the small intestine where they replicate by binary fission. Attachment is mediated via the AD. Detachment and entry into the lumen may result in encystation in the posterior part of the small intestine. Detailed descriptions of this process have been documented by several authors (24, 87). A cyst wall is elaborated and protects the organism as it passes outside the host and into the environment; cyst maturation occurs with the single division of the nuclei. A new host acquires infection by ingesting the cysts,

which pass through the stomach and excyst shortly after entering the small intestine. A tetranucleated trophozoite in the process of division emerges from the cyst wall (10) and promptly completes the division process, yielding two binucleated trophozoites.

B. Taxonomic status of Giardia species.

1. Taxonomic problems with agamous organisms.

Taxonomy provides a system of classification which permits the assignment of fresh isolates of an organism to defined categories. From such an assignment, the biologist may be able to consider the ecological and epidemiological interactions of the isolate. Therefore, the goal of taxonomy is to provide a set of descriptive criteria for classifying organisms. For Giardia, experiments related to the epidemiology of giardiasis have been confused by the inability of investigators to identify unequivocally the Giardia isolates with which they work.

The problem of Giardia speciation is analogous to that of other agamous agents such as bacteria and viruses. The fertility-linked species concept employed in classifying higher organisms is not applicable to agamous genera; as Dobzhansky (20) stated, "all criteria of species distinction utterly break down in such forms." Stanier et al. (89), who examined this problem in bacteria, developed a general definition of species

which may be applied to all organisms, including protozoa: "A species consists of an assemblage of individuals that share a high degree of phenotypic similarity, coupled with an appreciable dissimilarity from other assemblages of the same general kind."

Therefore, with agamous organisms, it is important to define characteristics which members of a species share and which enable one to distinguish them from other similar isolates. The more distinguishing characteristics these organisms possess, the clearer the separation.

Viral characteristics employed in classification include the content and organization of the genome; for bacteria, it is a series of biochemical and immunologic reactions, gross morphology, and Gram stain reaction. Although protozoa are classified on the bases of a number of characteristics, they are grouped primarily by distinctive internal and external morphologic characteristics (14) (for examples of intestinal flagellates, see Kulda [48]).

However, there are a number of important human pathogenic protozoa for which few differential morphological characteristics exist. The African salivarian trypanosomes and Leishmania spp. are examples of genera in which non-morphologic characteristics are used to define morphologically indistinguishable species.

Differences in the clinical course of disease in humans are used to distinguish Trypanosoma brucei rhodesiense from T. b. gambiense. Resistance to human serum in vitro and the inability to infect humans distinguishes T. b. brucei from T. b. rhodesiense and T. b. gambiense. T. b. evansi and T. b. equiperdum are distinguished from each other, and from the other subspecies as well, by differences in developmental behavior and the venereal transmission of T. b. equiperdum (109).

The taxonomy of Leishmania species has been based on the clinical picture of human disease, epidemiological differences, and geographic considerations (51). Leishmania donovani is distinguished from L. tropica by the site and nature of the infection in the human host. L. tropica is also known under synonymous species names which are based on geographic distribution: L. peruana, L. mexicana, L. guyanensis, etc. The "wet" oriental sore differentiates L. tropica major from L. t. minor, which causes the classical "dry" oriental sore. Further subspeciation within the L. tropica complex is based on the classification of the sores.

2. Speciation of Giardia.

Giardia was considered for many years to be a harmless commensal. Subsequent association with symptoms of epigastric pain, diarrhea, flatulence, and steatorrhea clarified its status as a pathogen. However, until the early 1970's, normal

transmission was thought to be fecal-oral, either directly or via food, and Giardia spp. were thought to be host specific.

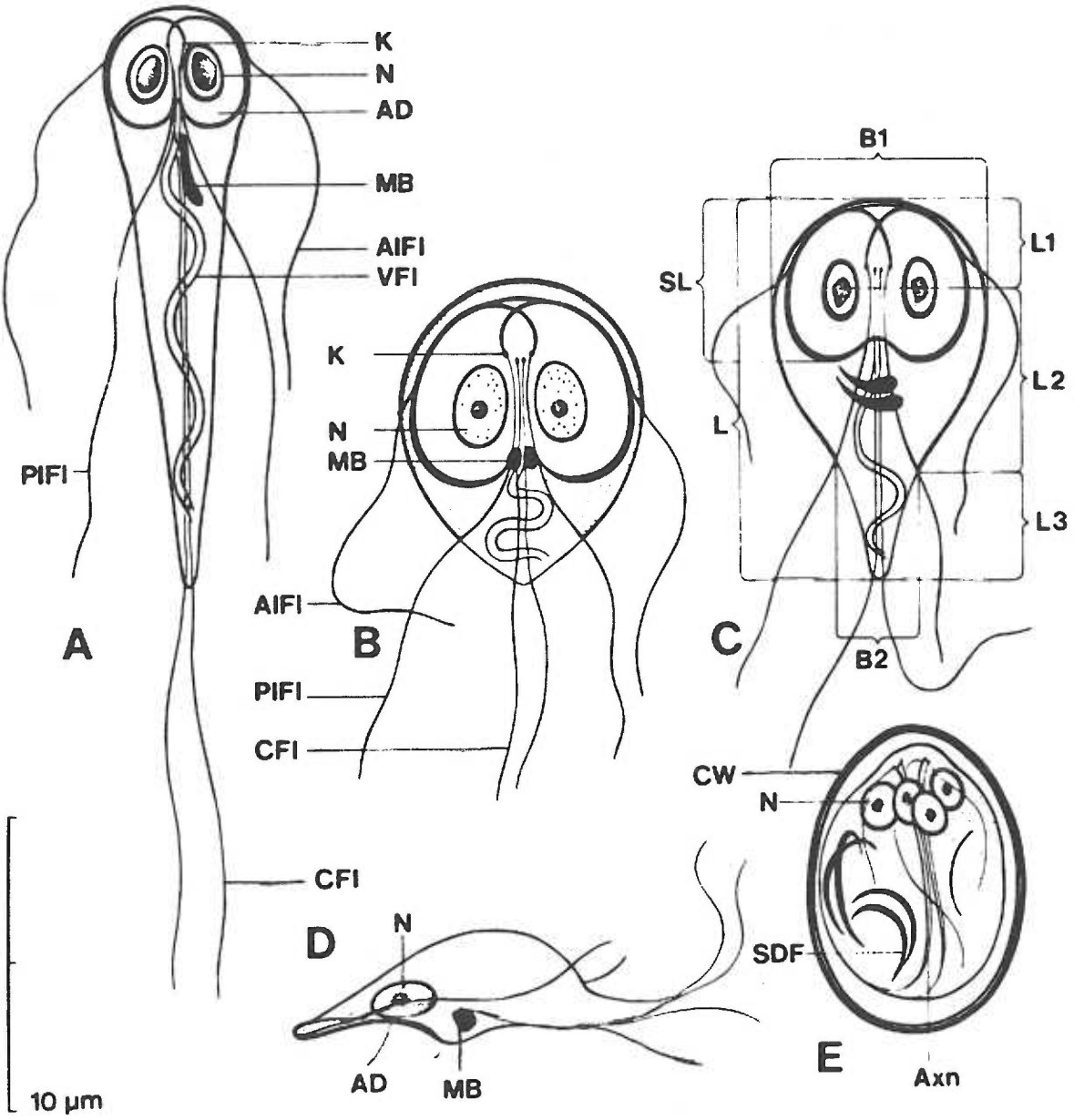
The incidence of epidemic outbreaks (16), associated with drinking water suggested that Giardia could be waterborne. (Although Rentdorf [68] had demonstrated in 1954 that waterborne cysts could cause infection, this was not thought to be an important epidemiological mode of transmission until the 1970's.)

Giardiasis among campers who drank from "pristine" streams in remote mountain regions further suggested that lower mammals could be a source of cysts for human infection (5). However, due to the morphologic similarity of mammalian Giardia trophozoites and cysts, direct confirmation of this latter hypothesis was not possible without laboratory experiments of feeding cysts to human subjects.

As Levine (51) has stated, "If it turns out that Giardia can be freely transmitted from one host to another, we shall have to revise our ideas about the danger to man of infections in the laboratory and domestic animals, and of infections of one domestic animal to others. Here is an area of ignorance that deserves exploration."

Giardia trophozoites may be speciated on the basis of three morphologic types. The types were originally described by Nieshulz (64) and later confirmed by Filice (24) (see Fig. 3 for diagramatic representation):

Figure 3. Semidiagrammatic representation of three morphological types of Giardia viewed in the light microscope. (A-C) Ventral view of tropozoites of Giardia agilis from amphibians (A), Giardia muris from mice (B), and Giardia duodenalis from man (C). (D and E) Giardia duodenalis, lateral view of a trophozoite (D) and cyst (E). Trophozoites of the Giardia agilis group (A) have a long and narrow body, a relatively short adhesive disk and long club-shaped median bodies (MB) situated parallel to the longitudinal axis of the cell. Trophozoites of the Giardia muris group (B) have a broad, short body with a large adhesive disk overlapping in length one-half of the body length. Median bodies (MB) are small and round situated parallel to the longitudinal axis of the cell. Trophozoites of the Giardia duodenalis group (C) have a pyriform body with an adhesive disk shorter than one-half of the body length. Fang-shaped median bodies are situated transversely across the cell. Cysts of Giardia (E) are enveloped by a thick wall (CW). The ripe cyst possesses four nuclei (N), a double set of flagellar axonemes (Axn), and crescent-shaped fragments of the striated disk (SDF). (C) Defines dimensions used in the differentiation of Giardia species: B1, maximum width; B2, breadth across site of emergence of posterolateral flagella; L, overall length; L1, distance between anterior end of cell and nuclei; L2, distance between nuclei and site of emergence of posterolateral flagella; L3, distance between site of emergence of posterolateral flagella and caudal tip of cell; SL, length of adhesive disk. AlFl, anterolateral flagella; PlFl, posterolateral flagella; VFl, ventral flagella; CFl, caudal flagella; K, complex of kinetosomes; N, nuclei; AD, adhesive disk. From Kulda (48).


K
N
AD
MB
AIFI
VFI
PIFI
A
K
N
MB
AIFI
PIFI
CFI
B
B1
L1
SL
L2
L
L3
B2
C
CW
N
SDF
E
CFI
N
D
AD
MB
Axn
10 µm

A) Giardia agilis, a parasite of tadpoles and frogs, has long, teardrop-shaped median bodies;

B) Giardia muris, a parasite of rodents, birds, and reptiles, has two small round median bodies; and

C) Giardia duodenalis, a parasite of warmblooded vertebrates, including humans and rodents, has median bodies which resemble the claw of a claw hammer.

Within the G. duodenalis complex, speciation has depended upon a variety of criteria employed by the individual investigators. Traditionally, it was believed that Giardia were host specific or confined to a limited host range. Thus, more than 40 species have been designated on the basis of host specificity. Furthermore, morphometics, the comparison of body dimensions, has been used as a means for confirming these designations.

a. Host specificity.

Historically, the division of Giardia isolates into groups began in 1908 with Bensen's (6) recognition of three species: G. muris (mouse), G. cuniculi (rabbit) and G. intestinalis (human) (the latter two are of the G. duodenalis morphologic type).

Between Lambl's description of Giardia in 1859 (50) and Bensen's work in 1908, investigators examined Giardia in many animals. However, until 1908, only one species of Giardia (G. lamblia) was recognized. Bensen introduced the host as a

criterion for speciation. This criterion subsequently became entangled with the concept of host specificity, an idea championed by Hegner, the most prolific Giardia researcher between 1922 and 1938 (34, 35, 36, 37).

Three categories of host specificity of parasites are recognized: monoxenous (single host species), stenoxenous (narrow host range), and euryxenous (broad host range). Levine (51) has concluded that there are "fewer (monoxenous organisms) than our present records indicate". A human malaria organism (P. malariae), once thought to be monoxenous, is now known to be capable of infecting apes and West African chimpanzees (51). A well-documented stenoxenous protozoan is Eimeria (a coccidian). Most trypanosomes, non-human plasmodia, and trichomonads are euryxenous protozoans (51).

Over the last 100 years, numerous reports have been published of experiments conducted to determine whether Giardia spp. are monoxenous, stenoxenous, or euryxenous. These investigators attempted to demonstrate cross-infection between animal host species by oral inoculation of one species with cysts obtained from another (see [37] for a summary of the earliest experiments). The issue of host specificity continues to be controversial; the results of most such experiments must be considered equivocal because the experiments were inadequately controlled.

Negative cross transmission experiments may result from host immunity, the immunity having been elicited by a prior infection (70), or the feeding of non-viable cysts (especially since it has been only recently possible to determine cyst viability *in vitro* [10]).

Positive infection may result from recrudescence of a pre-existing cryptic infection; such exacerbations in giardiasis are known to occur when female mice become pregnant (91). Natural acquisition may occur unexpectedly during the course of the experiment via contaminated food, bedding, or water; even insects may serve as carriers of cysts (73, 115). Despite these problems, there is evidence which suggests humans can be infected with cysts from other animals (17) and that cysts of the *G. duodenalis* type from one animal can infect a number of mammals (31).

It may well be possible that some *Giardia* spp. are monoxenous while others are stenoxenous or euryxenous. The resolution of this point must await adequately controlled studies of *Giardia* cross-transmission. It is clear, with the data presently available, that host specificity or source is at best an imprecise criterion on which to base *Giardia* speciation.

b. Morphometrics.

A review of the literature provides an equally unconvincing argument for the reliability of using *Giardia* tropho-

zoite body measurements alone as a species criterion. Those arguing for morphometric comparison maintain that there are small but constant differences in trophozoite body measurements which justify the creation of large numbers of species. Trophozoite measurements used by these proponents have included differences in overall length, maximum width, the length of the AD, as well as minor internal dimensions.

Trophozoite measurements are subject to technical problems which make reproducibility difficult. Many investigators have found, by analysis of variance, that the variation of minor internal dimensions (L1, L2, L3, B2, and SL in Figure 3) is greater within than between different Giardia "species" (24, 30). Trophozoites may be distorted by drying smears prior to fixation and staining. Grant and Woo (30) noted that differences in size, general morphology, density of cytoplasmic staining, and relative positions of organelles were produced by varying the prefixation drying time. The shape of the median body, an internal structure of unknown function, was the only characteristic that was relatively unaffected.

It is possible that uncontrolled environmental factors have influenced previous reports of trophozoite measurements. Trophozoites used for all published reports were obtained by duodenal aspiration or dissection since Giardia trophozoites are difficult to establish in axenic culture.

It has been demonstrated that the size of Giardia cysts is influenced by host environment. Tsuchiya (96, 98) found that the size of cysts appearing in the feces of a human subject could vary significantly from day to day. He also found that a size change of up to 15 percent could be induced in the cysts of a dog by varying the amounts of carbohydrate and protein in the animal's diet (97). These experiments have not been performed with trophozoites, and use of these data as an argument against morphometric species criteria has been criticized since what has been found with cysts may not be true with trophozoites.

However, similar experiments have been performed in vitro with another flagellated parasitic protozoan, Trichomonas tenax. One strain was measured after growth in two different media and in the presence of one or three contaminating bacteria (40). Significant differences in size were found between the strain in the two different media and in the presence of different numbers of species of bacteria. Organisms grown in a medium containing three different bacterial species were significantly smaller in length and width than the same strain in the same medium with only one bacterial species contaminant. The same strain, grown in two different media (yolk infusion vs. cysteine-peptone-liver-maltose), each with only one bacterial species contaminant, differed in length but not width. The authors concluded that "environmental factors may result in

significant size differences among strains of a single species and that hence such differences must be used with caution in specific speciation of closely related forms."

There are few examples within the literature of morphometrics as a sole criterion for speciation of protozoa. Organisms of the genus Leishmania, which are considered to be morphologically indistinguishable, are speciated on several criteria. Several recent investigations of size differences among Leishmania species (27, 80) classified on the bases of geography and clinical course of infection in humans have yielded significant differences in the organisms' length and width.

The nomenclature and taxonomy of the species of Entamoeba are described as "confused as it is possible to make them" (51). Entamoeba are placed into five groups on the bases of trophozoite and cyst structure. Within the groups, most of the species are essentially indistinguishable. However, in some of these groupings, organisms are further classified and thus speciated on the bases of size and/or pathogenicity.

In both of the above examples of Entamoeba and Leishmania, these questions of classification are being resolved by the application of biochemical taxonomy.

In spite of the problems encountered in its use, there remain numerous advocates of biometrics as a means for speciation of Giardia and as an index of host source and speci-

ficity. The differences in measurements which are used to define and differentiate species may be very small. Furthermore, the range of values obtained for one species by a single investigator is often substantial and may approach 30% (30, 87).

The available morphometric data have been examined in several formats. Tables 1-3 compare sizes of trophozoites of three Giardia "species" of the G. duodenalis type. It is apparent that a great variation exists in the measurements of each "species".

This lack of comparability of data reported by different investigators has been attributed by some to the use of different staining techniques. To examine this, the data of two investigators on several populations assigned to the same Giardia species were compiled (Table 4).

Filice (24) found statistically significant differences in both length and breadth measurements of G. peromysci from three different deer mice. He also observed significant differences in length and breadth measurements of two different populations of G. microti from meadow mice. Soloviev (87) measured G. lamblia from three infected humans. Retrospective analysis of his data yielded statistically significant ($p \leq 0.05$) differences for both the length and breadth of the trophozoites.

Table 1. Comparison of trophozoite measurements obtained by different investigators: *G. lamblia* (human).

Investigator	Date	Reference	Length (μm)	Width (μm)
Lambl	1860	Cited in 81	18-21	8.6-11
Perroncito	1887	Cited in 81	17	10-12
Salomon	1899	Cited in 81	10-18	7.5-15
Noc	1909	Cited in 81	16	10
Simon	1922	81	13.7	6.6
Simon	1922	82	13.7	7.46
Soloviev	1975	87	15.03	7.78

Table 2. Comparison of trophozoite measurements obtained by different investigators: *G. microti* (meadow mouse).

Investigator	Date	Reference	Length (μm)	Width (μm)
Simon	1922	82	13.25	7.49
Lavier	1924	cited in 24	17	9
Hegner	1924	37	11.1	7.58
Potter	1928	66	14	7.4
Filice	1952	24	11	5.72
Soloviev	1975	87	12.83	6.18
Grant and Woo	1978	30	13.4	6.7

Table 3. Comparison of Trophozoite measurements obtained by different investigators: G. simoni (rat).

Investigator	Date	Reference	Length (μm)	Width (μm)
Simon	1922	82	13.25	7.49
Filice	1952	24	12.07	6.68
Soloviev	1975	87	13.15	6.9
Grant and Woo	1978	30	15.5	7.5

Table 4. Comparison of measurements obtained by investigators on different populations of one species.

Species (host)	Date	Investigator (Reference)	Length (μm)	Width (μm)
G. peromysci (deer mouse)	1952	Filice (24)	12.08	6.28
			13.30	6.89
			16.28	8.29
G. microti (meadow mouse)	1952	Filice (24)	10.41	5.48
			11.6	5.92
G. lamblia (human)	1975	Soloviev (87)	14.20	7.47
			15.72	7.75
			15.25	8.08

Table 5 shows the measurements of different species of Giardia measured by the same investigator. It is apparent that very small differences in size are being used for assigning species. Retrospective statistical analysis of Simon's data (81, 82) is not possible, so the statistical validity of his species designations can not be determined.

Grant and Woo (30) statistically analyzed their data and concluded that: 1) G. peromysci and G. microti can be differentiated on the basis of a 0.4 µm difference in length ($p \leq 0.05$), 2) the 0.8 µm difference in breadth between G. simoni and G. microti was significant but the 2.1 µm difference in length was not, and 3) the 1.7 µm difference in length and the 0.7 µm difference in breadth between G. simoni and G. peromysci were significant. Retrospective analysis of these data agrees with all but one conclusion; the 2.1 µm difference in length between G. simoni and G. microti is significant ($p \leq 0.05$).

Filice (24) did not find the differences among G. simoni, G. chinchillae, and G. peromysci to be significant.

In summary, the taxonomy of the Giardia parasitic in mammals has been a continuing source of confusion and controversy. Although body shape and dimensions, and host specificity (determined by animal transmission experiments) have been employed as criteria for classifying these organisms, strict standardization of the conditions for determining these qualities is necessary. The lack of such standardization to

Table 5. Comparison of different "species" of trophozoites measured by the same investigator.

Species (host)	Date	Investigator (Reference)	Length (range*)	Breadth (range)
G. simoni (rat)	1921, 1922	Simon (81, 82)	13.25	7.49
G. lamblia (human)			13.70	7.46
G. simoni (rat)	1952	Filice (24)	12.07	6.68
G. chinchillae (chinchilla)			12.57	6.28
G. peromysci (deer mouse)			12.08	6.86
G. simoni (rat)	1978	Grant & Woo (30)	15.5 (14.7-17)	7.5 (6-9.5)
G. peromysci (deer mouse)			13.8 (10.1-19.6)	6.8 (4.1-10.9)
G. microti (meadow mouse)			13.4 (10.3-18.6)	6.7 (7.9-9.0)

*values in μm; range reported if given in the paper.

date has been one of the problems in comparing the available data. While these attributes may have useful taxonomic value, it is clear, as Filice (24) pointed out three decades ago, that Giardia "species should be named on more evidence than that of host and body size".

C. Chemotaxonomy.

Morphologic criteria are commonly used in classifying parasites; a species is defined as one that is consistently different morphologically from another (14). However, when minor morphologic variations occur between specimens, the question of what constitutes a species becomes more complex.

In recent years, advances in biochemical techniques have introduced new ways to differentiate species. As a result, new species criteria are being established. The rise of comparative biochemistry and serology have led to the synthesis of a new species concept--the biochemical species.

Chemotaxonomy, the use of biochemical characteristics in classification, is a consequence of the desire to determine the amount of genetic homology which exists among organisms. Although the central dogma of one cistron-one protein is undergoing modification, it is still true that an organism's proteins are a reflection of its genetic content; therefore the "protein systematist is close to the genotype and still concerned with the fundamental basis of phenotypes" (55).

Chemotaxonomy includes isozyme profile comparisons and membrane and soluble protein comparisons. The latter may be accomplished by examining total protein profiles in denaturing gels and immunologic differences.

1. Isozymes.

Isoenzymes or isozymes are defined as "different molecular forms in which proteins may exist with the same enzymatic activity" (41). This term was first applied to enzymes resolved by physico-chemical techniques (e.g. electrophoresis); it now includes the variety of physical and kinetic parameters which characterize enzymes. The physical parameters of size, charge, and isoelectric point, kinetic properties such as substrate and cofactor binding affinity (Km), dependence of Vmax on temperature (E_A), functional stability at various temperatures, pH optimum, and immunochemical properties have all been utilized to characterize isozymes.

The definitive demonstration of isozymes occurred with the publication of two papers in 1957 (41, 100). Prior to that, reports of possible enzyme heterogeneity were usually attributed to contaminants or partially denatured or degraded enzyme molecules. The development of starch gel electrophoresis (SGE) (85) provided a technique of high resolving capacity for separating different proteins on the bases of size and charge.

Medical application of the isozyme phenomenon preceded other biological uses. Vessel and Bearn (100) found multiple forms of lactate dehydrogenase (LDH) in human serum when it was examined by SGE; the pattern of the LDH isozymes was observed to change in various disease states, including myocardial infarction, leukemia, and hepatitis. For these experiments, it was necessary to cut the gel into multiple fractions after electrophoresis, elute the enzyme, and spectrophotometrically assay individual fractions for activity.

Hunter and Markert (41) developed a more simple and direct technique, the zymogram, which couples SGE with histochemical staining of the enzyme in situ to identify separated enzymes. These authors were able to show that isozymes have tissue, ontogenetic, and species specific patterns (41, 56). Specific isozymes may occur in a) various tissues from a single host, b) different developmental stages of the same host, and c) different animal species.

As a consequence of these technological and conceptual innovations, thousands of investigations have focused on isozymes. The ubiquity of isozymes is such that enzyme preparations are generally tested for physical heterogeneity before kinetic properties are studied.

Cells may generate molecular polymorphism by any of several mechanisms (45, 83). Gene duplication with subsequent

divergence by mutation or post-translation modifications of enzymes are two such possible mechanisms.

There is precedence for using enzyme differences for classifying organisms (*infra vide*). Physical properties have been examined by comparing enzyme mobility in starch and polyacrylamide gels and isoelectric point. Kinetic properties which have been studied in this regard include the effect of temperature on observed velocity, pH optima, Km, and Vmax.

The study of isozymes by SGE has been used to differentiate invasive from non-invasive *Entamoeba histolytica* (79), *E. histolytica* from non-pathogenic intestinal amebae (77), to demonstrate parasites in host tissue (116), and to distinguish forms of *Trypanosoma cruzi* involved in sylvatic and domestic cycles (60). Questions of species designation have been resolved with organisms that are difficult to distinguish morphologically. Examples of this include the African salivarian trypanosomes (28), murine plasmodia (13), schistosome cercariae (54), *Entamoeba* (67, 76, 78), and animal trypanosomes (4, 46, 47).

Enzymes of *Naegleria* spp. (104), *Leishmania* spp. (2, 26), *Paramecium* spp. (1, 95), and nematodes of marine fish (12) have all been studied using polyacrylamide gel electrophoresis (PAGE). Since migration in PAGE under non-denaturing conditions is dependent upon the size, asymmetry, and charge of the protein, it is possible to detect variation in protein confor-

mation by varying the percentage of acrylamide and thus the pore size of the gel (45). This method has been used successfully with Colias meadii enzymes and Drosophila xanthine oxidase (44, 45, 83). Furthermore, it is possible to examine differences in the ratios of charge to mass through Ferguson plot analysis of these data (33).

The isoelectric point of proteins can be determined by electrophoresis in the presence of ampholytes. Enzyme staining in situ allows for the direct detection of differences between alleles on the basis of pI. Schistosoma spp. (74), Bulinus snail parasites (111), and freshwater fish (15) have been differentiated on this basis. It is also of interest that schistosome hybrids have been detected using isoelectric focusing (112).

Most comparisons of enzymes have used physical properties; however, when isozymes cannot be detected electrophoretically, it may be possible to demonstrate kinetic differences between electrophoretically similar enzymes. Such experiments may permit the detection of otherwise cryptic variation between enzyme alleles. The effect of temperature on observed velocity has been studied in the α-glycerophosphate dehydrogenase (α-GPdH) allele of Drosophila melanogaster (61), hemoglobin and glucose-6-phosphate dehydrogenase alleles of humans (7), and Chlamydomonas arginosuccinate lyase (57). Moreover, the α-GPdH loci of Colias meadii and Drosophila melanogaster have

been examined for differences in Km (substrate binding affinity), Vmax (maximal reaction velocity), temperature dependence of Km, and E_A (Arrhenius constant, which expresses the dependence of Vmax on temperature) (8, 44).

Until recently, little was known of the enzymes or energy yielding metabolism (EYM) of Giardia isolates. These experiments were hampered by the absence of axenic cultures. However, with the development of pure cultures of several Giardia isolates (9, 25, 58, 59), such experiments have been possible. Lindmark (52) has observed the end products of the metabolism of glucose for G. lamblia to be ethanol, acetate and CO_2; energy production appears to result from substrate level phosphorylation and a flavin, iron-sulfur protein-mediated electron transport system. Furthermore, G. lamblia apparently lacks a functional tricarboxylic acid cycle and cytochrome mediated oxidative phosphorylation. Lipid and carbohydrate metabolism (43), in vivo DNA replication (42), pyrimidine salvage (53), and respiratory metabolism (108) have also been examined. All of these experiments have used a single isolate of Giardia (see Appendix B) and no comparisons of the metabolism or enzymes of different isolates have been published.

2. Membrane and soluble protein comparisons.

a. Total protein profiles-electrophoresis.

The membrane and soluble proteins of organisms have been compared directly by electrophoresis in polyacrylamide gels (PAG). Proteins, when subjected to an electric field in a retarding gel support, migrate on the basis of charge and size. Proteins can be solubilized with the negatively charged detergent sodium dodecyl sulfate (SDS), which binds extensively to the polypeptide chains of proteins and causes them to unfold and dissociate. Under these conditions, migration is dependent upon the size of the molecule and not the charge (71, 72, 107).

Helminths collected during predator control programs and biological studies have also been used in taxomonic studies. The subsequent identification of these helminths, which have often undergone morphologic deterioration, is difficult. Some parasitologists have approached the problem by the application of SDS-PAGE of whole bodies and soluble proteins.

Adult lung flukes of the genus Paragonimus are differentiated by a combination of morphologic features; the arrangements of the cuticular spines, size ratios of testes and ovaries, and morphologic features of the ovary (114). However, these criteria do not always yield reliable answers. Yoshimura (113, 114) has examined the whole body proteins of Paragonimus species for characteristic electrophoretic patterns. He was able to distinguish between P. ohirai and P. iloktsuenensis

(114) and among P. ohirai, P. miyazaki, and P. westermani species (113). Although there were many similarities, species specific proteins were observed.

Ruff et al. (75) examined adult worms of the dioecious trematode, Schistosoma japonicum. Soluble proteins obtained by sonication of three strains from Japan, Formosa, and the Philippines were compared. Quantitative and qualitative differences were found among the three strains and between sexes within the strains.

Taenia species (Cestoda) have also been examined by SDS-PAGE. Bursey et al. (11) were able to demonstrate that, for total proteins, all parts of the strobila were basically alike. Therefore, examination of a single proglottid by electrophoresis could provide identification. Species specific patterns were found. In contrast to results with Paragonimus, few common proteins were observed among Taenia taeniaformes, T. macrocysts, and T. pisiformis.

The species within two genera of facultative parasitic amebae, Hartmanella and Naegleria, have been compared by electrophoresis in non-SDS gels (32). Many common protein bands were found between the pathogen Naegleria fowleri and the non-pathogenic N. gruberi. Nevertheless, species specific patterns were observed. When soluble proteins extracted by freezing and thawing the four Hartmanella species were electrophoresed, significant differences in patterns were observed.

Comparisons of total protein profiles have been possible with helminths since individual worms may be selected for study. Experiments with protozoa have been and continue to be limited by the difficulty in developing axenic cultures.

b. Immunologic comparisons.

Immunologic comparisons of parasites have included immunodiffusion (Ouchterlony) analysis (ID), immunoelectrophoresis (IEP), and two-dimensional IEP. Fluorescein labeled-antibody has been used for quantitating differences among species. Immunologic methods permit the examination of fine differences among species since molecules with identical molecular sizes may have different antigenic determinants.

The relationship between antigenic composition and virulence of Trichomonas gallinae strains has been examined by ID (90). When freshly isolated avirulent strains or strains attenuated by prolonged in vitro culture were compared with virulent T. gallinae, some antigens observed in the avirulent strains were not seen in virulent strains and vice versa.

Small free-living amebae of the genera Hartmanella, Acanthamoeba, and Naegleria have stimulated interest in recent years due to their facultative parasitic nature. Distinction among species in these genera and the differentiation between Acanthamoeba spp. and some Hartmanella spp. is subject to some controversy. Immunologic comparison by IEP and ID (102) showed distinct differences between Acanthamoeba and Hartmanella which

along with structural and nutritional characteristics appeared to justify their classification into separate genera. Pathogenic and free-living Naegleria spp., which can be distinguished by electrophoresis of whole proteins, are also antigenically distinct (104).

Close antigenic relations have been found among Trichomonas spp., Histomonas spp., and Dientamoeba spp. Qualitative gel diffusion indicates some identical as well as a number of partially related antigens (22). IEP with absorbed antisera (23) have provided additional quantitative data on the number of common antigens and those with partial identity. These data, combined with quantitative immunofluorescence (21), have been used to examine the evolutionary relationships of these three genera. It is postulated that amebae arose from flagellated stocks that lost their flagella; these comparisons of a typical monophasic flagellate (Trichomonas), an ameboflagellate (Histomonas), and an ameba with some flagellar characteristics (Dientamoeba) do not necessarily represent the actual evolutionary steps, but do demonstrate that quantitative and qualitative antigenic similarities may be found.

The development of antibodies to plasma membrane-enriched fractions of Acanthamoeba species has permitted the analysis of cell surface antigens by quantitative immunofluorescence (93). Furthermore, these same sera enabled retrospective identifica-

tion of A. culbertsoni in a case of amebic meningioencephalitis (110).

There have been numerous studies of the pathogenesis of Giardia infections and host immune mediation of the disease process (3, 65, 69, 70, 91, 92, 94, 99). The development of assays for quantitation of serum antibody against Giardia has been very recent. Visvesvara et al. (106) detected a systemic antibody response by an indirect immunofluorescence test. A sensitive, enzyme-linked immunosorbent assay (ELISA) was developed by Smith et al. (84); this method demonstrated that 81% of symptomatic patients and 12% of uninfected controls had circulating IgG against G. lamblia.

Holberton et al. (38, 39) isolated the cytoskeleton material of three Giardia isolates and examined the arrangement of subunits in microribbons. Visvesvara et al. (105) examined the antigenic character of G. lamblia trophozoites grown in medium supplemented with human, bovine, or rabbit serum. Although, in some cases, the trophozoites appeared to incorporate some serum components, the overall precipitin pattern was similar regardless of the serum source. Moore et al. (62, 63) has examined somatic extracts and secretory-excretory products of G. lamblia by immunoelectrophoresis, PAGE, ELISA, and high-pressure liquid chromatography. Numerous distinct bands were observed and the most antigenic fraction in the ELISA test was the high molecular weight material.

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III. Manuscripts

Paper 1

A morphometric examination
of five *Giardia* isolates

Abstract

Morphometrics is a method of Giardia speciation which is still used by some investigators to support speciation based upon host source and presumed host specificity. All such prior studies have used organisms subjected to uncontrolled environmental factors. This study compared five axenic cultures of Giardia isolates. The length and width of trophozoites were measured both live and after fixing and Giemsa staining. These isolates, as named on the basis of host source, are classified as G. lamblia (3 isolates), G. felis (1 isolate), and G. caviae (1 isolate).

The size of unstained trophozoites from five isolates measured without regard to the presence or absence of median bodies, showed occasional significant differences in length and/or width. More frequent significant differences were observed among the isolates in stained preparations. These differences occurred between G. caviae and G. felis, and among the three G. lamblia isolates.

The significant differences in size among the three human isolates raises significant doubts concerning the use of morphometrics in Giardia speciation; such differences would, by the criterion of morphometric speciation, justify a separate species category for each of these isolates. Moreover, the absence of differences between isolates assigned to different species further questions the reliability of morphometrics.

Introduction

Those characteristics which define a Giardia species have been the object of continued debate. Among taxonomists, there exist two factions: the lumpers and the splitters. The lumpers recognize three Giardia species: G. agilis, G. muris, and G. duodenalis (3). This classification is based on trophozoite body shape and an internal structure, the median bodies. Splitters have divided Giardia into more than 40 species (11), based on host source (and presumed host specificity in some cases) and measurements (particularly of length and width [morphometrics]).

A primary problem in Giardia research is the difficulty in establishing axenic cultures. As a result, the morphometric research reported to date has been conducted on protozoan specimens obtained by duodenal aspiration or dissection of wild or laboratory animals. Because these organisms have been obtained from animals, it is impossible to determine whether the differences observed between Giardia trophozoites, often from animals of the same species, are the result of innate protozoan characteristics or of the environment provided by the host. The fact that the diet of the host can affect the size of Giardia cysts (19) indicates that the latter possibility cannot be excluded.

Therefore, it was decided to address the reliability of morphometrics by examining the dimensions of five axenically grown Giardia isolates from human, cat, and guinea pig hosts.

Materials and Methods

Isolates.

Five isolates of *Giardia* were utilized in this study. Three were Portland isolates from human, cat, and guinea pig hosts (4,12,13), one human isolate was kindly provided by John Ackers of the London School of Hygiene and Tropical Medicine (1), and another human isolate was kindly provided by Frances Gillin of the Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, Bethesda, MD (16).

For clarity, each *Giardia* isolate will be referred to on the bases of host source and geographic location; i.e., human-1/Portland (H/P), cat-1/Portland (C/P), guinea pig-1/ Portland (G/P), human-1/England (H/E), and human-1/Bethesda (H/B).

Culture method and preparation of cells.

Giardia isolates were grown axenically in TPS-1 medium supplemented with 10% heat inactivated calf serum and vitamins (Eagle's minimal essential medium) as described by Visvesvara (20). For these experiments, *Giardia* trophozoites from stock cultures were inoculated into tubes of fresh medium to a final concentration of approximately 10^4 cells per ml. After 48 hours of incubation, the supernatant was discarded and cold 250 mM sucrose, pH 7.2, added to each tube. Since *Giardia* trophozoites in culture attach to the wall of the culture vessel, tubes were placed in an ice bath for 30 min. to release adherent trophozoites, then centrifuged for 5 min. at 600 x g. The

resultant pellet was washed once in 0.15M saline (NS) then resuspended in a small volume of NS.

Preparation and measurement of fixed trophozoites.

Trophozoites were fixed and stained as described by Grant and Woo (7). Smears of trophozoites were wet fixed in absolute ethanol for 3 min. and air dried before secondary fixation for 5 sec. in 10% buffered formalin, pH 7.2. After air drying, fixed smears were stained 45 min. in a 1:45 dilution of Giemsa stain prepared as described by Garcia and Ash (5). Trophozoites were measured under oil immersion by light microscopy with an eyepiece micrometer at a total magnification of 1000X.

Preparation and measurement of unstained trophozoites.

Live trophozoites, from the same pellet that was used for fixed smears, were measured after photography. Cells were observed and photographed at 450X in a Zeiss inverted phase contrast microscope. For internal standardization, the cells were placed on a Petroff-Hausser grid (Hausser Scientific, Blue Bell, PA) which contains squares, 50 µm on a side. Photographic negatives were projected onto paper, the dimensions of trophozoites in the plane of focus and the companion 50 µm grid were marked and these values were used for computations of size.

Results

Stage of growth.

Giardia trophozoites in Diamond's TPS-1 medium have a generation time of 12.2 hours (20). Cultures with an initial inoculum of 10^4 cells/ml are in the mid-logarithmic phase of growth by 48 hours (2, 20). Adherent organisms were selected for these experiments since stationary phase and dead trophozoites tend to detach and settle to the bottom of the tube. It has been suggested (3,17), but not confirmed, that the presence of median bodies, an internal structure of unknown function, is related to the stage of trophozoite development. The number of organisms with visible median bodies was counted to determine the percentage of organisms in mid-logarithmic growth phase which possess median bodies. For each culture, 300 stained organisms on each of two slides were examined; the percentage of individual trophozoites possessing median bodies varied from 47.8 to 54.9%. Chi square of the analysis of the data did not reveal significant differences among the five cultures.

On the basis of these results, a representative population of stained trophozoites was constructed for comparison of live and stained trophozoite dimensions. This population, mb+,-, is 50% positive for median bodies.

Comparison of unstained trophozoites.

The length and width of unstained trophozoites (50 of each isolate) were measured without regard to the presence or ab-

sence of median bodies (Table 1). A photograph of trophozoites as they appear by phase contrast microscopy is presented in Figure 1. The dimensions used for length and width measurements for all experiments are presented in Figure 2. The resultant measurements were compared statistically (Table 2); a p value of ≤ 0.05 was considered significant for these experiments.

H/B was significantly different from C/P and G/P in length and width, and from H/E in width. H/P was significantly different from C/P in length and from G/P in width. There were no other significant differences in the sizes of unstained trophozoites.

Comparison of stained trophozoites.

Some authors (3,7) have noted that Giardia trophozoites without median bodies (mb-) are smaller in length and width than those with median bodies (mb+). The length and width of stained mb+ and mb- trophozoites of the five isolates were determined (Tables 3 and 4) and statistically compared (Table 5). In all cases, the mb+ trophozoites were longer than mb- trophozoites; for three isolates, C/P, H/B, and H/E, this difference was significant ($p \leq 0.05$). The width of four isolates was wider in mb+ than mb- trophozoites; this was significant for C/P and H/B.

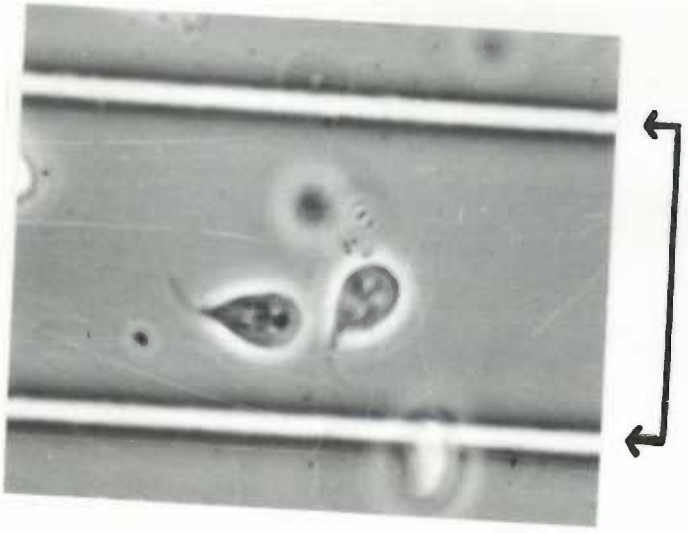
Table 1. Dimensions of unstained Giardia trophozoites from five isolates (n=50).

		ISOLATES				
		C/P	G/P	H/P	H/B	H/E
LENGTH	average	12.9[a]	12.7	12.3	12.0	12.3
	std. dev.	±1.5	±1.7	±0.9	±0.9	±0.8
	range	(9.4-16.5)	(11.6-15.2)	(10.4-14.8)	(10-14.4)	(10.6-15.4)
WIDTH	average	8.9	9.0	8.7	8.3	8.9
	std. dev.	±1.1	±0.5	±0.5	±1.3	±0.7
	range	(6.7-11.0)	(7.9-10.6)	(7.2-9.6)	(7.3-10.4)	(7.5-10.1)

[a] Dimensions in μm

Figure 1. Trophozoites as observed by phase contrast microscopy.

Figure 2. Diagrammatic representation of G. duodenalis from Kulda (11). Dimensions used: L used for length and Bl used for width.



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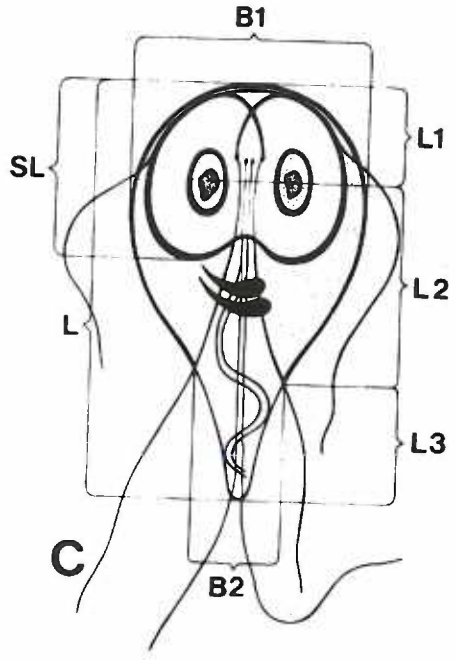

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Table 2. Statistical analysis of differences between unstained *Giardia* trophozoites from five isolates.

		C/P	G/P	H/P	H/B
LENGTH	G/P	ns[a]			
	H/P	S	ns		
	H/B	S	S	ns	
	H/E	ns	ns	ns	ns

		C/P	G/P	H/P	H/B
WIDTH	G/P	ns			
	H/P	ns	S		
	H/B	S	S	ns	
	H/E	ns	ns	ns	S

[a]ns: not significant

S: significant at $p \leq 0.05$.

Table 3. Length measurements of stained Giardia trophozoites from five isolates with respect to the presence and absence of median bodies.

Median Body[a]		ISOLATES				
		C/P	G/P	H/P	H/B	H/E
mb- n=40	average	12.6[a]	13.6	12.4	12.0	11.0
	std. dev.	±1.2	±1.2	±2.3	±2.0	±1.3
	range	(10.9-15.6)	(12-16.6)	(9.9-15.6)	(9.4-14.5)	(9.4-14.6)
mb+ n=40	average	13.3	14.0	13.1	13.0	12.2
	std. dev.	±1.3	±0.8	±1.1	±1.0	±2.0
	range	(9.4-15.6)	(12-15.6)	(10.9-15)	(10.9-15.6)	(10.4-14.6)
mb+,- n=40	average	13.0	13.8	12.7	12.7	11.8
	std. dev.	±1.3	±1.0	±1.8	±1.0	±2.1
	range	(9.4-15.6)	(12-16.6)	(9.9-15.6)	(9.4-15.6)	(9.4-14.6)

[a]mb-, median bodies absent; mb+, median bodies present; mb+,-, equal numbers of organisms with and without median bodies.
[b]Dimensions in µm.

Table 4. Width measurements of stained Giardia trophozoites from five isolates with respect to the present and absence of median bodies.

Median body[a]		ISOLATE				
		C/P	G/P	H/P	H/B	H/E
mb- n=40	average	7.6[b]	9.0	7.8	7.0	7.5
	std. dev.	±0.8	±0.9	±0.9	±0.6	±0.7
	range	(5.7-8.8)	(7.3-10.4)	(6.8-9.4)	(6.2-8.3)	(6.2-8.3)
mb+ n=40	average	8.2	9.1	7.5	7.3	7.6
	std. dev.	±0.9	±0.9	±0.9	±0.7	±0.6
	range	(6.9-9.9)	(7.8-11.4)	(6.9-9.4)	(6.2-8.8)	(6.2-8.8)
mb+,- n=80	average	7.9	9.1	7.7	7.2	7.6
	std. dev.	±0.9	±0.9	±0.9	±0.6	±0.6
	range	(5.9-9.9)	(7.3-11.4)	(6.8-9.4)	(6.2-8.8)	(6.2-8.8)

[a] mb-, median bodies absent; mb+, median bodies present; mb+,-, equal numbers of organisms with and without median bodies.
[b] Dimensions in µm.

Table 5. Comparison of the size differences between trophozoites (with vs without median bodies) from the same culture.

	LENGTH [mb+ vs mb-]		WIDTH [mb+ vs mb-]	
Isolate	Difference	Significance	Difference	Significance
C/P	longer[a] (0.7 µm)	S[b]	wider (0.6 µm)	S
G/P	longer (0.4 µm)	ns	wider (0.1 µm)	ns
H/P	longer (0.7 µm)	ns	narrower (0.3 µm)	ns
H/B	longer (1.0 µm)	S	wider (0.3 µm)	S
H/E	longer (1.2 µm)	S	wider (0.1 µm)	ns

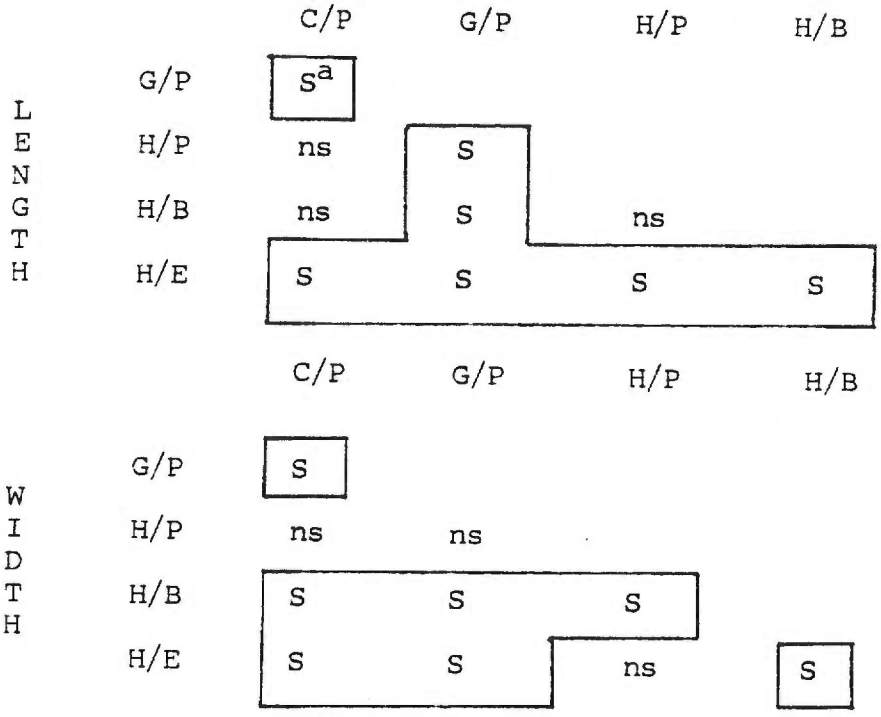
[a] i.e. The mb+ trophozoites were longer than the mb- trophozoites by 0.7 µm.
[b] S: significant ($p < 0.05$)
ns: not significant

Some authors have chosen to compare only mb+ organisms since comparing the combination of mb+ and mb- could result in wide variations in the dimensions obtained. However, as tables 3 and 4 demonstrate, the ranges of values within both groups were similar. Furthermore, a comparison of the standard deviations and ranges of values obtained by combining equal numbers of mb+ and mb- organisms (mb+,-) with mb+ and mb- groupings show few significant differences (Table 5).

The choice of organisms, mb+, mb- or mb+,-, for statistical comparisons, has been based on the assumption that the combination of mb+ and mb- would obscure significant differences between isolates. To examine this, a comparison was made of the statistical data obtained with these three different combinations (Tables 6, 7, and 8). The significant differences ($p \leq 0.05$) in length, observed when the representative populations (equal numbers of mb+ and mb- trophozoites) of the isolates were compared, were also seen when mb+ organisms were examined. Some differences, observed in these two groupings, were not apparent when mb- organisms were compared. More differences in the statistical results were observed among three groupings for width comparisons than were seen in length comparisons.

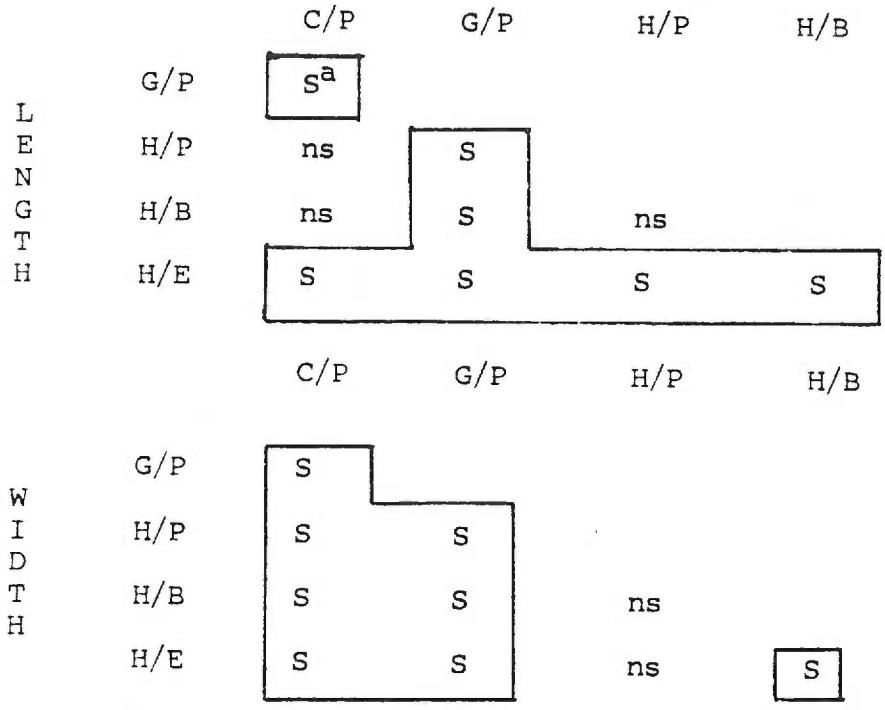
There were statistically significant size differences between C/P and G/P in both length and width as well as differences between these two and the three human isolates. More

Table 6. Statistical analysis of size differences between trophozoites when equal numbers of organisms with and without median bodies are used.



[a]S: significant at $p \leq 0.05$
ns: not significant

Table 7. Statistical analysis of size differences between stained trophozoites with median bodies.



[a]S: significant at $p \leq 0.05$
ns: not significant

Table 8. Statistical analysis of size differences between stained trophozoites without median bodies.

LENGTH	C/P	G/P	H/P	H/B
G/P	S[a]			
H/P	ns	S		
H/B	ns	S	ns	
H/E	S	S	ns	ns

WIDTH	C/P	G/P	H/P	H/B
G/P	S			
H/P	ns	S		
H/B	S	S	S	
H/E	ns	S	ns	S

[a]S: significant at $p \leq 0.05$
ns: not significant

importantly, there were significant differences among all three human isolates and no significant differences between C/P and H/P, isolates assigned to different species.

Stained vs. unstained trophozoites.

An examination of the absolute values (Tables 1, 3, and 4) for length and width measurements demonstrates the apparent effect of fixation and staining on trophozoite dimensions. For statistical evaluation, stained mb+,- trophozoites were compared with unstained trophozoites (Table 9). In four isolates, the organisms were longer and in four isolates the organisms were narrower after fixing and staining. Length differences in G/P and H/B were significant; width differences in C/P, H/P, H/B, and H/E were significant.

Discussion

The need for widely accepted criteria for *Giardia* speciation is well recognized. Although Grant and Woo (7) and Soloviev (18) have recently revived morphometrics as a reproducible criterion for *Giardia* identification and speciation, the concept has not been widely accepted.

Historically, results from different laboratories have resulted in widely divergent measurements for the same *Giardia* "species". For example, the values for the length of *G. simoni* (rat host) range from 12.07 to 15.5 µm (3,7,14,15,18). Values

Table 9. Statistical analysis of size differences between stained (equal numbers of organisms with and without median bodies) and unstained trophozoites of the same isolate.

	LENGTH (Stained vs Unstained)		WIDTH (Stained vs Unstained)	
Isolate	Difference	Significance	Difference	Significance
C/P	longer[a] (0.1 µm)	ns[b]	narrower (1.0 µm)	S
G/P	longer (1.1 µm)	S	wider (0.1 µm)	ns
H/P	longer (0.4 µm)	ns	narrower (1.0 µm)	S
H/B	longer (0.7 µm)	S	narrower (0.9 µm)	S
H/E	shorter (0.5 µm)	ns	narrower (0.7 µm)	S

[a] i.e. the stained tropozoites were longer than unstained trophozoites by 0.1 µm, this is the order of comparison for the entire table.

[b] ns: not significant
S: significant ($p \leq 0.05$)

for the length of G. microti (meadow mouse) range from 11 to 17 μm and for width from 5.72 to 9 μm.

These differences have been explained as being due to the use of different staining techniques by different investigators. However, when the measurements of single investigators on different populations of an apparent single Giardia species are examined, statistically significant differences may be found. Thus, Filice in 1952 (3) found significant differences among the three populations of G. peromysci and the two populations of G. microti he examined. Soloviev measured G. lamblia (synonym for Lamblia intestinalis) from three patients from different hospitals in Moscow (18). Although he claimed the values obtained for length (14.2, 15.25, and 15.75 μm) and breadth (7.45, 7.75, 8.08 μm) were comparable at a $p \leq 0.01$, retrospective Student's t-test analysis of the data indicates significant differences in length and width measurements among these three isolates.

Another criticism which can be made of morphometrics is that relatively small differences, sometimes of a single dimension, are utilized for species designations. Grant and Woo (7) support the construction of separate species on the basis of small statistically significant differences in single dimensions. For example G. peromysci is distinguished from G. microti on the basis of a 0.4 μm difference in trophozoite length. This difference is further substantiated by host

specificity data reported in a subsequent paper by the same authors (8). However, host specificity experiments include many potential pitfalls and well-controlled experiments require detailed and expensive protocols, not available in most laboratories as a routine technique available for confirmation.

Uncontrolled environmental effects may have influenced previous reports of trophozoite measurements. In most published experimental data, the range of values for a particular dimension may be very significant. Since Giardia trophozoites are difficult to establish in axenic culture, trophozoites used in these earlier experiments were obtained by duodenal aspiration or dissection. The observed variation has been considered, by some, to be due to host environmental factors (3). Host diet has been shown to affect the size of Giardia cysts (19). The effect of environment on the size of Giardia trophozoites has not been examined. This has been done, however, with another flagellated protozoan, Trichomonas tenax (10). One strain was measured after growth in two different media and in the presence of one or three contaminating bacterial species. Significant differences in size were found between the strain in the two different media and in the presence of different numbers of species of bacteria. Organisms grown in a medium containing three bacterial species were significantly smaller in length and width than that strain in the same medium with only one bacterial species contaminant. The same strain,

grown in two different media (yolk infusion vs. cysteine-peptone-liver-maltose), each with only one bacterial species contaminant, differed in length but not width.

Even in this present study of axenic isolates, ranges of ± 20% were observed for some dimensions of stained trophozoites. It may eventually be possible to determine whether the observed variation in dimensions is a result of innate protozoan characteristics or due to a mixed population. Gillin (6) has developed a method for cloning Giardia. The measurement of several cloned populations could aid in the resolution of this question.

The data reported herein represent the first measurement and comparison of Giardia trophozoites in axenic culture. Two methods for measurement were chosen. Live organisms were measured since it has been shown that fixation and staining may result in distortion. Stained trophozoites were measured to permit comparison with previous reports in which stained organisms were studied. The data obtained by either method are comparable with but not identical to that reported by other investigators (9,11,18).

Measurement of the same cultured organisms, both alive and stained, permits determination of the effect of fixation and staining on trophozoite size. Significant differences in size were observed between stained and unstained organisms. In general, the stained cells were longer and narrower. Such

differences may well result from dehydration during fixation with ethanol. Whether these differences include a change of total volume cannot be determined from these data.

The measurement of living trophozoites is a better reflection of size than the measurement of stained organisms. Photographs were taken of unstained trophozoites to obviate the possibility that the wet mount would dry during the tedious process of measuring. Few significant differences were found among the isolates by this method. Most interesting was the significant difference in width between H/B and H/E, two isolates presently assigned to the same species.

Greater significant differences were found among the isolates when stained trophozoites were examined. The results were grouped into three sets based upon the presence or absence of median bodies: mb+, mb-, and equal numbers of mb+ and mb- cells (mb+,-). The latter combination was chosen since it was shown that approximately 50% of the trophozoites in each of the populations have median bodies. Statistical analyses demonstrated that the dimensions of mb+ and mb+,- trophozoites were comparable for length, and, with the exception of H/P, comparable for width. This difference for H/P may be attributable to the apparent increase in both dimensions observed in this sample as compared with the decrease observed for all other isolates. The statistical data obtained with mb- cells were different in several ways and are not further considered.

It is highly significant that differences at $p \leq 0.05$ could be found in length and width measurements of organisms which are presently considered the same species. Furthermore, in some cases, no significant differences were found between some isolates assigned to different species. These data indicate that morphometrics alone cannot serve as a reliable criterion for the identification and speciation of *Giardia* isolates. It is also clear that morphometrics may not give sufficient information to even warrant its use as one of several criteria for definition of a species.

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Paper 2

A comparison of the zymogram patterns of five *Giardia* isolates

Abstract

The relative mobilities of six enzymes from the trophozoites of five axenically cultured Giardia from human, cat, and guinea pig hosts were compared by starch and polyacrylamide gel electrophoresis. Three isolates from humans and one each from cat and guinea pig hosts were studied. The six enzymes compared were malate dehydrogenase (NAD+) (EC 1.1.1.37), malate dehydrogenase (decarboxylating) (EC 1.1.1.40), hexokinase (EC 2.7.1.1), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and α-glycerophosphate dehydrogenase (EC 1.1.1.8). The latter three enzymes have not been previously reported in Giardia.

All five Giardia isolates are of the morphologic type described by earlier workers as G. duodenalis. They have been classified previously, based on host source and presumed host specificity, as G. lamblia, G. felis, and G. caviae. On the basis of reproducible enzyme patterns, the five Giardia isolates can be divided into three zymodemes. Zymodeme I comprises three isolates with common enzyme patterns: human-1/England, human-1/Bethesda, and cat-1/Portland. Zymodeme II consists of the guinea pig-1/Portland isolate and Zymodeme III comprises the human-1/Portland isolate.

These results, in which Giardia isolated from different mammalian hosts share multiple isoenzymes, question the valid-

ity of the practice of assigning Giardia species names on the basis of the animal host from which the protozoan was obtained.

Introduction

Giardia is the most commonly reported human intestinal protozoan parasite in the U.S.A.; the incidence of giardiasis is reported to average about 4% of stool specimens submitted to public health laboratories, with a state to state range of 1.1 to 16.7% (5). Giardia are also intestinal parasites of a variety of animals (19). Until the 1970's, most Giardia spp. were considered to be host specific (26). However, recent epidemic outbreaks of giardiasis, in which drinking water was the probable vehicle for spread, implicated lower animals as a likely source of some human Giardia infections (7, 9). Furthermore, recent cross transmission studies suggest that Giardia spp. are not as host specific as once believed (8, 15). These findings have led to a controversy concerning the interrelationship and taxonomic status of Giardia from different animal hosts. The trophozoites of most mammalian Giardia are essentially morphologically indistinguishable. The characteristics used for their speciation have included host source and specificity (19), trophozoite shape and dimensions (14, 36), and morphology of median bodies (microtubular structures inside Giardia) (10, 29).

Because there is no general agreement regarding the characteristics used to define species in the genus Giardia, I have

looked for biochemical differences by which Giardia trophozoites from different host species may be differentiated. I report here the results of a comparison of the electrophoretic mobility of six enzymes from five Giardia isolates from human, cat, and guinea pig hosts.

Materials and Methods

Isolates.

Five axenic isolates of Giardia were utilized in this study. Three were Portland isolates from human, cat, and guinea pig hosts (11, 24, 25); one human isolate was kindly provided by John Ackers of the London School of Hygiene and Tropical Medicine (1), and another human strain was kindly provided by Frances Gillin of the Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, Bethesda, MD (35).

For clarity, each Giardia isolate will be referred to on the bases of host source and geographic location; i.e., human-1/Portland (H/P), cat-1/Portland (C/P), guinea pig-1/ Portland (G/P), human-1/England (H/E), and human-1/Bethesda (H/B).

Culture method and preparation of lysates.

Giardia isolates were grown axenically in TPS-1 medium supplemented with 10% heat inactivated calf serum and vitamins (Eagle's minimum essential medium) as described by Visvesvara (37). Cells were harvested after 96 hours of incubation at

37°C by chilling the culture tubes for 2 hours at 4°C and centrifuging the culture medium at 1,000 x g for 10 min. The cell pellet was washed two times with phosphate buffered saline, pH 7.0, once in 250 mM sucrose, pH 7.2, then resuspended in the 250 mM sucrose. Trophozoites were lysed by freezing at -70°C then thawing at 4°C. The lysed suspension was centrifuged for 2 min. at 10,000 x g in a Microfuge (Beckman Instruments, Palo Alto, CA); the resulting supernatants were utilized for electrophoresis. The protein concentration of the lysates was estimated by the method of Bradford (3), modified by using a commercial reagent (Bio-Rad Laboratories, Richmond, CA).

Chemicals.

Reagents, including all enzymes used as assay controls, were obtained from Sigma Chemical Co. (St. Louis, MO). Assay controls were from the following hosts: hexokinase and 6-phosphogluconate dehydrogenase (yeast, genus not identified); glucose-6-phosphate dehydrogenase (Torula yeast); malate dehydrogenase (decarboxylating) (chicken liver); malate dehydrogenase (pig heart, cytoplasmic); and α-glycerophosphate dehydrogenase (rabbit muscle).

Electrophoresis.

Starch gel electrophoresis (SGE) was performed in horizontal gels. The electrode buffer was 0.687M Tris (hydroxymethyl)

aminomethane [Tris-HCl] --0.157M citric acid, pH 8.0, and the gel was solubilized in 0.0229M Tris-HCl--0.009M citric acid, pH 8.0 (33). Polyacrylamide gel electrophoresis (PAGE) in a vertical slab apparatus was as described by Laemmli (20) except that sodium dodecyl sulfate was deleted from the solution. Cell free lysates (50-100 µg of protein), 5-20 µl of calf serum, and dilutions of standard enzymes were applied to wells, then electrophoresed for 4-8 hours at 4°C at constant current.

Detection of enzyme activity in gels.

After electrophoresis, hexokinase (EC 2.7.1.1), malate dehydrogenase (EC 1.1.1.37), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) and α-glycerophosphate dehydrogenase (EC 1.1.1.8) were visualized as described by Shaw and Prasad (33). Malate dehydrogenase (decarboxylating) (EC 1.1.1.40) was detected by using the concentrations of nucleotides, substrate, and buffer described by Ochoa (30). Indicator dye concentrations (phenazine methosulfate and thiazoyl blue) were as described in Shaw and Prasad (33) for malate dehydrogenase (NAD+). Purified enzymes (Sigma Co., St. Louis, MO) were used as assay controls. Giardia lysates did not exhibit substrate-independent reduction of either nicotinamide adenine dinucleotide (NAD+) or nicotinamide adenine dinucleotide phosphate (NADP) after electrophoresis.

Results

Activities corresponding to malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), and malate dehydrogenase (decarboxylating) (ME) were detected after electrophoresis of lysates in polyacrylamide gels (PAG). Activities corresponding to α-glycerophosphate dehydrogenase (αGP), hexokinase (HK) and 6-phosphogluconate dehydrogenase (6PG) were detected after electrophoresis of the lysates in starch gels since those enzymes could not be detected after electrophoresis in PAG. Pre-electrophoresis of the separating gel in the separating gel buffer did not remove the apparent inhibitors of these three enzymes. It should be noted that artifactual isozymes were observed when samples were frozen and thawed more than once.

MDH activity was observed in calf serum, a medium component; however, calf serum MDH exhibited a faster mobility than that of Giardia isolates (data not shown). The other five enzymes were not detected in calf serum. All six control enzymes were detected by the electrophoretic methods utilized for the Giardia enzymes, and all had different electrophoretic mobilities than the comparable Giardia enzymes (data not shown).

Electrophoretic heterogeneity was found in Giardia enzymes; the results are summarized in Table 1. In four of the six systems, MDH, G6P, αGP and HK, two isozymes were found

Table. Summary of isozyme patterns.

		ENZYME					
Zymodeme	Isolate	ME	6PG	MDH	αGP	G6P	HK
I	C/P	0.33[a]	0.17	0.21	0.15	0.11	0.44
I	H/B	0.33	0.17	0.21	0.15	0.11	0.44
I	H/E	0.33	0.17	0.21	0.15	0.11	0.44
II	G/P	0.30	0.13	0.21	0.15	0.11	0.44
III	H/P	0.23	0.25	0.30	0.25	0.15	0.51

[a]Relative mobility of the enzyme with respect to the mobility of bromophenol blue in 12% starch gels (6PG, αGP, and HK) or 7% polyacrylamide gels (ME, MDH, and G6P).

(Fig. 1-4). The slower migrating isozyme was shared by four of the isolates: H/B, H/E, G/P, and C/P. The faster migrating isozyme was only found in H/P. Three different isozymes were detected in ME and 6PG (Fig. 5 and 6). The fast migrating ME isozyme was common to C/P, H/B, and H/E, the middle isozyme was only found in G/P, and the slow band in H/P. In 6PG, the fast migrating isozyme was found in H/P, the middle isozyme in C/P, H/B, and H/E, and the slow isozyme in G/P. Admixtures of cell free lysates yielded the same relative patterns, after electrophoresis, as individually electrophoresed lysates.

When two samples exhibit enzyme bands at the same position on the gel, one may not conclude that these are identical. Electrophoresis in non-denaturing gels is dependent upon charge and size, and the contributions of these factors may result in apparent homogeneity at one concentration (pore size) of starch or acrylamide (18). Variation in pore size of the gel aids in the detection of otherwise cryptic heterogeneity (17, 18). Samples were examined in 5, 6, 7, 8, and 9% polyacrylamide gels (MDH, G6P and ME) and in 10 and 12% starch gels (HK, 6PG, and αGP) in an effort to detect differences in relative mobilities. None were found.

Figure 1. Zymogram pattern in polyacrylamide gel of malate dehydrogenase (NAD+) in Giardia isolates.

Figure 2. Zymogram pattern in polyacrylamide gel of glucose-6-phosphate dehydrogenase in Giardia isolates.


H/P
C/P
G/P
H/B
H/E
origin
slow
fast
1

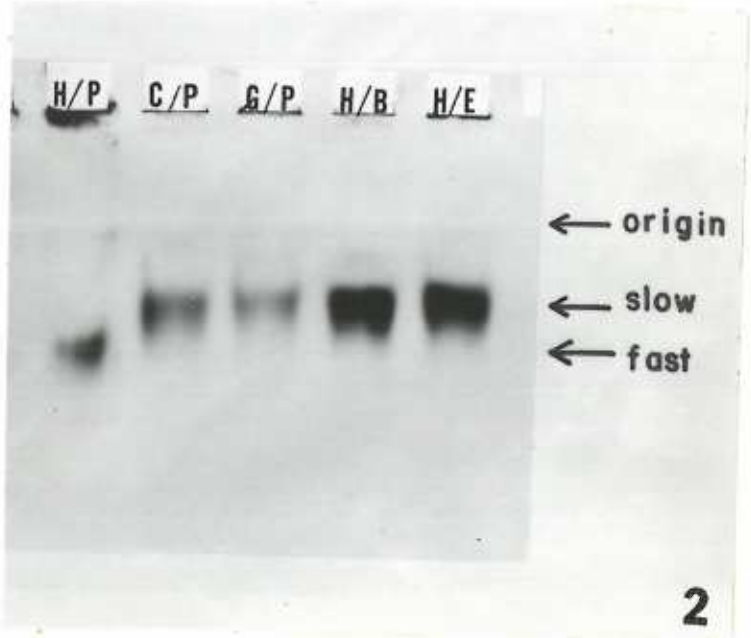
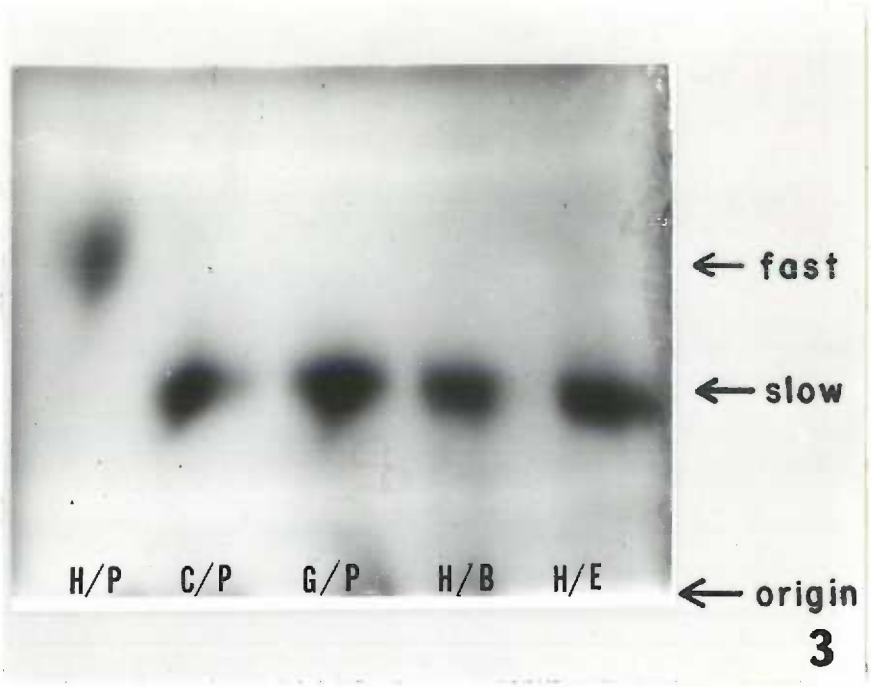
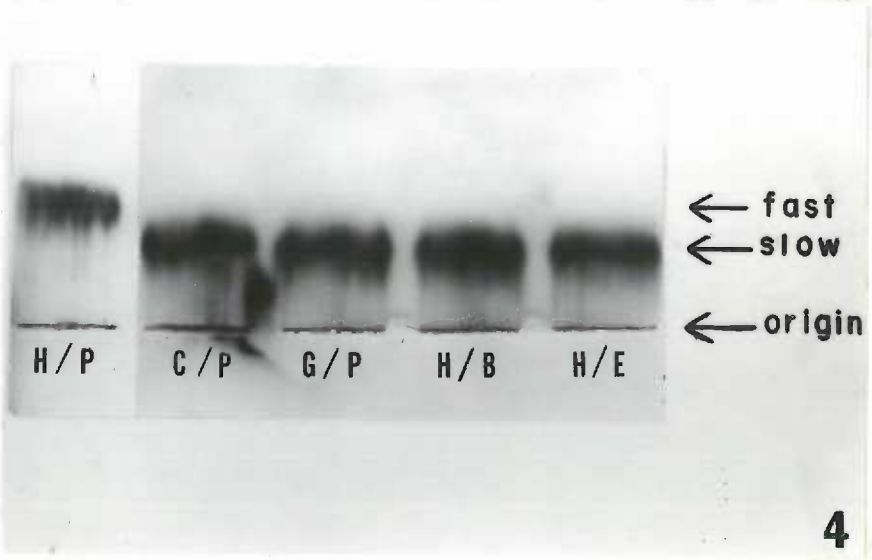

H/P
C/P
G/P
H/B
H/E
origin
slow
fast
2

Figure 3. Zymogram pattern in starch gel of α-glycerophosphate dehydrogenase in Giardia isolates.

Figure 4. Zymogram pattern in starch gel of hexokinase in Giardia isolates.



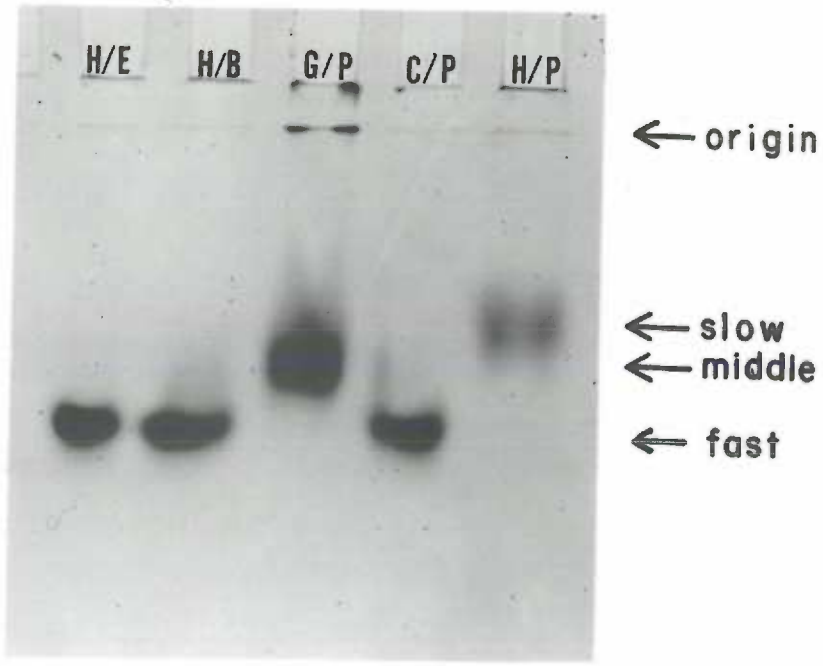
3



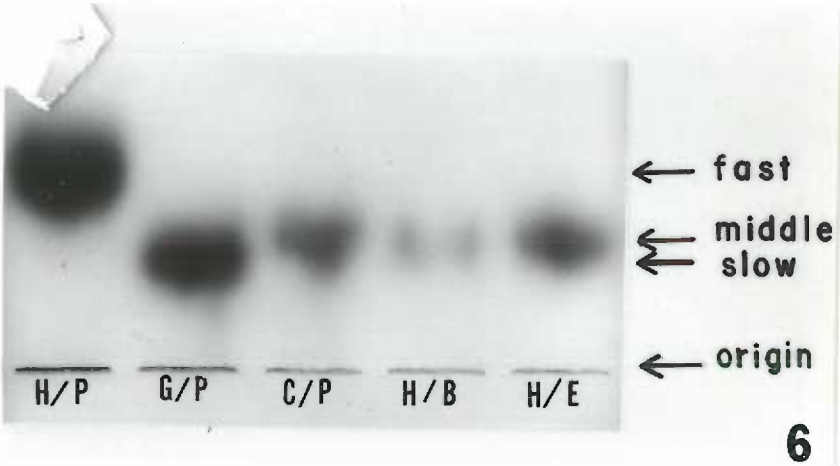
4

Figure 5. Zymogram pattern in polyacrylamide gel of malate dehydrogenase (decarboxylating) in Giardia isolates.

Figure 6. Zymogram pattern in starch gel of 6-phosphogluconate dehydrogenase in Giardia isolates.


H/E
H/B
G/P
C/P
H/P
origin
slow
middle
fast

5


fast
middle
slow
origin
H/P
G/P
C/P
H/B
H/E

6

Discussion

Biochemical taxonomy has been used to address the problems of classification of a number of protozoan parasites. The comparison of the electrophoretic mobility of enzymes has frequently proven to be a successful approach. Electrophoresis of enzymes has been used to differentiate between invasive and non-invasive Entamoeba histolytica (32), to distinguish forms of Trypanosoma cruzi involved in sylvatic and domestic cycles (27), and to resolve speciation problems among African salivarian trypanosomes (12), plasmodia (4), and schistosomes (23).

This paper is the first report of the application of a biochemical approach to Giardia speciation. The isolates examined in this study are of the G. duodenalis type described by Nieschulz (29) and Filice (10). These isolates are presently considered three distinct species: G. lamblia, G. felis, and G. caviae (19), based on host source, implied host specificity, and body shape and measurements.

The strict host specificity of Giardia spp. is in question with the introduction of recent evidence suggesting that humans can be infected with cysts from lower mammals (8, 9) and that some duodenalis-type Giardia can be transmitted among different species of small mammals (15). The significance of many negative cross transmission experiments is difficult to assess since prior infection may result in rejection (31), and many early workers failed to exclude the possibility of prior

Giardia infection in test animals. Furthermore, it is only recently that Bingham (2) devised an *in vitro* technique by which cyst viability can be determined; this test should be included in all protocols to ensure that the cysts being tested are viable.

Recent investigators (14, 36) have promoted the use of morphometrics as a criterion for *Giardia* identification and speciation. Although statistically significant differences in length and breadth measurements between trophozoites of different "species" have been noted, there are grave problems with this methodology. Historically, different investigators have reported widely divergent measurements for the same *Giardia* species; for example, values for the length of *G. simoni* (rat host) range from 12.07 to 15.5 µm (10, 14, 34, 36). A morphometric comparison of the isolates in this study (Paper 1) reveals statistically significant differences among the human *Giardia* isolates, further clouding the validity of this technique for *Giardia* species identification.

The need for a biochemical means of identifying *Giardia* isolates is acute, and will intensify as more strains of these organisms are isolated. Zymograms have proven useful in preventing the misidentification of cell lines (28) and protozoan cultures (13). Furthermore, the isozyme phenotypes of human tumor cell lines (38) as well as those of parasites (4, 12) have proven stable during long term maintenance in the labora-

tory. All of the isolates in this study had stable isozyme patterns over the 15 months of this study.

The zymogram technique has been applied successfully to the identification of parasites in clinical samples contaminated with bacteria (32) or host enzymes (4, 12, 39). In this respect, I have successfully examined a monoxenic Giardia culture from a rabbit (rabbit-1/Portland [24]). The pattern of the enzymes of the contaminating Pseudomonas spp., determined in the absence of Giardia, was distinguishable from the pattern of the Giardia enzymes. The pattern of rabbit-1/Portland was indistinguishable from that of the human-1/Portland isolate (data not shown).

Three of the six enzymes investigated in this study (6PG, G6P, and αGP), have not been previously reported in Giardia species. It is only recently that Giardia physiology and metabolism have been examined (16, 21, 22); as more is understood, it may prove possible to examine more enzymes as potential isozyme phenotypic markers for Giardia isolates.

These data do not in themselves provide the criterion for Giardia speciation. Although there are individuals who claim microbial speciation to be a myth (6), the classification of microorganisms, including their assignment into genera and species, would seem to be a practice here to stay. For a given group of organisms, a successful taxonomy should provide a system of classification which permits the assignment of new

isolates of the grcup to definite categories. The use of such a system may also enable the biologist to consider the ecological and epidemiological interaction of the isolates. Investigations of Giardia species have been hampered by the inability to assign unequivocal species names to isolates of this organism with which they work.

The isozyme data presented suggest that this approach yields data of value in classifying organisms in the genus Giardia. Because isozymes were demonstrated with all 6 enzymes studied, it is suggested that these enzymes be characterized in other isolates as they become available. The study of enzymes of Giardia other than those examined here may provide additional means of differentiating organisms in this genus.

The data presented herein demonstrate that Giardia isolates in this study of one morphological group (G. duodenalis), can be separated, by the patterns of their isozymes, into three groups as follows:

Isozyme Group	Giardia Isolate
I	human-1/England
	human-1/Bethesda
	cat-1/Portland
II	guinea pig-1/Portland
III	human-1/Portland
	rabbit-1/Portland

The practice of assigning Giardia species names solely on the basis of body dimensions or host specificity should be discouraged for the following reasons:

1) Trophozoite dimensions have been shown by Filice (10) to be an unsatisfactory means of identifying Giardia. Once separated from the animal host, it is not possible to identify the source of any organisms of the G. duodenalis type.

2) There is recent evidence, from feeding experiments, that at least some of the Giardia are capable of infecting more than one animal species (8, 15). Further, circumstantial evidence suggests that human Giardia infection may be acquired from cysts excreted by lower mammals (9).

3) Isozyme data presented in this paper indicate that Giardia of the same morphologic type and isozyme pattern may infect different animal species.

4) These data further indicate that Giardia isolates of the same morphologic type, from a single host species (human), may be differentiated biochemically.

This study offers a reproducible means of characterizing Giardia isolates of the G. duodenalis-type. The isolation of additional strains of these organisms and the characterization of their enzymes should yield further insights into Giardia classification. Until more such information is available,

however, I suggest the adoption of the proposal made by Filice in 1952 (10) of recognizing three Giardia species: G. agilis, G. muris, and G. duodenalis.

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Paper 3

Kinetic and physical properties of selected *Giardia* isozymes

Abstract

The physical and kinetic properties of the enzymes malate dehydrogenase (decarboxylating) (ME) (EC 1.1.1.40), malate dehydrogenase (NAD+) (MDH) (EC 1.1.1.37), and glucose-6-phosphate dehydrogenase (G6P) (EC 1.1.1.49) from the cat-1/Portland (C/P), guinea pig-1/Portland (G/P), and human-1/Portland (H/P) isolates of Giardia have been examined.

Previous work grouped five Giardia isolates into three zymodemes on the basis of isozyme patterns after electrophoresis. The isolates selected for this study represent each zymodeme: C/P, Zymodeme I; G/P, Zymodeme II; and H/P, Zymodeme III.

The selected enzymes were further characterized on the bases of the physical properties of molecular weight, isoelectric point and charge to mass ratio, and the kinetic properties of pH optima, Q_{10}, Km, and specific activity.

Isozyme groupings established in the original study were substantiated on the basis of observed differences of physical and some kinetic parameters. Furthermore, no differences were shown between the MDH and G6P isozymes shared by Zymodemes I and II.

Introduction

Until recently, little was known of the enzymes or energy yielding metabolism (EYM) of *Giardia*. This lack of knowledge was hampered by the absence of axenic cultures of this organism. However, the isolation of axenic cultures of several *Giardia* (4, 11, 25, 26) has stimulated research in this area.

Lindmark (20) has reported that the end products of glucose metabolism by *Giardia* were ethanol, acetate, and CO_2. Jarroll et al. have also examined lipid and carbohydrate metabolism (16) and the *in vivo* DNA replication (15) of *Giardia*. Pyrimidine salvage (21) and respiratory metabolism (37) have also been studied.

All of these studies have examined the same *Giardia* isolate and no comparisons of the metabolism or enzymes of different isolates have been published. I have demonstrated that *Giardia* isolates may be grouped according to reproducible isozyme patterns (Paper 2); these isozymes were identified by characteristic migration during electrophoresis in non-denaturing polyacrylamide and starch gels. This paper describes the physical and kinetic properties of several of these isozymes.

Materials and Methods

Isolates.

Three axenic isolates of *Giardia* were used in this study. The three cultures were isolated in Portland from human, cat,

and guinea pig hosts (11, 25, 26). For clarity, each Giardia isolate is referred to on the bases of host source and geographic location; i.e., human-1/Portland (H/P), cat-1/Portland (C/P), and guinea pig-1/Portland (G/P). Previous electrophoretic studies of the enzymes of these isolates indicated that the three Giardia belonged to different zymodemes (Paper 2).

Chemicals.

Reagents, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture method and preparation of cell lysates.

Giardia isolates were grown axenically in test tubes containing six ml of TPS-1 medium supplemented with 10% heat inactivated calf serum and vitamins (Eagle's minimum essential medium) as described by Visvesvara (35).

Late log phase cells were harvested after 96 hours of incubation at 37°C. The tubes were centrifuged at 600 X g for five min. after chilling the culture tubes for one hour at 4°C to release the viable trophozoites adhering to the sides. The cell pellet was washed twice with phosphate buffered saline, pH 7.0, once in 250 mM sucrose, pH 7.2, then resuspended in the 250 mM sucrose, pH 7.2. Trophozoites for most experiments were lysed by freezing at -70°C then thawing at 4°C. Cell free extracts were prepared (20) by centrifugation of lysed cells at 104,000 X g for 10 min. in an Airfuge (Beckman Instruments,

Palo Alto, Ca.). The protein concentration of the lysates was estimated by the method of Bradford (5), modified by using a commercial reagent (Bio-Rad Laboratories, Richmond, Ca.). Bovine gamma globulin was used for the standard curve.

Electrophoresis.

Polyacrylamide gel electrophoresis for molecular weight determination was carried out as described by Laemmli (18) except that sodium dodecyl sulfate was deleted from all solutions; gels were run at 4°C. Enzymes were visualized in the gels as described by Shaw and Prasad (31) or as modified in Paper 2. The molecular weight of the enzymes was estimated by comparing their mobility in gels of different acrylamide concentrations with that of protein standards of known molecular weight, as described by Hedrick and Smith (14). The slope of a plot of the log of protein mobility relative to the dye front versus acrylamide concentration is related to the molecular weight. The molecular weight to slope relationship is established by utilizing well-characterized proteins as standards. Standards for these experiments were: thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; and bovine serum albumin, 67,000. Differences in the molecular weight of the isozymes were statistically analyzed by comparing the slopes of the lines as described by Phillips (29).

Isoelectric point.

Isoelectric point was determined by using LKB Ampholine gels pH 3.5-9.5 (LKB Instruments, Inc., Pleasant Hill, CA). The pH gradient was determined by slicing the gel into 0.5 cm sections and eluting into 0.5 ml deionized H_2O. The pH of each section was determined and the pI of the enzymes calculated by comparing their migration relative to this pH gradient.

Enzyme assays.

All reaction mixtures were contained in a final volume of 1 ml. Assay mixtures used to determine the effect of temperature on observed velocity contained 100 mM Tris (hydroxymethyl) aminomethane [Tris-HCl] buffer mixed as recommended by the manufacturer (Sigma, St. Louis, Mo.) to maintain a constant pH of 7.5 over a temperature range of 25 to 45°C. Saturating substrate concentrations were used. All other enzyme assays were measured at 30°C in a buffer consisting of 100 mM Tris-HCl, 100 mM sodium acetate, and 100 mM sodium cacodylate, pH adjusted as appropriate. The initial velocity kinetic experiments were measured at pH 7.5.

Malate dehydrogenase (decarboxylating) (ME) (EC 1.1.1.40), malate dehydrogenase (NAD+) (MDH) (EC 1.1.1.37), and glucose-6-phosphate dehydrogenase (G6P) (EC 1.1.1.40) activities were determined spectrophotometrically at 340 nm by following (Beckman model 25 spectrophotometer, Beckman Instruments, Inc., Palo Alto, Ca.) the formation of reduced nicotinamide-adenine

dinucleotide phosphate (NADPH) (G6P and ME enzymes) or the oxidation of reduced nicotinamide adenine dinucleotide (NADH) (MDH enzyme).

The standard G6P reaction mixture contained 30 mM $MgCl_2 \cdot 6H_2O$, 40 mM glucose-6-phosphate, monosodium salt, 0.02 mM nicotinamide-adenine dinucleotide phosphate (NADP), monosodium salt, and 0.5% Triton X-100. The standard ME reaction mixture contained 0.66 mM $MnCl_2$, 3.33 mM malic acid, 0.33 mM NADP, monosodium salt, and 0.5% Triton X-100. The MDH reaction mixture contained 0.15 mM oxaloacetic acid, 0.23 mM NADH, and 0.05% Triton X-100. Reactions were started by the addition of cell free extract.

Enzyme units (U) are defined as the amount of enzyme necessary to form 1 µmol of product or to degrade 1 µmol of substrate per minute under the assay conditions described.

Km determination.

Km values were determined from 1/s vs 1/v plots (9) of seven substrate concentrations assayed, in duplicate, in 3-5 different experiments.

pH optima.

The pH optima of the enzymes was determined by assaying relative activity at 0.25 intervals over a pH range of 6.0 to 9.0. Activity at each pH was assayed in duplicate and 2-4

experiments were performed. Optimal pH was defined as greater than 90% relative maximal activity.

Specific activity.

The specific activity was determined at saturating substrate concentrations. Cells were harvested, washed, and resuspended as previously described; however, the trophozoites were used immediately for determination of activity. Cells were lysed by the additon of Triton X-100 to a final concentration of 0.1% (v/v).

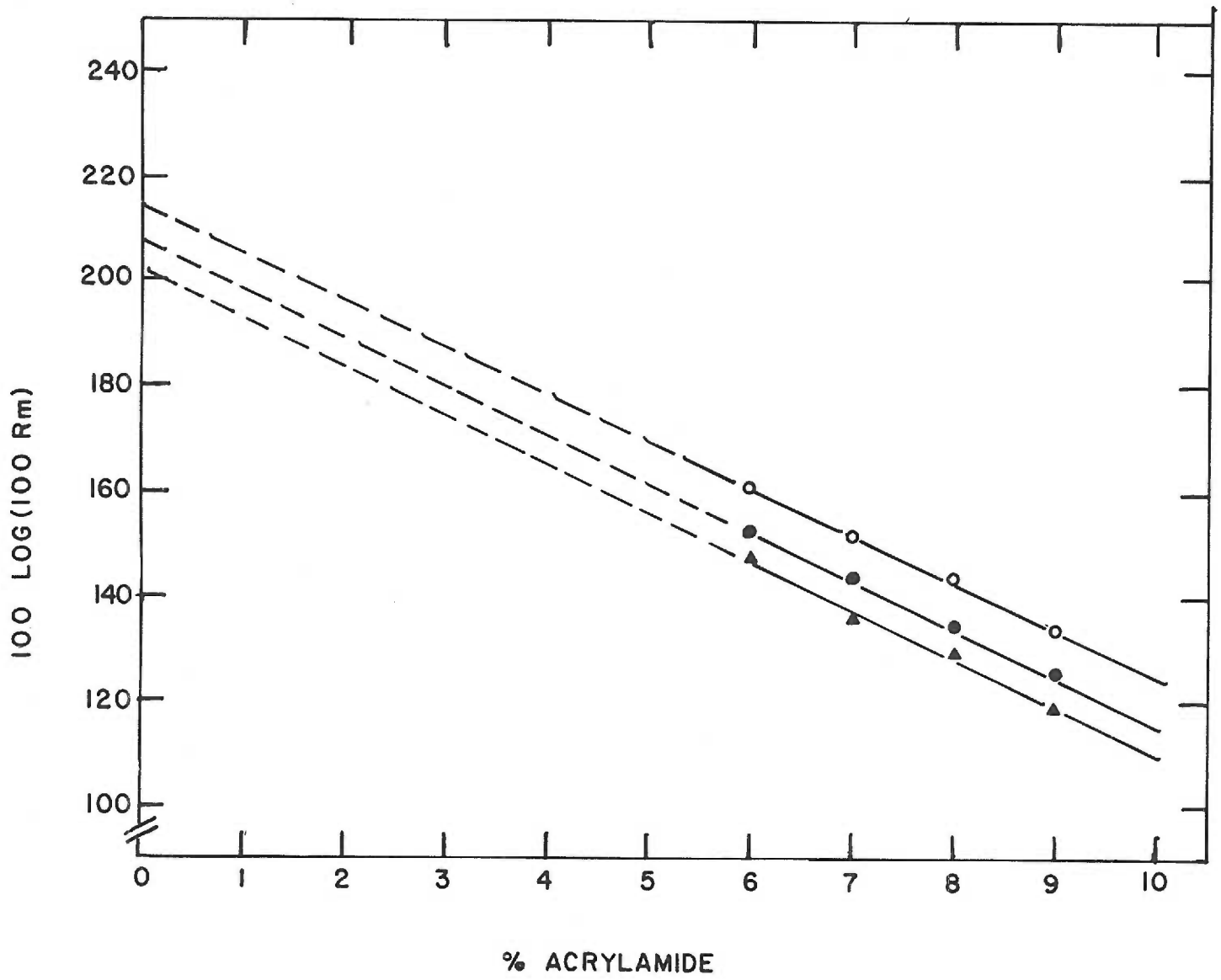
Results

A. Malate dehydrogenase(decarboxylating) (ME).

Physical properties.

A Hedrick and Smith (14) plot of the relative migration of the ME isozymes in acrylamide revealed lines whose slopes were not statistically different (29) and which intersected at different points on the ordinate (Fig. 1). These data suggest that the three isozymes are charge isomers (9). The MW's were 147,000 $\pm$ 11,000 for H/P, 140,000 $\pm$ 13,000 for C/P, and 138,000 $\pm$ 8,000 for G/P (Table 1). The differences in molecular weight (MW) are not statistically significant. The observed pI's, determined after isoelectric focusing, were: H/P, 7.6; C/P, 6.9 $\pm$ 0.1; and G/P, 7.2. These followed the prediction from the Hedrick and Smith plot.

Figure 1. The relative mobility of malate dehydrogenase (decarboxylating) isozymes at different acrylamide concentrations: △, H/P; ○, C/P; ●, G/P.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% ACRYLAMIDE

Table 1. Physical and kinetic parameters of ME from Giardia.

Parameter	ISOLATE		
	H/P	C/P	G/P
Molecular weight[a]	147,000 ± 11,000	140,000 ± 13,000	138,000 ± 8,000
pI	7.6 ± 0	6.9 ± 0.1	7.2 ± 0
pH optima[b]	6.75-8.0	7.0-8.0	7.25-7.75
Km (NADP)[c]	7.0 ± 0.2	5.3 ± 0.6	2.1 ± 0.4
Km (malic acid)	19.3 ± 2.8	24.6 ± 0.7	38.1 ± 0.8

[a] molecular weight ± standard deviation.
[b] greater than 90% relative activity.
[c] μM ± standard deviation.

General properties.

Maximal enzymatic activity was observed over a pH range of 6.75-8.0 for H/P, 7.0-8.0 for C/P, and 7.25-7.75 for G/P (Figure 2). Enzyme activity required the addition of a divalent cation (Mn^{2+}, Co^{2+} or Mg^{2+}) and was inhibited by 1 mM ethylene diaminetetraacetic acid (EDTA). ME was not able to utilize NAD as a cofactor.

Initial velocity.

The apparent Km values (Table 1) for NADP were: H/P, 7.0 $\pm$ 0.2 µM; C/P, 5.3 $\pm$ 0.6 µM; and G/P, 2.1 $\pm$ 0.4 µM. The apparent Km values for malic acid were: H/P, 19.3 $\pm$ 2.8 µM; C/P, 24.6 $\pm$ 0.7 µM; and G/P, 38.1 $\pm$ 0.8 µM.

B. Malate dehydrogenase (MDH).

Physical properties.

A Hedrick and Smith (14) evaluation of the relative mobility of isozymes versus the concentration of acrylamide predicted that the H/P MDH isozyme would differ from the G/P and C/P MDH isozyme in both size and charge as the lines (Figure 3) were not parallel (the difference in slopes was statistically significant [29]) and did not intersect at a gel concentration of 0%. The molecular weight and pI (Table 2) for G/P and C/P MDH isozymes were indistinguishable and were 197,000 $\pm$ 16,000 and 6.6 $\pm$ 0.2, respectively. The MW and pI for the H/P MDH isozyme were 150,000 $\pm$ 16,500 and 6.0 $\pm$ 0.1, respectively.

Figure 2. Profile of the relative activity of malate dehydrogenase (decarboxylating) isozymes at different pH values: △, H/P; ○, C/P; and ●, G/P.

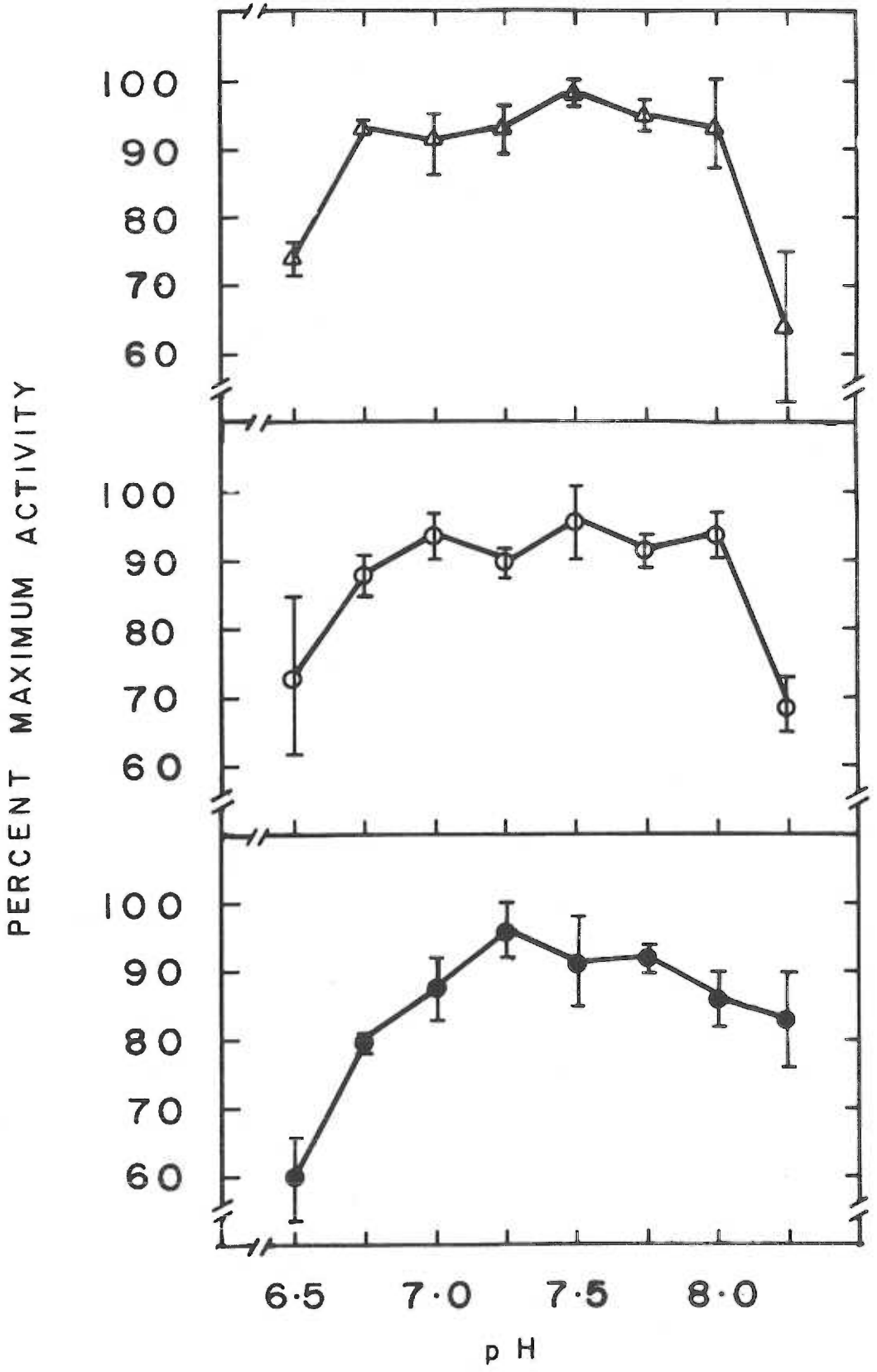
PERCENT MAXIMUM ACTIVITY
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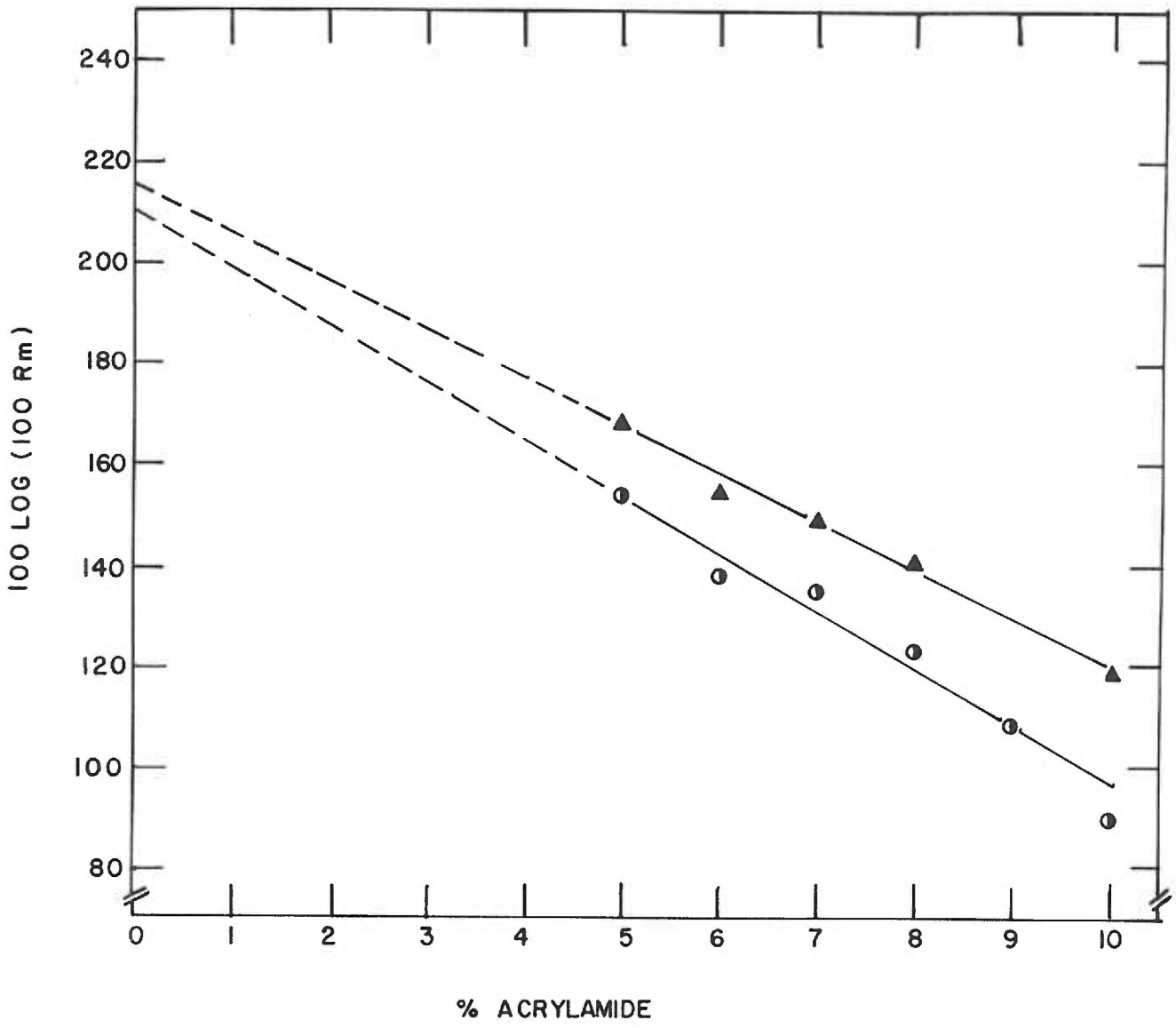
Table 2. Physical and kinetic parameters of MDH from *Giardia*.

	ISOLATE		
Parameter	H/P	C/P	G/P
Molecular weight[a]	150,000 ± 16,500	197,000 ± 16,000	197,000 ± 16,000
pI	6.0 ± 0.1	6.6 ± 0.2	6.6 ± 0.2
pH optimum[b]	7.5 - 8.5	7.25 - 8.0	7.5 - 8.0
Km (NADH)[c]	41.4 ± 4.6	22.8 ± 4.8	20.7 ± 2.8
Km (OAA)	16.8 ± 3.8	37.8 ± 5.2	35.0 ± 3.5

[a] molecular weight ± standard deviation.
[b] greater than 90% relative activity.
[c] μM ± standard deviation.

Figure 3. The relative mobility of malate dehydrogenase isozymes in different acrylamide concentrations: ▲, H/P: and ◑, C/P and G/P.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% ACRYLAMIDE

The observed difference in size was statistically significant ($p \leq 0.05$).

General properties.

Maximal enzymatic activity (greater than 90% relative activity) was observed over a pH range of 7.5 - 8.5 for H/P, 7.25 - 8.0 for C/P, and 7.5 - 8.0 for G/P (Figure 4). Enzyme activity did not depend upon the addition of a divalent cation and was not inhibited by EDTA. NADPH was utilized at less than 1% of the efficiency of NADH.

Initial velocity.

Steady state initial velocity measurements were consistent with typical Michaelis-Menton first order kinetics. The apparent Km values (Table 2) for NADH were: H/P, 41.4 $\pm$ 4.6 µM; C/P, 22.8 $\pm$ 4.8 µM; and G/P, 20.7 $\pm$ 2.8 µM. The apparent Km values for oxaloacetic acid (OAA) were: H/P, 16.8 $\pm$ 3.8 µM; C/P 37.8 $\pm$ 5.2 µM; and G/P, 35.0 $\pm$ 3.5 µM.

C. Glucose-6-phosphate dehydrogenase (G6P).

Physical properties.

A Hedrick and Smith evaluation (14) of the mobility of the G6P isozymes versus the concentration of acrylamide predicted that the H/P G6P isozyme would differ from the G/P and C/P G6P isozyme in both size and charge as the lines (Figure 5) were not parallel and did not intersect at a gel concentration of 0%. The MW and pI (Table 3) for the G/P and C/P G6P isozymes

Figure 4. Profile of the relative activity of malate dehydrogenase (NAD+) isozymes at different pH values: △, H/P; ○, C/P; ●, G/P.

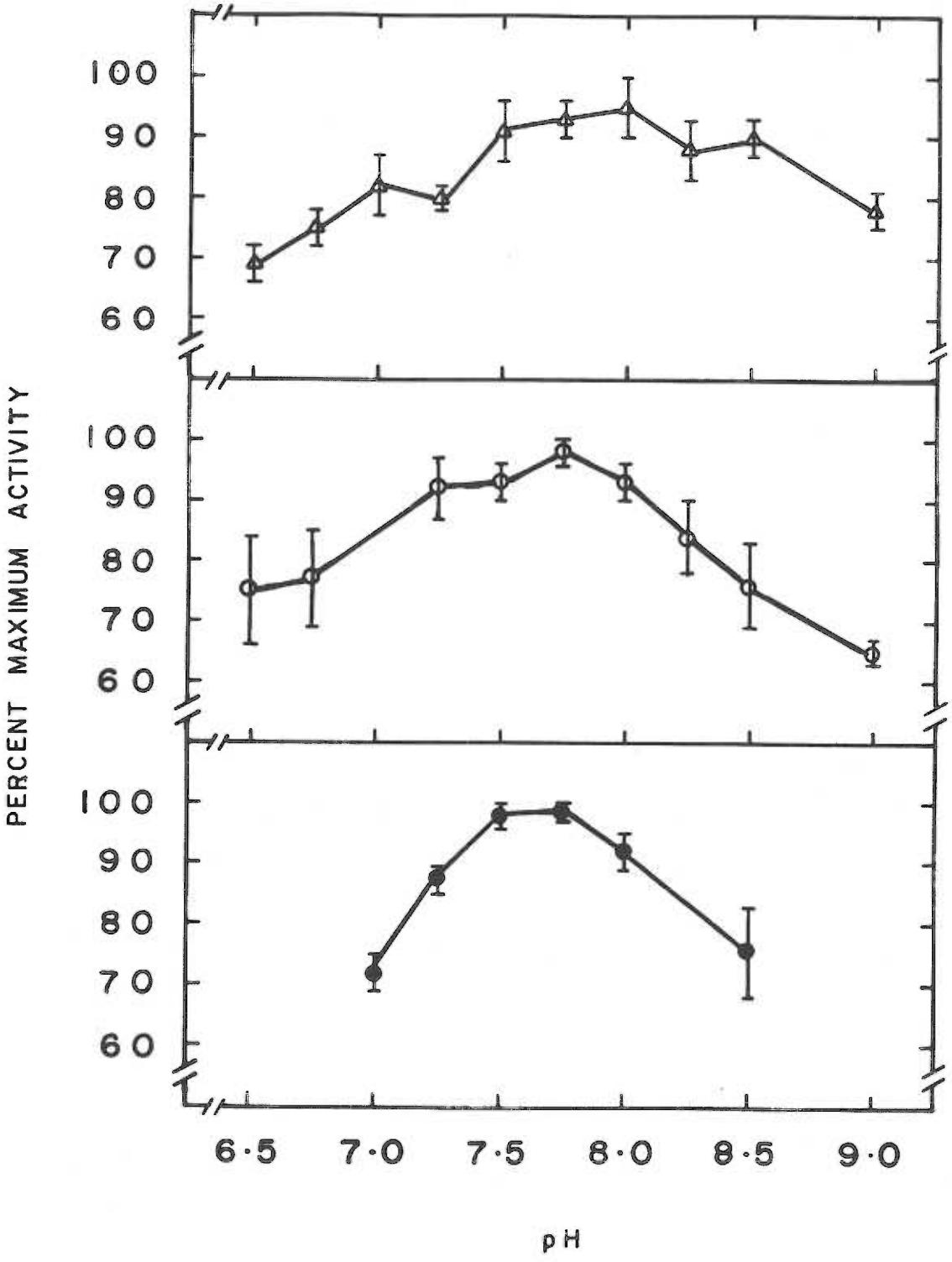
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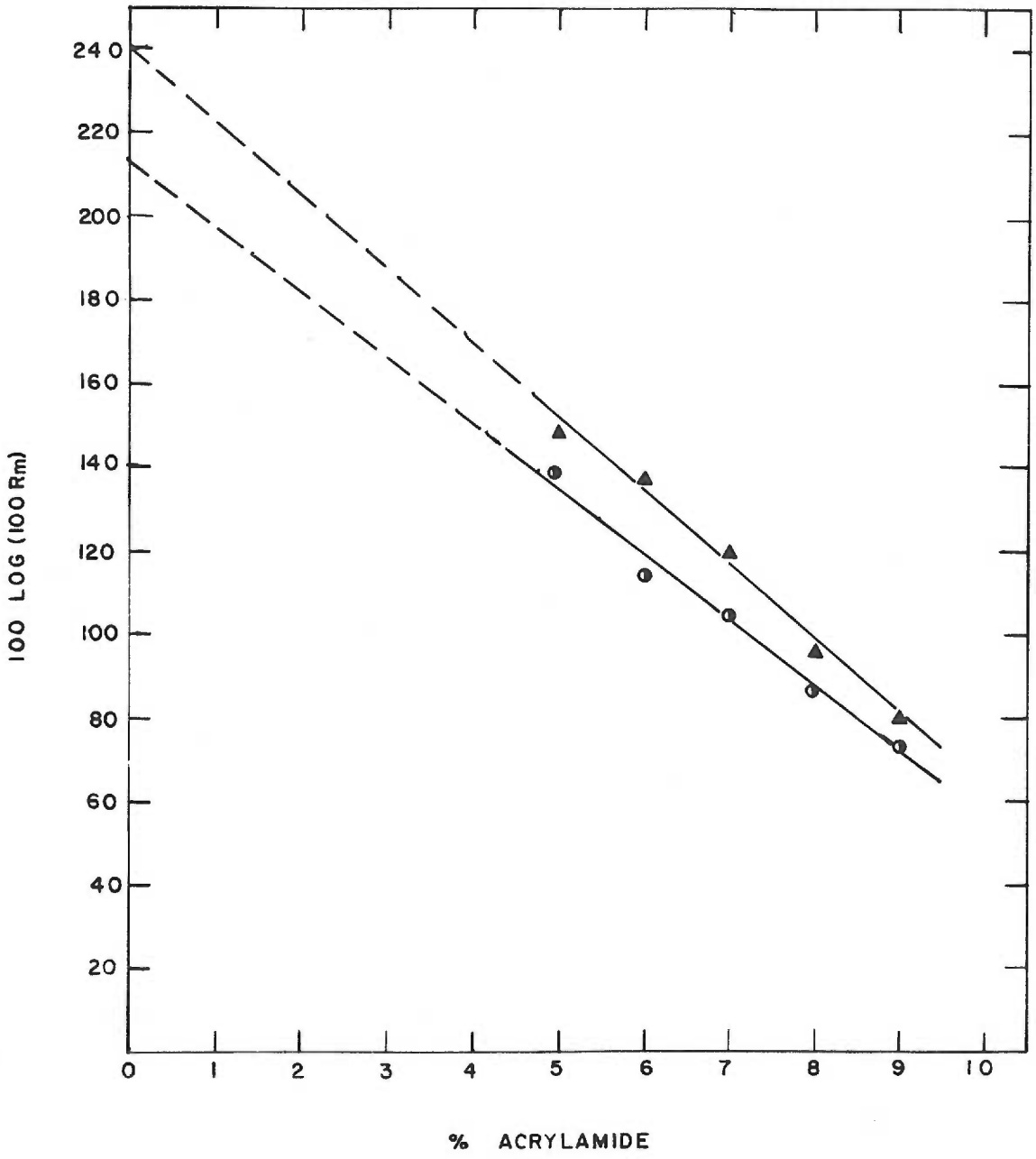
Figure 5. The relative mobility of glucose-6-phosphate dehydrogenase isozymes at different acrylamide concentrations: ▲, H/P; ◑, C/P and G/P.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% ACRYLAMIDE

Table 3. Physical and kinetic parameters of G6P from Giardia.

Parameter	ISOLATE H/P	C/P	G/P
Molecular weight[a]	185,000 ± 31,000	253,000 ± 23,000	253,000 ± 23,000
pI	n.d.[d]	6.9	6.9
pH optima[b]	7.0 - 8.25	7.25 - 8.0	7.5 - 8.25
Km (NADP)[c]	2.4 ± 0.3	2.1 ± 0.2	2.3 ± 0.3
Km (glucose-6-PO_4)	360 ± 76	691 ± 77	565 ± 80

[a] molecular weight ± standard deviation.
[b] greater than 90% relative activity
[c] μM ± standard deviation
[d] n.d.: not determined

were indistinguishable and were 253,000 ± 23,000 and 6.9 respectively. The MW for H/P G6P enzyme was 285,000 ± 31,000;the pI could not be determined. The observed difference in size between the two isozymes was statistically significant.

General properties.

Maximal enzymatic activity was observed over a pH range of 7.0 - 8.25 for H/P, 7.25 - 8.0 for C/P, and 7.5 - 8.25 for G/P (Figure 6).

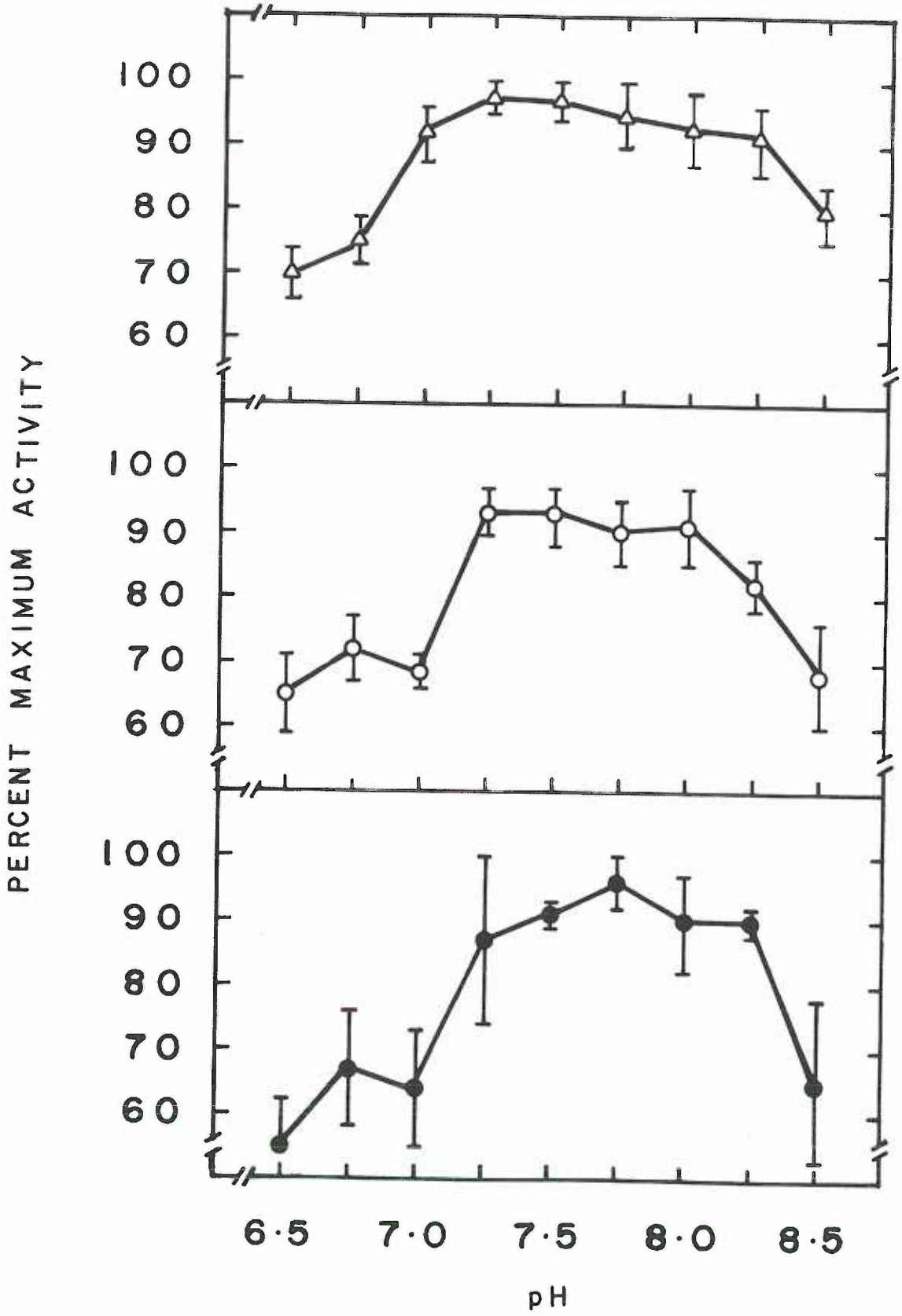
Initial velocity.

Steady state initial velocities were consistent with typical Michaelis-Menton first order kinetics. The apparent Km values (Table 3) for NADP were: H/P, 2.4 ± 0.3 μM; C/P, 2.1 ± 0.2 μM; and G/P, and 2.3 ± 0.3 μM. The apparent Km values for glucose-6-phosphate were: H/P 360 ± 76 μM; C/P, 691 ± 77 μM; and G/P, 565 ± 80 μM.

Specific activity

The most commonly utilized method for monitoring the purification of an enzyme and for expressing the quantity of an enzyme in microorganisms and cells is mU per mg protein. However, when the mg protein per 10^7 cells was determined for each isolate, significant differences were found among the three: H/P, 1.0 ± 0.1 mg/10^7 cells; C/P, 2.3 ± 0.4 mg/10^7 cells; and G/P, 2.3 ± 0.4 mg/10^7 cells. When mU/mg protein was the unit

Figure 6. Profile of the relative activity of glucose-6-phosphate dehydrogenase isozymes at different pH values: △, H/P; ○, C/P; ●, G/P.


PERCENT MAXIMUM ACTIVITY
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for comparison (Table 4), the only statistically significant difference was that the activity of ME in H/P was significantly different from C/P and G/P. The difference observed in specific activity of MDH between the H/P isozyme and C/P and G/P isozymes was not statistically significant. Evaluation of the specific activity, when expressed as $mU/10^7$ cells (Table 4), yielded significant differences between H/P isozymes and C/P and G/P isozymes for MDH and G6P.

Q_{10}

The effect of temperature on observed relative velocity was examined. The results are presented in Figures 7 - 9.

Statistical comparison of the slopes (29) indicated that there were no significant differences between isozymes of MDH or G6P. A significant difference ($p \leq 0.05$) was found between H/P and G/P for ME. The differences between H/P and C/P ME, and C/P and G/P ME were not significant.

Q_{10}, an expression of the rate of increase of enzyme activity for each $10^{\circ}C$ rise in temperature, was determined (Table 5). The values are in the range reported for most enzymatic reactions (18, 32).

Table 4. Specific activities of selected Giardia enzymes.

Enzyme	Isolate	mU/mg protein ± S.D. (no. determinations)	mU/10^7 cells
ME	H/P	25.2 ± 4.4 (3)	25.2 ± 4.4
	C/P	48.1 ± 10.6 (2)	20.9 ± 4.6
	G/P	53.2 ± 8.3 (3)	23.1 ± 3.6
MDH	H/P	2278 ± 500 (3)	2278 ± 500
	C/P	1760 ± 177 (3)	765 ± 77
	G/P	1640 ± 320 (3)	713 ± 139
G6P	H/P	8.4 ± 1.6 (3)	8.4 ± 1.6
	C/P	8.3 ± 1.8 (3)	3.6 ± 0.8
	G/P	8.5 ± 1.3 (3)	3.3 ± 0.6

Figure 7. The effect of temperature on observed relative velocity of malate dehydrogenase (decarboxylating) in Giardia: A, H/P; B, C/P; and C, G/P.

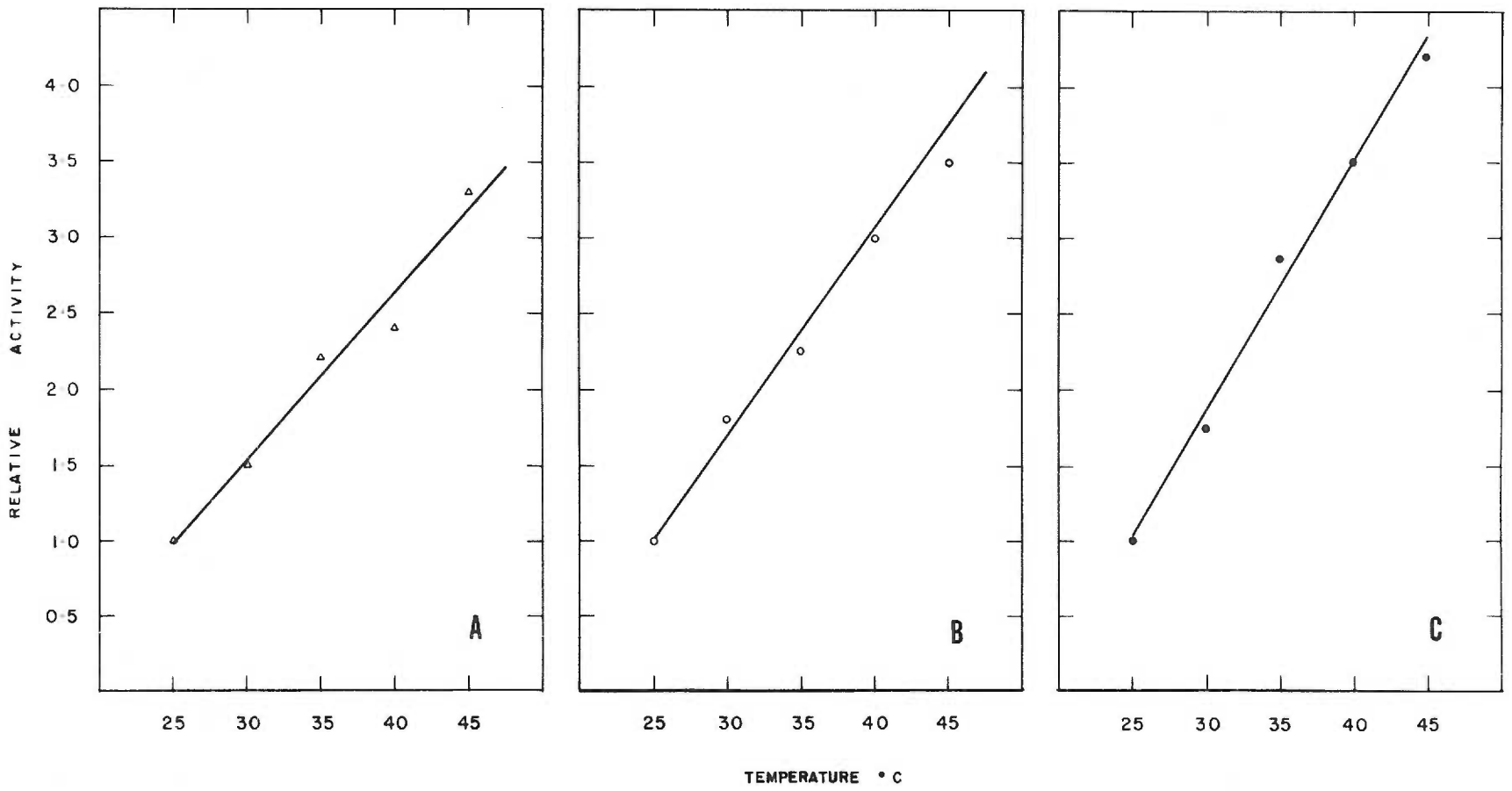
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Figure 8. The effect of temperature on observed relative velocity of malate dehydrogenase (NAD+) in Giardia: A, H/P; B, C/P; and C, G/P.

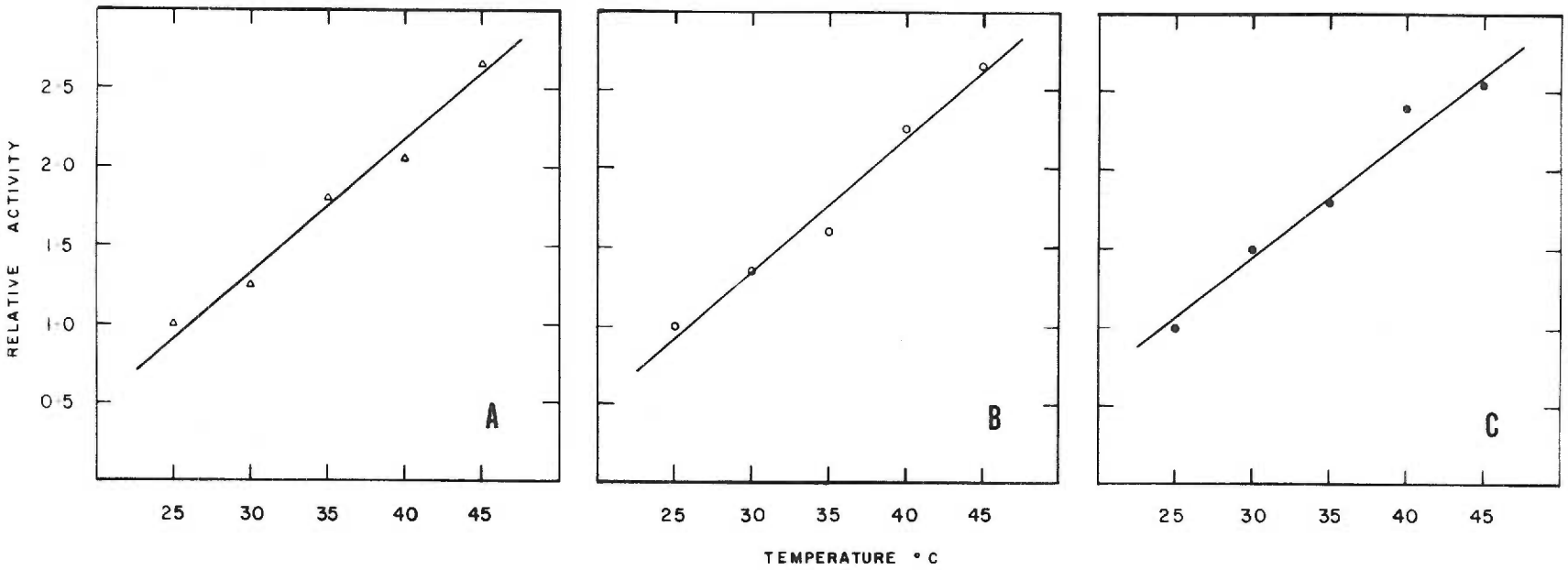
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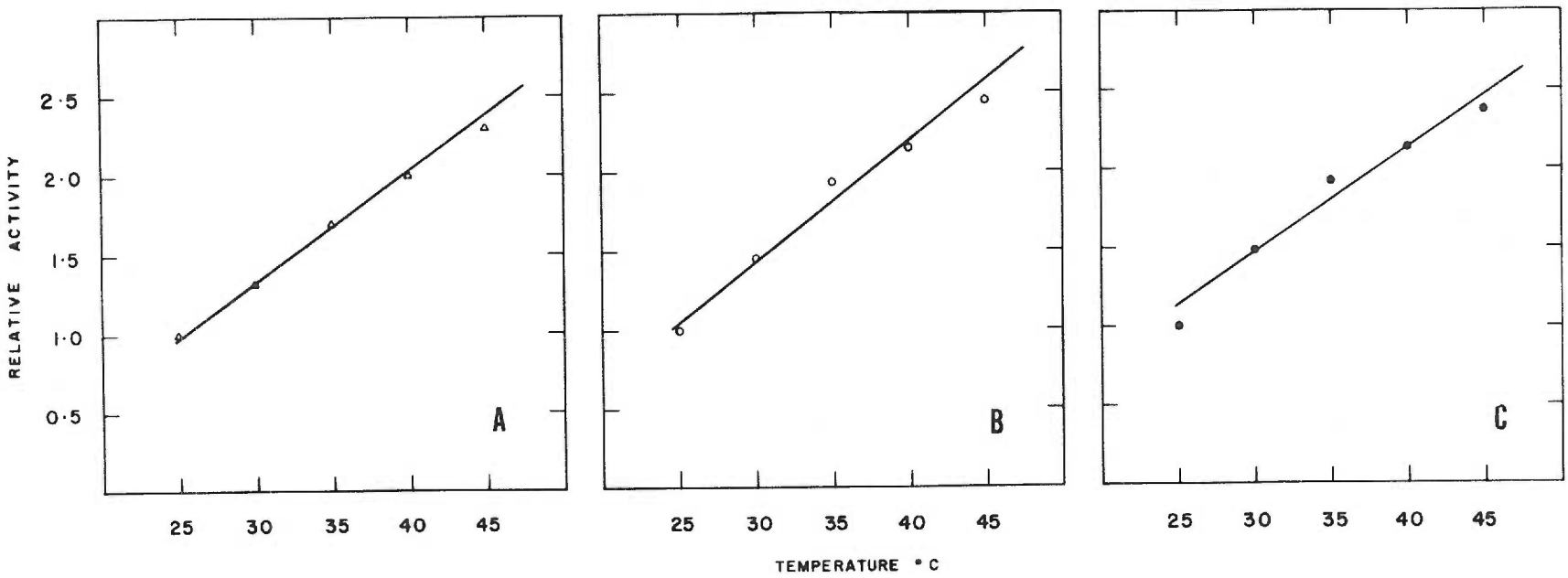
Figure 9. The effect of temperature on observed relative velocity of glucose-6-phosphate dehydrogenase in Giardia: A, H/P; B, C/P; and C, G/P.


RELATIVE ACTIVITY
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TEMPERATURE °C

Table 5. Q_{10} for selected Giardia isozymes.

Enzyme	Isolate	Q_{10}[a]
ME	H/P	1.72
	C/P	1.85
	G/P	1.89
MDH	H/P	1.60
	C/P	1.61
	G/P	1.56
G6P	H/P	1.43
	C/P	1.48
	G/P	1.38

[a] rate of increase of enzyme activity for each 10^{o} rise in temperature

Discussion

This paper is the first comparison of the physical and kinetic properties of metabolically important enzymes of several Giardia isolates.

In an earlier study of axenic cultures of five Giardia isolates (Paper 2), the isolates were grouped into three zymodemes on the basis of isozyme patterns after electrophoresis. Since each isolate within a zymodeme demonstrated only single isozymes for each enzyme examined, these enzymes were determined to be suitable for kinetic studies. Isolates representing each zymodeme (H/P, C/P, and G/P) were chosen for further physical and kinetic characterization of several metabolically important enzymes for which isozymes were found.

Enzymes which perform the same catalytic function but exist in different forms are isozymes: isozymes may show differences in physical and/or kinetic properties.

Physical properties of isozymes have been examined by comparing mobility during PAGE and isoelectric focusing. Enzymes of Naegleria spp. (36), Leishmania spp. (2, 12), Paramecium spp. (1, 34), and nematodes of marine fish (6) have all been compared using PAGE. Protein migration in PAG (without denaturing agents such as sodium dodecyl sulphate) is dependent upon the size, asymmetry, and charge of the protein. Variation in protein size and presumably weight (14) is detected by varying the percentage of acrylamide and thus the pore size of

the gel. A linear regression analysis of the log of the relative mobility (Rm) of a protein at various acrylamide concentrations, the "Ferguson plot" (10), provides an estimation of molecular weight when this slope is compared with the MW to slope relationship established with standard proteins (14). In addition to determining MW, this comparison can detect otherwise cryptic variation in proteins since examination of proteins at a single percentage of acrylamide may not reveal size differences (17).

The Ferguson plot also allows for the comparison, in native gels, of the charge to mass ratio of proteins (14). Proteins with different molecular weights and similar charges (size isomers) are characterized by nonparallel lines which intersect at 0% acrylamide; proteins of identical molecular sizes but different pI (charge isomers) are characterized by nonparallel lines which intersect at 0% acrylamide; proteins which differ in size and charge yield nonparallel lines which do not intersect at 0% acrylamide. The three ME isozymes were found to be charge isomers (Figure 1); MDH and G6P isozymes were demonstrated to be of different size and charge (Figures 3 and 5).

Schistosoma spp. (30), Bulinus snail parasites (38), and freshwater fish (8) have been differentiated on the basis of isoelectric point as determined by electrophoresis in the presence of ampholytes. An examination of the pI of these

enzymes confirmed the predictions of the Ferguson plots. None of the lines in the plots intersected at 0% acrylamide, indicating that the enzymes would not have the same pI. This prediction was confirmed when the enzymes were subjected to isoelectric focusing.

Kinetic properties of isozymes are sometimes examined in an attempt to fully characterize the differences between isozymes. The effect of temperature on maximal velocity of the α-glycerophosphate dehydrogenase (α-GPdH) allele of Drosophila melanogaster (27), hemoglobin and G6P alleles of humans (3) and Chlamydomonas arginosuccinate lyase has been studied (24). Furthermore, the effect of temperature on Km for various substrates of the α-GPdH alleles in D. melanogaster (27) and Colias meadii (17) have been examined. Differences in the Km values of induced thymidine kinases can be used to distinguish HSV-1 from HSV-2 and to distinguish the thymidine kinase of these viruses from that of the host's tissue (36). Differences in substrate specificity (13, 23), specific activity (22), and pH optima (23, 28) have also been used for comparing kinetic properties of isozymes.

For some kinetic parameters (pH optima, Km, and the effect of temperature of maximal velocity) the isozymes were essentially indistinguishable. However, a clear cut difference was seen for one parameter (specific activity).

Two of the enzymes in this paper, MDH and ME, have been examined in Giardia. Lindmark (20) studied the enzymes of one isolate (see Appendix B). The apparent Km values for ME substrates obtained in this study are similar to those which Lindmark observed; the apparent Km values for OAA and NADH of the MDH isozymes differ substantially. Lindmark obtained Km values of 300 μM for OAA and 330 μM for NADH as compared with 16.8 to 35 μM for OAA and 20.7 to 41.4 μM for NADH in this study. The reasons for this discrepancy are unclear. Lindmark did not describe the systems used for the enzyme assays; differences in buffer, how the reaction is started, and pH, may affect the apparent Km.

As much as a 2.5 to 5-fold difference in specific activities of MDH and ME, respectively, were observed between this study and Lindmark's. This could also a reflection of differences in the assay conditions. For example, the activity observed in a 0.1 M phosphate buffer was 40% of that observed in the Tris-acetate-cacodylate buffer used for these experiments (data not shown).

Two enzymes, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase, unique to the pentose phosphate pathway (PPP) have now been detected and described in Giardia. These data confirm radiorespirometry and glucose incorporation data obtained by Jarroll et al. (16) which suggested the presence of an active PPP.

Finally, whether the observed differences have physiological significance is uncertain. However, it is interesting that these organisms, which are classified as different species on the bases of host source and presumed specificity, share important enzymes which are similar on the bases of physical and kinetic properties.

References

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Appendix A. An examination of the total protein profile of *Giardia* isolates: SDS-PAGE and Ouchterlony double diffusion.

Giardia isolates are ubiquitous parasitic protozoa observed in a wide variety of animals. Recent evidence suggests the importance of the waterborne mode of transmission (1, 4) and laboratory experiments have demonstrated cross infection among a variety of mammals (5, 6). These data suggest the need for more precise field-applicable methods for distinguishing among *Giardia* isolates. Such methodology might facilitate the identification of the source of outbreaks of waterborne giardiasis.

Morphology and morphometrics are unreliable means for discrimination among *Giardia* trophozoites (paper 1) and probably for cysts. Isozyme techniques, while very informative (papers 2 and 3), require large numbers of organisms. This study is a preliminary investigation of the possibility for using electrophoretic and immunologic methods for discriminating among *Giardia* isolates.

The *Giardia* isolates for these experiments are as previously described (papers 1, 2, and 3). Trophozoites (cat-1/Portland) for inoculation of rabbits were grown in HSP-3 (9) supplemented with 20% normal human serum. Cells for all other

experiments were grown in TPS-1 (10) supplemented with 10% calf serum.

The sodium dodecyl sulfate polyacrylamide gel (SDS-PAG) electrophoresis system used was as described by Laemmli (8). Gels with a 6-18% gradient of acrylamide were used. Samples (100 µg protein) of calf serum and washed whole trophozoites of each isolate were diluted to 25 µl with deionized water then solubilized with an equal volume of solubilizing buffer which contains 31.25 mM Tris-HCl (pH 6.8), 2% 2-mercaptoethanol, 20% glycerol, and 4% SDS. Samples were heated at 100°C for 5 min. The following proteins (with corresponding molecular weights) were used as molecular standards: phosphorylase B, 93,000; bovine serum albumin, 68,000; pyruvate kinase, 57,000; glutamate dehydrogenase, 50,000; ovalbumin, 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,000. Slab gel electrophoresis was performed at room temperature using a constant voltage of 100 volts over 10cm for 8 hours. Gels were stained overnight in a solution of 0.05% (w/v) Coomassie Blue R250, 25% isopropanol, and 10% (v/v) glacial acetic acid. Gels were then decolorized in 10% acetic acid.

Four Giardia-free New Zealand white rabbits were used as a source of antiserum. Prior to inoculation, the feces of the rabbits were examined for Giardia cysts three times a week for three weeks; rabbits were examined at weekly intervals there-

after. Control sera were drawn from the rabbits prior to the initial antigen inoculation. Antigen was prepared by washing trophozoites (cat-1/Portland) three times with phosphate buffered saline, pH 7.2, then sonicated for two minutes (Biosonik IV, Bronwill, Rochester, N.Y.). The sonicate (4 mg protein) was homogenized in Freund's complete adjuvant for the first injection; for subsequent injections, sonicate was homogenized in Freund's incomplete adjuvant. Rabbits were inoculated subcutaneously at weekly intervals for five weeks. Blood was drawn weekly and anti-Giardia titers were determined by indirect hemagglutination (IHA) (7). Typical IHA titers ranged from 1:1024 to 1:4096; control sera titers were less than 1:8. Antisera from rabbits were collected 6 weeks after the initial inoculation, pooled, and the immunoglobulins precipitated with saturated ammonium sulfate at a final concentration of 33%, and dialyzed against borate buffered saline. The dialysed immunoglobulins were used in subsequent gel diffusion (ID) experiments.

Antigen for ID experiments was prepared by sonication of washed trophozoites for two minutes. The material was centrifuged at 10,000 X g for 2 minutes. ID was performed in 1% agarose (Sigma Corp., St. Louis, MO) in a buffer consisting of 73 mM Tris-HCl, 22 mM Sodium Barbital, 0.47 mM $CaCl_2$, and 0.2 mM NaN_3, (pH 8.6) (3).

Protein was estimated by the method of Bradford (2) modified by using a commercial reagent (Bio-Rad, Richmond, CA). Bovine gamma globulin was used as the standard.

An SDS-PAG of the five isolates, calf serum (a medium component for these trophozoites) and molecular weight standards is presented in Figure 1. A minimum number of major protein bands are observed in calf serum--approximately ten. The major protein in calf serum appears to be bovine serum albumin (BSA) (68 K), a band which is absent from C/P, G/P, H/B, and H/E. BSA may be a minor band in H/P. More important, it does not appear that the Giardia isolates share many major bands with calf serum.

Substantial homogeneity is seen among H/B, H/E, C/P, and G/P. The protein profile of H/P is missing some bands present in the other four isolates and contains some not seen in those four.

The Ouchterlony data is presented in Figure 2. The center well contains antiserum to cat-1/Portland, the outer wells contain 60 g (protein) of antigen. No reaction was observed between the antiserum and calf serum, a component of the medium used to grow the cells for antigen in ID but not the cells used for immunization (data not shown). No reaction was observed between pre-inoculation sera and any of the antigens.

Far more variation was observed among the five isolates when compared by immunodiffusion than when compared by total

Figure 1. SDS-PAG of calf serum (CS), and whole trophozoites, and molecular weight standards. (A) Three protein bands observed in CS (1, 2, and 3) were also observed in the trophozoites. Bands 1 and 2 are found in all five isolates. Band 3 appears to be bovine serum albumin and was only observed in human-1/Portland (HP). (B) At least two bands, Bands 9 and 10, observed in CP, guinea pig-1/Portland (GP), human-1/Bethesda (HB), and human-1/England (HE) were not seen in HP. Bands which may be common with the other four isolates but migrated to a slightly different location (4-8) were observed in H/P.

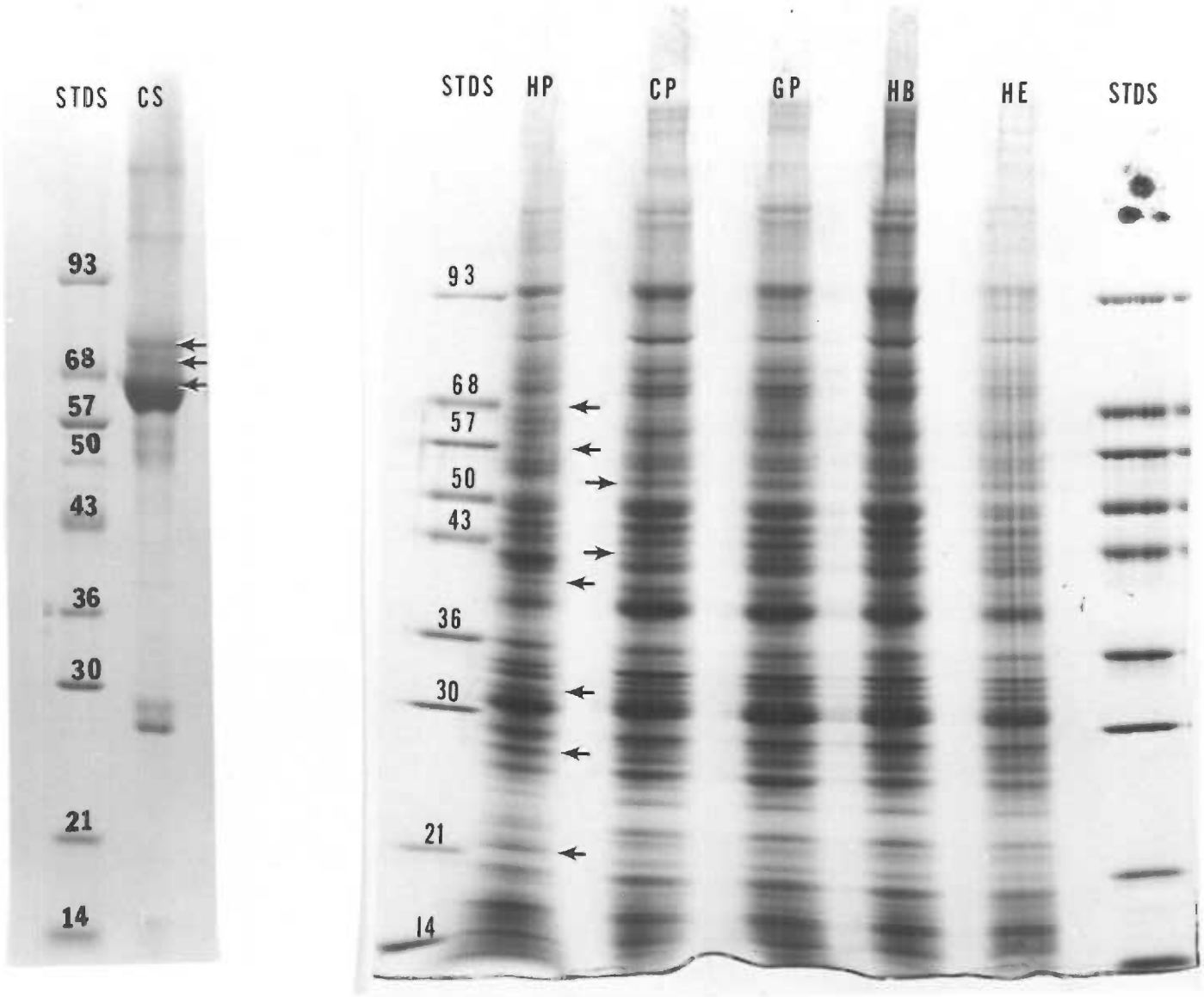
STDS
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Figure 2. Ouchterlony double diffusion; S, antiserum to cat-1/Portland; cs, calf serum; CP, cat-1/Portland antigen; HP, human-1/Portland antigen; GP, guinea pig-1/Portland antigen; HB, human-1/Bethesda antigen; HE, human-1/England antigen.

protein profile. H/P has lines of identity and of non-identity with all four other isolates. These data correspond with the substantial isozyme differences and the protein profile differences, in PAG, already reported. Minor immunologic differences were observed between C/P and G/P; primarily lines of identity were seen with one spur towards G/P. Even more interesting are the lines of identity and non-identity between H/E and C/P and the strong spurs between C/P and H/B, G/P and H/B, and G/P and H/E.

The data suggest the following: 1) isolates which share no isozymes may share many proteins as demonstrated by total protein profile, yet may have substantial antigenic differences; 2) isolates which appear identical on the basis of total protein profile, may share some isozymes and have minor antigenic differences; and 3) enzymatically identical isolates (at least of the enzymes examined) may appear identical on total protein profile, and yet differ substantially on the basis of antigenic comparison.

These results and the recent development of hybridomas to Giardia trophozoites (S. Kattarri, personal communication) suggest that antigenic studies may prove a fruitful means for distinguishing among Giardia isloates. Such studies may include the exploration of antigenic differences and similaries by cross absorption of antiserum raised against these isolates and the use of these sera in immunofluorescence studies.

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Appendix B. Cross contamination of Giardia cultures.

The possibility for cross contamination of cell lines has been well-established by Nelson-Rees et al. (13). For tissue culture lines, this problem is circumvented by the use of isozymes, karyotype, and other phenotypic markers of cells. Until this study, there were no unambiguous phenotypic markers by which Giardia cultures could be identified.

The trophozoite cultures originally designated for these studies had been caried in continuous culture in E.A. Meyer's laboratory for ten years. Frozen culture stocks were not available due to a laboratory accident. These trophozoites were identified on the basis of host: human, cat, and guinea pig.

Initial experiments identified suitable enzymes for the study of isozymes (at that time nothing was known of Giardia physiology). Attempts to demonstrate differences in migration of enzymes in starch and polyacrylamide gel were unsuccessful. Rabbits were immunized with the three isolates and the serum used in Ouchterlony double diffusion, immunoelectrophoresis (IEP), and two dimensional IEP. No significant differences were found.

Serendipity intervened. In November 1980, while looking through a freezer in J.V. Hallum's laboratory, E.A. Meyer found some old frozen stocks of these same Giardia cultures. The

culture stocks were dated as follows: guinea pig-1/Portland, 1971; cat-1/Portland, 1972; and human-1/Portland, 1973.

These are the cultures used for the experiments in this thesis. Differences among the isolates were found upon examination for isozymes.

The isozymes of the continuous culture strains (CCS) were compared with the recovered frozen stocks. The patterns of the CCS were identical with that of cat-1/Portland, 1972. On the basis of these data, I believe that the cat-1/Portland isolate is the contaminating strain in the CCS.

The strain identified in the literature as a Giardia isolate from a human host in Portland (1-12, 14-23) was obtained from E.A. Meyer's laboratory and is suspected to be the cat-1/Portland isolate. A letter to this effect is to be sent to the investigators and to the Journal of Parasitology.

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V. Discussion and Summary

Since van Leeuwenhook first observed *Giardia* in his own feces in 1681 (5), the organism has been described in many vertebrates (11). This flagellated protozoan parasite has been the subject of continued disputes regarding nomenclature and concommitant taxonomy since the late 1880's. These disputes are inspired by the presence of relatively few characteristics which can be used to differentiate *Giardia* trophozoites from different mammalian hosts.

Giardia isolates are traditionally described and speciated on the basis of host (and presumed host specificity), the dimensions of the trophozoites (morphometrics), and the morphologic type of the trophozoites.

The most frequently used criteria for speciation, host source/implied host specificity, and morphometrics, are difficult to reproduce. Demonstration of host specificity by cross infection studies is difficult. False negative results may occur from immune rejection or the use of non-viable cysts or trophozoites. False positive results may occur from recrudescence of cryptic infections or natural acquisition of giardiasis during the course of an experiment. Morphometrics is subject to numerous technical problems. Cell distortion occurs during fixation and staining. In addition, environmental influences are uncontrolled since trophozoites are obtained by

duodenal aspiration or dissection of an animal host and not from axenic cultures.

Giardia trophozoites are also grouped by some authors (6, 17) into three different species based on morphologic type. These categories were originally described by Nieshulz (18) and later confirmed by Filice (6):

1) Giardia agilis, a parasite of tadpoles and frogs, has long, teardrop-shaped median bodies;
2) Giardia muris, a parasite of rodents, birds, and reptiles, has two small round median bodies; and
3) Giardia duodenalis, a parasite of warmblooded vertebrates, including humans and rodents, has median bodies which resemble the claw of a claw hammer.

While this scheme is simple and reproducible, it is not a popular method for speciation since it does not provide a means for further characterizating the isolates within the three morphologic groups.

Axenic (pure) cultures of Giardia of the G. duodenalis morphologic type from several mammalian host species (2, 15, 16) have allowed the use of techniques not previously available. Studies on the physiology and metabolism of Giardia (10, 11, 12) and in vivo DNA replication (9) have been initiated in recent years.

The five isolates examined in this thesis are axenic cultures of Giardia of the G. duodenalis type. Based on host

zoites of the five isolates demonstrated significant differences in length and breadth between many isolates assigned to different species categories. However, in some cases, only length or breadth differences could be found and, in one case, no statistically significant differences were found between two isolates assigned to different species. Moreover, significant differences in the length and/or breadth between organisms assigned to the same species were found.

These data indicate that morphometrics alone cannot serve as a reliable criterion for the identification and speciation of Giardia isolates even when axenic cultures are examined. It may also be that morphometrics may not give sufficient information to even warrant its use as one of several criteria for speciation.

Paper 2 is the first report of the application of a biochemical approach to Giardia taxonomy. Since the isolates used in this study are of the same morphologic type, there is a need for a simple and reproducible means to distinguish between isolates. Isozyme phenotypes are stable during long term laboratory maintenance of parasites (3, 7) and human tumor cell lines (19).

The electrophoretic mobility of six different enzymes in starch and polyacrylamide gels was examined. The enzymes were malate dehydrogenase (NAD+) (MDH), malate dehydrogenase (decarboxylating) (ME), glucose-6-phosphate dehydrogenase (G6P), α-

glycerophosphate dehydrogenase (αGP), hexokinase (HK), and 6-phosphogluconate dehydrogenase (6PG). Electrophoretic heterogeneity was found among these Giardia enzymes. Three different isozymes were detected for ME and 6PG; two isozymes were found for MDH, G6P, αGP, and HK. In none of the enzymes examined were multiple isozymes present in a single isolate. Based on the observed electrophoretic patterns, the five axenic cultures as well as one monoxenic culture (rabbit-1/Portland) were divided into three groups: Zymodeme I, human-1/England, human-1/Bethesda, and cat-1/Portland; Zymodeme II, guinea pig-1/Portland; and Zymodeme III, human-1/Portland and rabbit-1/Portland. These results demonstrate that Giardia isolated from different mammalian hosts share multiple isozymes and further question the validity of speciation based on the animal host from which the protozoan was obtained.

The enzymes ME, MDH, and G6P were selected for further characterization (Paper 3). The physical properties of molecular weight, isoelectric point, and charge to mass ratio, and the kinetic properties of pH optima, Q_{10}, Km, and specific activity were examined. Each zymodeme, as defined by isozyme patterns after electrophoresis, was represented by the isolates selected for this study: Zymodeme I, cat-1/Portland; Zymodeme II, guinea pig-1/Portland; and Zymodeme III, human-1/Portland.

The three ME isozymes were found to be charge isomers: the isozymes of G6P and MDH were demonstrated to be of different

size and charge. The isozymes of each enzyme were essentially indistinguishable with respect to pH optima, Km, and Q_{10}. Clear cut differences were seen only in specific activity.

Isozyme groupings established on the basis of electrophoretic mobility were substantiated on the basis of differences in the physical and some kinetic parameters. No differences were shown in the MDH and G6P isozymes shared by Zymodemes I and II.

Less variation among the five isolates was observed when the total protein profile was examined by SDS-PAGE (Appendix A). The total protein profile of the human-1/Portland isolate was qualitatively different from that of the other four isolates (cat-1/Portland, human-1/Bethesda, human-1/England, and guinea pig-1/Portland). No differences were observed among the other four isolates.

There are two possible reasons for the paucity of observable variations in SDS-PAGE when compared with the number of differences seen with electrophoresis of enzymes. Electrophoresis of proteins in a non-denaturing gel will detect differences in amino acids which result in changes of charge; about 30% of amino acid substitutions result in a change in charge. SDS-PAGE obviates differences in charge; migration is based on size. Size differences of at least 500-1000 daltons are required for detection; thus single amino acid substitutions would escape detection.

The location of the proteins examined in these two studies (enzymes vs. total protein) differ in relative abundance and in subcellular origin. Structural protein components of membranes are more abundant than enzymes. Furthermore, it can be argued that protein to protein interactions such as occur in membranes place constraints in the number of mutations which may occur.

Therefore, while substantial differences have been observed in some parasites when examined by SDS-PAGE, few were observed among the five Giardia isolates examined.

The immunodiffusion data demonstrated far more variation among the five isolates than did the total protein profiles. The results suggest the following: 1) isolates, which share no isozymes, may share many proteins as demonstrated by total protein profile, yet may have substantial antigenic differences; 2) isolates which appear identical on the basis of total protein profile, may share some isozymes and have minor antigenic differences; and 3) enzymatically identical isolates (at least of the enzymes examined) may appear identical on total protein profile, and yet differ substantially on the basis of antigenic comparison.

The objectives of biological classification have been recently summarized by Mayr (14). They are: 1) to serve as the basis of biological generalizations in all sorts of comparative studies and 2) to serve as the key to an information storage system. Biological classification is generally based on gene-

tic differences which are observed as phenotypic characteristics.

It is important that Giardia classification serve these goals of biological classification, and this thesis sought to address some of those questions. From the data within this thesis and other recent studies, it appears that the practice of assigning Giardia species names solely on the basis of body dimensions or on host source/specificity should be discouraged:

1) Trophozoite dimensions are unsatisfactory means of identifying Giardia. Once separated from the animal host, it is not possible to identify the source of any organism of the G. duodenalis type on the basis of size.
2) There is recent evidence, from feeding experiments, that at least some Giardia are capable of infecting more than one animal species (4, 8).
3) Isozyme data presented herein indicate that biochemically indistinguishable Giardia of the same morphologic type, may infect different animals, and that isolates from a single host species (human), may be differentiated biochemically.

It is obvious that additional isolates of Giardia need to be studied. However, while the amount of genetic difference which is required to constitute a separate species varies widely, it is clear that the reliance on minor and uncertain

differences does not provide a useful format for Giardia speciation. Therefore, until more information is available, it is suggested that Filice's proposal (6) of recognizing three Giardia species: G. agilis, G. muris and G. duodenalis be adopted. It is also proposed that these species designations be subdivided into zymodemes (1) (a population with a unique combination of isozyme patterns). This scheme will permit the further characterization and identification of Giardia isolates and help prevent misidentificaiton of axenic cultures (Appendix B).

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