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Behavior of *Leishmania* in Panamanian Phlebotomine Sandflies Fed on Infected Animals[1]

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JOHNSON, PHYLLIS T., AND HERTIG, MARSHALL. 1970. Behavior of *Leishmania* in Panamanian Phlebotomine Sandflies Fed on Infected Animals. *Experimental Parasitology* 27, 281–300. Two species of Panamanian sandflies, *Lutzomyia sanguinaria* and *Lu. gomezi*, were fed on hamsters infected with various strains of *Leishmania*. Repeated trials proved that all Panamanian strains of *Leishmania braziliensis*, whether isolated from man, other mammals, or wild sandflies, produced infections in both sandfly species that were characterized by growth of leptomonad (promastigote) flagellates in the hindgut, especially the "hind triangle," with or without growth in the midgut. Over 90% of the flies had attached flagellates in the hindgut, and almost half of these had attached flagellates only in the hind triangle. A strain of *L. braziliensis* from Peruvian espundia reacted similarly in the sandflies. On the other hand, two strains of *L. mexicana* from Guatemala and British Honduras typically caused midgut infections alone in *Lu. sanguinaria* and *Lu. gomezi*. In over 70% of the flies, flagellates were confined to the midgut alone, without even free flagellates in the hindgut.

These studies indicate that position of flagellates in the sandfly gut may serve as a reliable taxonomic character for the separation of strains or species of *Leishmania*.

INDEX DESCRIPTORS: *Leishmania mexicana, Leishmania braziliensis,* phlebotomine sandflies, *Lutzomyia,* cutaneous leishmaniasis, *Leishmania* growth patterns in phlebotomines.

A short time after the discovery in 1924 of the striking behavior of the parasite causing Indian kala azar in the gut of infected *Phlebotomus* sandflies, it was found that essentially the same phenomena and growth pattern characterized all sandfly infections with *Leishmania* of Old World kala azar and oriental sore. The flagellates tended to accumulate at the anterior part of the midgut, the cardia, where they multiplied and many became attached by their flagella to the gut wall. At times there was growth forward into the foregut,–esophagus, pharynx, and mouthparts. It became the general belief that this growth pattern at the "anterior station," which provided an obvious mechanism for transmission by the sandfly bite, would be found in all the leishmaniases of man and other mammals, and indeed, it seemed that this pattern would hold for the New World leishmaniases as well (see review by Adler, 1964).

In early work with some lizard leishmaniae, the flagellates adopted a posterior position, i.e., in the hindgut. This gave rise to the belief that natural infections with a

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posterior station indicated a reptile as the vertebrate host. As Adler (1964) pointed out, this view proved untenable since certain other lizard leishmaniae were found to adopt an anterior position.

Intensive studies on phlebotomine sandflies and leishmaniases, which have been carried on in various parts of the world and are still in progress, have built up an impressive body of data supporting the generally accepted view that sandflies are the chief vectors of all the leishmaniases. Sandflies of many species have been shown experimentally to be susceptible to infection with almost any species of *Leishmania*. Sandflies have been found naturally infected. Many attempts at transmission have been made, with few successes and many failures, the reasons for which are not understood. With regard to the behavior of the flagellates in infected sandflies, collective attention, so to speak, has been focused on the anterior growth pattern while the rest of the sandfly gut seems to have been largely neglected. For example, in several studies by Pessoa and associates (summarized by Johnson *et al.*, 1963), out of a total of over 11,000 Brazilian sandflies, 26 were found naturally infected. Pessoa and Barretto (1948) reviewed these findings but gave no information about the location of the flagellates except in the case of two pharyngeal infections. In one case, Coutinho (1940) described and figured the flagellates in the pharynx but did not mention whether there were any elsewhere in the gut.

It is interesting to note, however, that in the very first studies in India, flagellates from some of the midgut infections extended into the hindgut. Knowles *et al.* (1924) noted one instance out of 25 infected sandflies. Christophers *et al.* (1925) found 5 out of 15 infected sandflies with flagellates in the hindgut, quite numerous in some and in one case extending to the rectum. These five were sandflies with heavy midgut infections, dissected on the fifth day after feeding on kala azar patients. One 4-day infection had one organism in a Malpighian tubule, few in the midgut, and none in the hindgut. In the midgut, the flagellates were commonly attached to the gut wall but those in the hindgut were free forms.

In 1958, the Leishmaniasis Group at Gorgas Memorial Laboratory, Panama, began the experimental infection of sandflies by feeding them by the pipet technique on mixtures of blood and flagellates from cultures (Hertig and McConnell, 1963). Over 800 sandflies, mostly laboratory reared and belonging to five species, were fed leptomonad forms (promastigotes) of nine Panamanian human and two spiny rat strains. The behavior of all strains in the five species was essentially similar. By the third day multiplication had taken place in the blood meal, at which time, and occasionally by the second day, flagellates had begun to congregate near or become attached to the proventricular valve. Also, beginning at the third day, the hindgut was invaded, with flagellates singly or in patches and rosettes attached to the epithelium of the short, thin-walled portion of the hindgut just posterior to the opening of the Malpighian tubules. This portion, an elongated triangle in outline, was called the "hind triangle" (Fig. 1). By the fourth or fifth day its wall was often completely covered, with very few flagellates attached elsewhere in the hindgut. The lumen might contain flagellates limited to the triangle or extending to the rectal ampulla. These hindgut infections tended to persist throughout the life of the sandfly. This unexpected and "unorthodox" departure from the "anterior-station" doctrine by no means invalidated the basic assumption that anterior-station growth provides the mechanism of transmission by bite, since

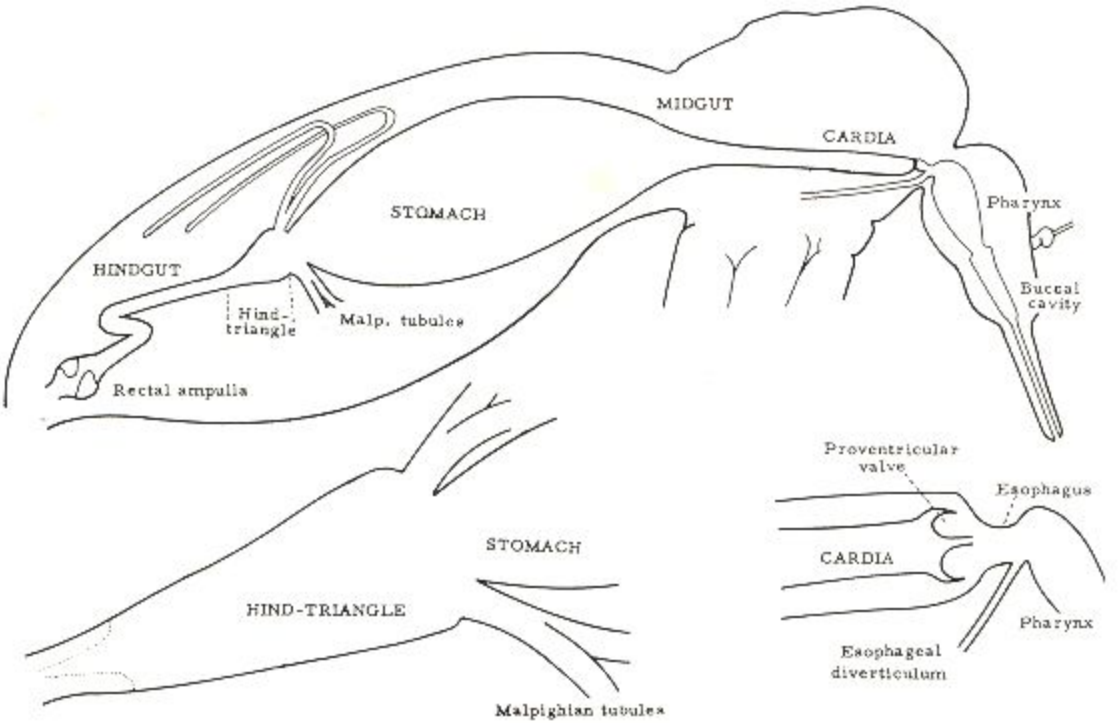


FIG. 1. Diagram of sandfly gut.

the hindgut infections merely accompanied the "standard" anterior growth phenomena in these experimental infections.

When Panamanian sandflies were first found naturally infected with leptomonad flagellates in 1961 (Johnson *et al.* 1963) it was not surprising to find flagellates attached in the hind triangle. This pattern proved to be characteristic of the 400-odd infections found in over 5000 females of seven species (including the five species of the artificial feedings). Pure cultures were obtained about 100 times (McConnell 1963); 19 of these strains were tested in hamsters and 5 produced lesions indistinguishable from those following inoculation of Panamanian human strains (McConnell 1963; Schneider and Hertig 1966). The natural infections, however, differed from those of the artificial feeding series in that, while anterior infections did occur, the flagellates were often confined to the hindgut, particularly the hind triangle, and many infections consisted of relatively few flagellates, at times only 12 or less. One possible explanation was that the natural infections were the result of ingesting relatively few L-D bodies (Leishman-Donovan bodies, amastigotes) with the infecting blood meal from the unknown vertebrate host. They could thus be regarded as incipient infections, with a few organisms having transformed to the flagellate stage with perhaps some multiplication in the blood meal in the midgut and as being in the process of establishing themselves at the first point of attachment, the hind triangle. On the other hand, the artificially fed sandflies received large numbers of flagellates to begin with, which were thus able to bypass some of the steps which had to be followed by the much smaller number of L-D bodies.

Some support for this view was provided by the increasing proportions of midgut infections, probably indicating forward growth from the hindgut, in wild-caught sandflies held for varying periods up to 5 days before dissection.

The examination of wild-caught sandflies was accompanied by feeding laboratory-reared sandflies on hamsters infected with various strains of *Leishmania* from Panama and elsewhere (Johnson *et al.* 1963). In general, the Panamanian strains produced the same growth pattern as the natural infections, especially with regard to flagellates in the hind triangle. It was found, however, that the growth pattern of certain other strains was consistently different in that flagellates rarely became attached in the hind triangle. This seemed to offer what could be in effect a taxonomic character, possibly useful in the still unsettled nomenclature of the American species of *Leishmania*. This work is the subject of this report.

Materials and Methods

The strains of *Leishmania* used included a Guatemalan human strain, a human strain of *L. mexicana* from British Honduras received from Prof. P. C. C. Garnham, a strain of *L. braziliensis* from Peruvian espundia (mucocutaneous) received from Dr. A. Herrer, and several Panamanian strains isolated from man, a spiny rat (*Proechimys*), and wild-caught phlebotomine sandflies. The lesions that developed in hamsters inoculated with the spiny-rat and sandfly strains were indistinguishable from those produced by Panamanian human strains. The Panamanian human strains were: Monteza, Vega, Rodríguez, Nuñez, and Calixto. The spiny-rat strain was No. 202, and the sandfly strains were WC-60 (from *Lutzomyia trapidoi*), WC-6103 (from *Lu. yelephiletrix*), and WC-6217 (from *Lu. trapidoi*). The Vega, Monteza, WC-6103, and WC-6217 strains are immunologically related (Schneider and Hertig 1966). The last three strains were examined by electron microscopy (Wallace and Hertig 1968); the ultrastructure of the kinetoplast was leishmanial in all three. The immunological affinities of the Rodríguez, Nuñez, Calixto, spiny rat 202, and WC-60 strains have not been tested. The Guatemalan human strain is immunologically closely related to the human strain from British Honduras (Schneider and Hertig 1966), and provisionally we use the name *L. mexicana* for the Guatemalan strain. The Peruvian espundia strain had been carried in culture for about 1 year at Gorgas Memorial Laboratory before being inoculated into hamsters. In attempts to find by xenodiagnosis the source of the sandflies' natural infections, a few feeding trials were made using wild-caught woolly opossums (*Caluromys*) and kinkajous (*Potos*).

Sandflies used in the tests were *Lu. gomezi* and *Lu. sanguinaria*, reared and maintained in the laboratory and handled by methods outlined by Hertig and Johnson (1961). The *Lu. sanguinaria* were from the 1st to 23rd laboratory generations (mainly 12th to 23rd), and the *Lu. gomezi* were from the 6th to 19th laboratory generations. The flies were fed on golden hamsters with known active leishmanial lesions of 1 month to over a year in duration.

The hamster lesions were produced experimentally by the inoculation of leishmanial cultures into the skin and subcutaneous tissues of the nose (McConnell 1963). With Panamanian strains the lesion is typically a nonulcerated swelling with abundant L-D bodies. In most cases flies were fed on the primary lesions of the nose, but with the Guatemalan strain, secondary lesions on the ears and feet also served as sources of infection.

Bolting-cloth cages were constructed over globular wire frames; a plastic entry tube for the flies was cemented to the bolt-

ing cloth; the cage was fitted over the entire head or leg of the hamster, and fastened with a drawstring around the neck or leg. Thus, the flies were confined to the general area of the lesion, lessening chances of their imbibing a noninfected blood meal. Hamsters were not attractive to Panamanian sandflies and reacted violently to bites. When restrained only by being fastened with gauze strips to a board, their active movements effectively prevented the already reluctant sandflies from feeding. Therefore, hamsters were anesthetized with Kemithal, S. A.[4] administered intracoelomically. This had the undesirable effect of lowering the hamster's body temperature, not only further decreasing attractiveness of the animal to the flies but endangering the life of the hamster. To keep the body temperature more nearly normal, the hamster was tied to a "warm board" made by winding resistance wire around the board, covering the whole first with aluminum foil and then with broad strips of adhesive tape and connecting the wire to a rheostat. The setting which maintained the most nearly normal skin temperature was determined empirically.

Once flies had been introduced into the bolting-cloth cage, a frame supporting damp turkish towelling was placed over the hamster and flies to provide darkness and high humidity. Flies were given ½–1 hr to feed. Most fed flies had taken blood but some fed on tissue juice alone. Fed flies were maintained in plaster-lined pots or, if only a few fed, in individual plaster-lined vials. Often, flies were also anesthetized with Kemithal. In almost every instance anesthetized flies recovered within an hour after feeding. Dead flies were removed daily, dissected, and the guts and mouthparts examined for the presence of flagellates according to methods outlined (Johnson *et al.* 1963).

In the case of *Caluromys* and *Potos*, the animal was anesthetized with Kemithal, placed on the "warm board," and put into a large releasing cage. The flies were either released directly into the large cage or into a bolting-cloth cage fitted over the head, tail, or other parts. Flies fed only on lightly haired or bare surfaces such as the snout, feet, and ears. In the case of *Caluromys*, they also fed on the tail and on young still in the pouch.

Some infected guts were teased out on slides, fixed with Carnoy's, and stained with Giemsa at pH 7.2. The dissecting medium of physiological saline causes distortion of the flagellates during drying. Dr. R. B. Heisch suggested placing the guts in human serum or fresh whole blood before spreading them on the slide or allowing them to dry. This method provided entirely satisfactory preparations for staining. Use of a pH 7.2, 1/15 *M* Sorenson phosphate buffer also gave good results.

Photographs of both fresh and fixed and stained preparations were taken with the aid of a Leitz Mifilmca apparatus.

Observations and Results

The percentages of infections in sandflies fed on animals infected with Panamanian, Peruvian, Guatemalan, and British Honduran strains are given in Table I. Despite the opportunity to feed on lesions which were, for the most part, quite heavily infected with L-D bodies, less than 50% of the flies that fed on Panamanian and Peruvian strains became infected. The infection rate in groups fed on the Guatemalan strain was greater, but even here only slightly more than half the fed flies were infected. In the one trial using a hamster infected with the British Honduras strain, two of nine flies (22%) were infected.

Lu. gomezi consistently had a higher in-

[4] Intranarcon: 5-allyl-5(2-cyclohexenyl)-2-thiobarbituric acid (sodium salt). Fort Dodge Laboratories, Fort Dodge, Iowa.

TABLE I

Infection Rate in Phlebotomine Sandflies Fed on Hamsters with Leishmanial Lesions

Strain	Species of sandfly, *Lutzomyia*	No. of positive trials	No. fed	No. infected	% Infected
Panamanian[a] (human and other)	*sanguinaria*	25	406	153	37.7
	gomezi	14	174	78	44.8
Total		39	580	231	39.8
Peruvian espundia[b]	*sanguinaria*	4	232	104	44.8
	gomezi	2	12	4	33.3
Total		6	244	108	44.2
Guatemalan[c]	*sanguinaria*	10	239	117	49.0
	gomezi	11	190	126	66.3
Total		21	429	243	56.6
British Honduran	*sanguinaria*	1	9	2	22.2
All strains	*sanguinaria*	40	886	376	42.4
	gomezi	27	376	208	55.3
Total		67	1262	584	46.2

[a] There were 8 negative trials, i.e., in which sandflies fed but none became infected: 5 trials with *Lu. sanguinaria*, 2 with *Lu. gomezi*, 1 with *Lu. trapidoi*; total flies fed and negative, 13. (Omitted from table.)

[b] There were 6 negative trials: 3 with *Lu. sanguinaria*, 2 with *Lu. gomezi*, one with various wild-caught flies; total negative flies, 36.

[c] There were 2 negative trials; 1 with *Lu. sanguinaria*, 1 with *Lu. gomezi*; total negative flies, 17.

fection rate than did *Lu. sanguinaria*, except in the two feedings on hamsters infected with the Peruvian strain. Total infection rates in flies fed on all strains of *Leishmania* were: *sanguinaria*, 376 of 886 (42%); *gomezi*, 208 of 376 (55%).

Infection with flagellates had no apparent effect on longevity of the flies, as the majority of both infected and noninfected flies died during or after oviposition, 3–7 days after feeding.

Position of Flagellates in the Gut

In Table II is summarized the results of examining flies infected with all the leishmanial strains. When the gut was broken during dissection, or the fly had been dead for some time, it was sometimes difficult to ascertain the true position of the flagellates and the final destination of infections in the blood meal alone (50/584, 8.5%) was unknown. Such infections are not included in the totals.

Panamanian strains. Flies infected with human, sandfly, or spiny-rat strains developed similar infections. Hindgut infections, often only in the hind triangle, were characteristic of the Panamanian strains and were found on days 2–12 after feeding. Roughly 50% (107/212) of all infected flies had flagellates only in the hindgut; 91% (192/212) had flagellates attached somewhere in the hindgut, and 50 percent (97/192) of this latter group had parasites attached only in the hind triangle (Fig. 2).

In the midgut, there were more stomach infections (95/212, 45%) than cardial infections (42/212, 20%) and even fewer flies had flagellates attached at the proventricular valve (36/212, 17%). Flagellates were never seen in the mouthparts, only once in the pharynx, and seldom in the

TABLE II

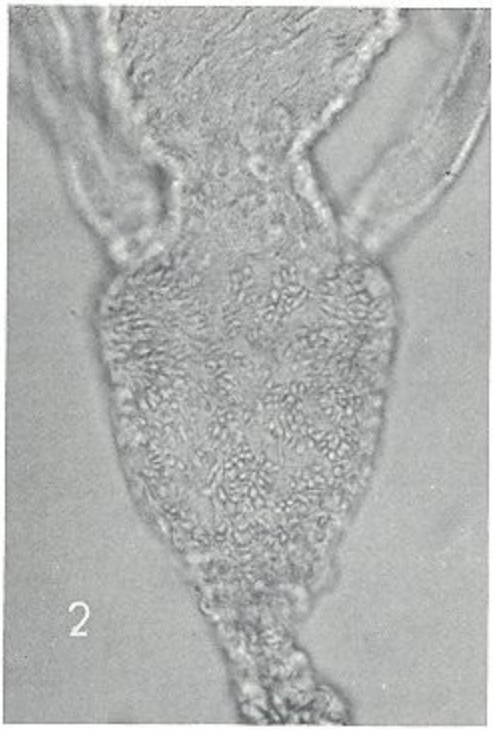
Location of Flagellates in the Gut of Sandflies Infected by Feeding on Hamster Lesions

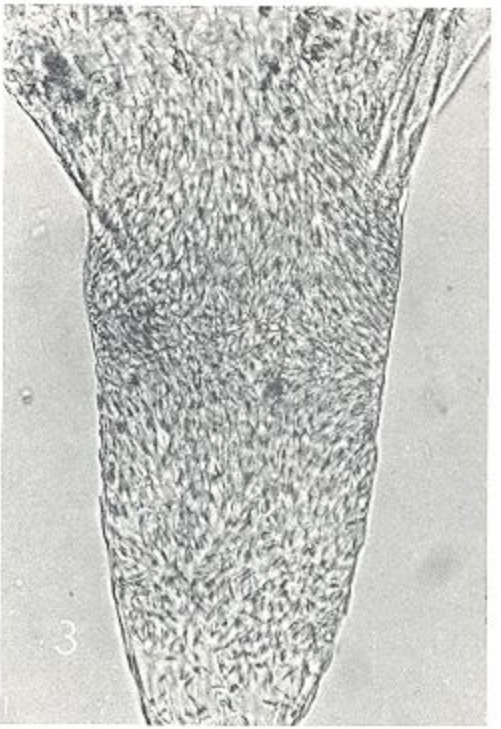
Strain	Species of *Lutzomyia*	Total[a] positive flies	Foregut: Pharynx and forward	Foregut: Esoph. and cs. divert.	Midgut: Proventr.	Midgut: Cardia	Midgut: Stomach[a]
Panamanian human	*sanguinaria*	92	0	3 (3.3%)	15 (16.3%)	18 (19.6%)	39 (49.5%)
Panamanian human	*gomezi*	64	0	1 (1.6%)	6 (9.4%)	8 (12.5%)	28 (43.8%)
Panamanian sandfly	*sanguinaria*	44	1 (2.3%)	2 (4.5%)	9 (20.5%)	9 (20.5%)	18 (40.9%)
Panamanian sandfly	*gomezi*	12	0	0	6 (50%)	7 (58.3%)	10 (83.3%)
Totals		212	1 (0.5%)	6 (2.8%)	36 (17.0%)	42 (19.8%)	95 (44.8%)
Peruvian espundia	*sanguinaria*	104	3 (2.9%)	7 (6.7%)	37 (35.6%)	49 (47.1%)	69 (66.3%)
Peruvian espundia	*gomezi*	4	0	0	1 (25%)	2 (50%)	3 (75%)
Totals		108	3 (2.8%)	7 (6.5%)	38 (35.2%)	51 (47.2%)	72 (66.7%)
Guatemalan	*sanguinaria*	83	0	3 (3.6%)	27 (32.5%)	60 (72.3%)	70 (84.3%)
Guatemalan	*gomezi*	101	1 (1.0%)	4 (4.0%)	47 (46.5%)	58 (57.4%)	60 (59.4%)
Totals		184	1 (0.5%)	7 (3.8%)	74 (40.2%)	118 (64.1%)	130 (70.6%)

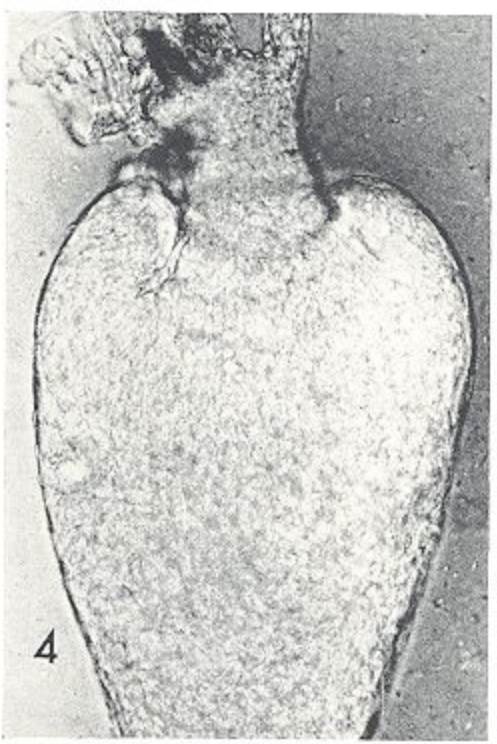
Strain	Species of *Lutzomyia*	Hindgut: Attached in hind triangle	Hindgut: Attached in hind triangle only[b]	Hindgut: Attached in any part of hindgut	Hindgut: Free only	Hindgut only	Malpighian tubules
Panamanian human	*sanguinaria*	72 (78.3%)	39 (42.4%)	79 (85.9%)	0	48 (52.2%)	0
Panamanian human	*gomezi*	57 (89.1%)	40 (62.5%)	61 (95.3%)	1 (1.6%)	35 (54.7%)	0
Panamanian sandfly	*sanguinaria*	34 (77.3%)	17 (38.6%)	40 (90.9%)	0	22 (50.0%)	0
Panamanian sandfly	*gomezi*	11 (91.7%)	1 (8.3%)	12 (100%)	0	2 (16.7%)	0
Totals		174 (82.1%)	97 (45.6%)	192 (90.6%)	1 (0.5%)	107 (50.5%)	0
Peruvian espundia	*sanguinaria*	99 (95.2%)	38 (36.5%)	101 (97.1%)	1 (1.0%)	31 (29.8%)	0
Peruvian espundia	*gomezi*	4 (100%)	4 (100%)	4 (100%)	0	0	0
Totals		103 (95.4%)	42 (38.9%)	105 (97.2%)	1 (0.9%)	31 (28.7%)	0
Guatemalan	*sanguinaria*	3 (3.6%)	3 (3.6%)	3 (3.6%)	20 (24.1%)	0	4 (4.8%)
Guatemalan	*gomezi*	11 (10.9%)	7 (6.9%)	11 (10.9%)	17 (16.8%)	0	19 (18.8%)
Totals		14 (7.6%)	10 (5.4%)	14 (7.6%)	37 (20.1%)	0	23 (12.5%)

[a] Although flagellates in the blood meal alone are not considered established infections and are not included in "Total positive flies," blood-meal infections in stomach are included in the total stomach infections if flagellates also occurred elsewhere in the gut.

[b] I.e., within the hindgut, attached only in the hind triangle.







FIGS. 2–5. *Leishmania* in gut of sandflies fed on infected hamsters; fresh dissections. Figs, 2, 3, 5; hind triangle with midgut at the top, above insertion of the Malpighian tubules.

esophagus and esophageal diverticulum (6/212, 2.8%).

Possibly because only a small number of infected flies survived beyond egg laying, the figures do not indicate the forward progression of flagellates from the hindgut to the stomach that appeared to occur in infected wild-caught sandflies (Johnson *et al.* 1963). Three *Lu. gomezi* and one of four *Lu. sanguinaria* dissected on days 9, 10, and 12 after infection had flagellates at the proventriculus, but also one proventricular infection (outside the blood meal) was found on day 2. However, in a heavy 5-day-old infection in the hind triangle of a *Lu. gomezi* fed on the Monteza strain, the constriction between the midgut and hind triangle was obliterated by a mass of long, large flagellates which extended into the stomach. At the periphery of the mass, motile flagellates were escaping into the stomach lumen (Fig. 3), and flagellates did not appear to be moving to the posterior part of the hindgut.

In the experimental series, flagellates were never observed in the Malpighian tubules, although 20% of the naturally infected flies had flagellates in the tubules.

In the one trial using the spiny-rat 202 strain, 1 of 17 *Lu. sanguinaria* became infected. Only three nonmotile, singly attached flagellates were found. They were in the hindgut.

Peruvian espundia strain. In general, the pattern of infection in *Lu. sanguinaria* approximated that of the Panamanian strains except that the proportions of proventricular, cardial, and foregut infections were markedly greater. The Peruvian-strain flies also had a greater percentage of hindgut infections (97% as opposed to 91% in Panamanian strains), and only 1.9% (2/105) of flies with hindgut infections lacked parasites in the hind triangle. While the four *Lu. gomezi* infected with the Peruvian strain hardly provide an adequate sample, these few infections fell into the general pattern developed in *Lu. sanguinaria*.

One of the four positive trials was highly successful; 84 of 128 flies dissected were positive (66%) (Table 1). Five of these infected flies fed on a noninfected hamster the 6th day after the original infective meal. At that time, heavy infections occurred in dissected females, not only in the proventriculus but in the esophagus as well. When dissected on days 9–13 after the original infective meal, four of the refed flies had heavy infections in the cardia, proventriculus, and posteriorly (Fig. 4); two of these had parasites in the esophagus and esophageal diverticulum; and in one fly, parasites had extended into the mouthparts. The hamsters used for refeeding the flies did not become infected although a hamster inoculated with the gut of a refed female dissected on the 9th day after the original feeding did become infected. The higher percentages of flies with anterior infections in this series may be because they lived long enough to develop heavy anterior infections, unlike the case in the Panamanian and Guatemalan strains where almost all the flies died before the 8th day after feeding.

The reasons for the successful feeding of *Lu. sanguinaria* on the Peruvian-strain hamster were not clear. The flies were of

FIG. 2. *Lutzomyia gomezi*; hind triangle, flagellates attached to wall (3-day infection, Monteza strain); ×315.

FIG. 3. *Lutzomyia gomezi*; same lot as Fig. 2 (5-day infection); hind triangle distended with mass of flagellates bulging into midgut; ×315.

FIG. 4. *Lutzomyia sanguinaria*; proventriculus and cardia distended with flagellates (9-day infection, Peruvian espundia strain; fly was refed at 6 days); ×315.

FIG. 5. *Lutzomyia sanguinaria*; same lot as Fig. 4; hind triangle with attached flagellates (7-day infection); ×525.

the 17th laboratory generation, the hamster had a lesion of 7 months' duration, conditions of feeding, the age of the flies, and other factors seemed to be similar to those in other feeding trials.

As in the Panamanian strains, parasites were never observed in the Malpighian tubules of flies fed on the Peruvian strain.

Guatemalan strain. Location of the flagellates was strikingly different from that in the Panamanian and Peruvian strains. Less than 8% of the flies had attached flagellates in the hind triangle, and only 2.2% had parasites attached posterior to the hind triangle. In over 70% of the infected flies, the infection was confined to the midgut and foregut, with not even free flagellates in the hindgut, and 40% had parasites at or near the proventricular valve. However, the percentage of foregut infections (4.3%) was not as high as in the Peruvian strain (9.2%), and only slightly more than in the Panamanian strain. Parasites occurred in the esophagus by the 3rd and 4th days and also were found attached in the hind triangle by the 3rd day.

Only in the Guatemalan strain were parasites found in the Malpighian tubules, where they occurred 3–8 days after the infective feed in 12.5% of the infected flies. Thus, infections of the Malpighian tubules were more common than infections of attached flagellates in the hindgut.

British Honduras strain. In the one trial using a hamster infected with the human strain from British Honduras, two of nine *Lu. sanguinaria* were positive on the 5th day. Both flies had heavy infections in the proventriculus and cardia, and in one there were parasites in the esophagus. Neither fly had flagellates in the hindgut.

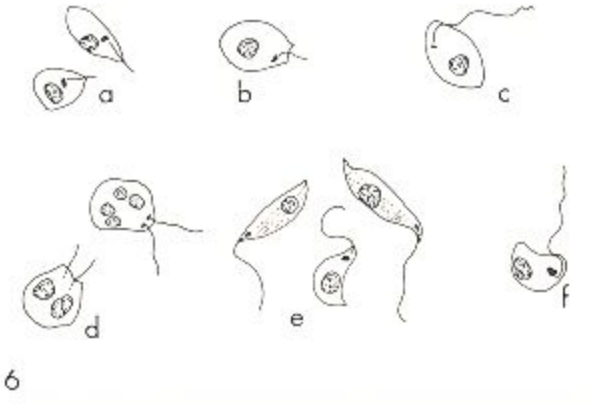
Morphology of the Flagellates, Living and Stained

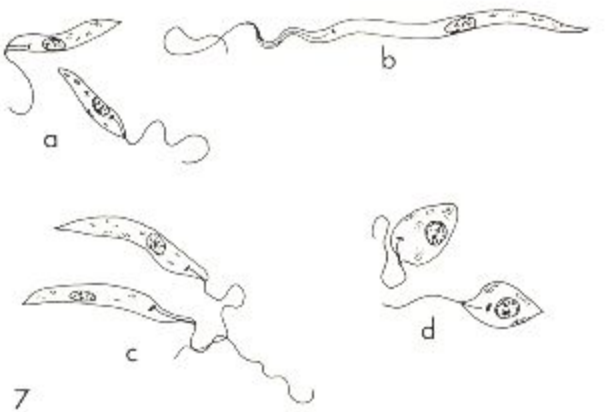
Panamanian strains (fresh dissections, living). Typical hind-triangle infections consisted of distinct rosettes of variable numbers of round to oval, seemingly aflagellar organisms which often were nonmotile. The rosettes were attached to the gut wall. In older infections, flagellates on the outer rim of the rosettes were sometimes thin, small, and very active. The thin organisms were attached by an obvious flagellum. Occasionally, rosettes were made up of larger spindle-shaped organisms. The rosettes were found in 3- to 9-day infections. Rosettes or single organisms posterior to the hind triangle were of similar structure.

In the midgut (cardia, stomach, blood meal, and proventriculus) all sizes and shapes of flagellates were found. There were usually various spindle-shaped to long, rather broad organisms that occurred singly or in free rosettes. Organisms were attached to the gut wall or free in the lumen. Flies infected with the Monteza, Vega, and WC-6103 strains often had flattened, lanceolate to oblanceolate organisms in the midgut. In the stomach of a *Lu. gomezi* with a 6-day infection of the Monteza strain, there was one organism that moved like a blastocrithidia. That is, the body anterior to the nucleus underwent a "shimmering" type of vibratory motion unlike the usual leptomonad type of motility in which movement involves mainly the flagellum, with some flexing of the entire body.

Organisms attached at the proventricular valve were round, oval or spindle shaped in earlier infections, but after 8–10 days, very small, thin active forms predominated.

Panamanian strains (Giemsa-stained smears). In round-to-oval forms typical of the hind triangle, the kinetoplast was round to rod shaped, and at varying distances from the nucleus (Fig. 6a). Generally, the flagellum was very short and often it appeared only as a small amorphous mass of pink acidophilic material at the anterior end of the organism. Flagellates from the hindgut occasionally had a truncate anterior end; more rarely, the same type was seen in the blood meal (Fig. 6b).





FIGS. 6–7. Experimental infections of *Leishmania* in sandflies; drawings of stained flagellates from sandfly gut; ×1175.

FIG. 6. Panamanian strains: a–c, *Lutzomyia sanguinaria;* f, *Lu. gomezi.* a. From hind triangle (WC-6103 strain, 6-day infection); b. From blood meal (Monteza strain, 5-day infection); c. From hind triangle (Monteza strain, 6-day infection); d. From blood meal (Monteza strain, 6-day infection); e. From midgut (Monteza strain, 6-day infection); and f. From proventriculus (Vega strain, 8-day infection).

FIG. 7. Guatemalan strain; *Lutzomyia gomezi,* a. From stomach (10-day infection); b. From stomach (5-day infection); c. From blood meal (3-day infection); d. From proventriculus (4-day infection).

Seldom was the flagellum of any morphological type longer than the body. There was a tendency, sometimes marked, for the flagellum to exit from what appeared to be the side of the organism rather than from the anterior end (Fig. 6c). At times the flagellum ran along the periphery of the body before exiting. Living flagellates never were observed to move as though the flagellum exited from the side.

In old blood meals monstrous round forms sometimes occurred. These parasites had as many as four nuclei and two to four flagella (Fig. 6d).

Parasites which may have been the flattened lanceolate forms occurring in living Monteza, Vega, and WC-6103 infections, appeared to have a "keel" running the length of the body and were half-moon shaped or lanceolate (Fig. 6e). In those

forms, with one side strongly convex and the other straight, or even slightly concave, which also showed the tendency for the flagellum to exit on the straight or concave side, a very distinctive flagellate resulted (Fig. 6f). These bizarre forms were seen in smears of the Monteza, Vega, and especially the WC-6103 strains. Not enough smears were made of other strains to determine whether such forms are of universal occurrence in Panamanian strains.

Peruvian espundia strain (living). The flagellates were similar to those of the Panamanian strains except that the hindtriangle rosettes were commonly composed of slightly larger, more spindle-shaped organisms (Fig. 5). As in the Panamanian strains, in older infections there was a tendency for flagellates on the edge of the rosettes to be thinner and more active. In *Lu. sanguinaria,* heavy infections involving the entire midgut as well as the esophagus and/or mouthparts were composed mainly of small, thin, very active organisms although in one 8-day infection, spindle-shaped as well as small thin flagellates were observed in the mouthparts.

Peruvian espundia strain (stained smears). In the few stained preparations made, no departure from "standard" leptomonad morphology was noted.

Guatemalan strain (living). These flagellates were larger and longer than those seen in Panamanian and Peruvian infections. In the midgut, they varied from large and spindle-shaped to long and thin. Flagellates at the proventricular valve appeared to be large and long to almost round when sparse enough so that their structure could be made out, but in stained smears of proventriculi packed with flagellates, they were thin and sometimes short. In the two flies infected with the British-Honduran strain, the flagellates were very large and long.

It was common on days 4–5 to see long, attenuated forms which were expanded in the nuclear area. Twice, on days 3 and 4, several parasites exhibited a blastocrithidiform movement similar to that seen once in a fly infected with a Panamanian strain.

In the Malpighian tubules organisms were of all shapes, and either single or in free rosettes.

In the hindgut, the rarely observed rosettes consisted of oval to spindle-shaped organisms. More usual were single, larger, spindle shaped to long, thin flagellates attached only by the flagellar tip.

Guatemalan strain (stained smears). The kinetoplast was rod shaped in the majority of flagellates of all morphological types (Fig. 7a–d). The flagellum was often longer than the body except in the round or oval forms. In long, thin forms the nucleus was often closer to the posterior than the anterior end and separated from the kinetoplast by a third of the total body length. In round forms, on the other hand, the kinetoplast was often close to the nucleus and in a few flagellates in a 6-day infection, the kinetoplast was either at the same level as the nucleus or, like some Panamanian flagellates, the flagellum appear to exit on the side. Rosettes of distinct morphological types often occurred in the same infection (Fig. 8). In old blood meals, monstrous forms like those seen in Panamanian strains sometimes occurred. There was a general tendency for flagellates of all shapes to have numerous cytoplasmic inclusions.

When one of the sandfly guts that contained flagellates with a blastocrithidiform movement was smeared and stained, no blastocrithidiae were seen despite the presence of many flagellates. In a small proportion of the flagellates there was an extensive acidophilic area anterior to the kinetoplast and extending to the anterior end. Flagellates of like structure were not seen in any other preparations from any of the strains, including other Guatemalan infections.

FIG. 8. *Lutzomyia sanguinaria;* Guatemalan strain, 7-day infection; stained flagellates from midgut, showing rosettes formed of two types of flagellates; ×1180.

Feeding Trials with Wild Panamanian Mammals

In the attempt to find the source of the leptomonad infections of wild-caught sandflies, xenodiagnostic tests were made by feeding reared sandflies on various live-trapped forest mammals. In 7 trials, 317 *Lu. sanguinaria* were fed on 4 woolly opossums, *Caluromys.* In a thick blood smear from one of the animals there were numerous microfilariae, but trypanosomes were not seen. In 36 of 67 flies fed on this animal, nematodes were present free in the hemocoel or associated with the ovaries.

Nineteen flies of the 317 fed (6.0%) had flagellates in the gut. Infected flies occurred in each group fed on the four opossums. Typically, infections consisted of rosettes or singly attached, round to oval, nonmotile organisms. Attached flagellates were found in the hindgut only below the hind triangle, in locations varying from just below the triangle to just anterior to the rectal ampulla. In at least 8 of the 19 infected flies, flagellates occurred only in the middle portion of the hindgut (in 4 of the remaining 11 the position was not recorded except that the flagellates were posterior to the hind triangle). In one case, free flagellates were seen in the hind triangle, and in the stomach of a second fly there were two free flagellates with "long tails" similar to flagellates occurring in cultures of some strains of *Leishmania* isolated from sandflies (McConnell 1963). In this infection, some definite trypanosomes were seen in the fresh dissection, but in the other 18 infections the form of the flagellates was not ascertained. Leptomonad flagellates were never cultured from *Caluromys,* and Thatcher *et al.* (1965a) were unable to produce infections of two human strains of *Leishmania* in seven *Caluromys* inoculated. However, these opossums are commonly infected with trypanosomes. Therefore, the sandfly infections probably involved mostly trypanosomes.

Two feeding trials, one with *Lu. sanguinaria,* the other with *Lu. gomezi,* were made using a female kinkajou, *Potos,* as the blood source. A blood culture of this

animal was positive for both trypanosomes and leptomonads. One of 20 *Lu. gomezi* and 1 of 84 *Lu. sanguinaria* fed on the kinkajou were positive for flagellates. Each was dissected on the 5th day after feeding. In both flies there were flagellates attached in the hind triangle, and in one a few organisms were attached below the triangle. They occurred singly and in rosettes and were not noticeably different from parasites found in flies infected with Panamanian strains of known *Leishmania*.

Discussion

The evidence is clear that Panamanian strains of *Leishmania* isolated from human cases as well as sandflies have a predilection for the hindgut, especially the hind triangle, of two Panamanian species of sandflies, *Lu. sanguinaria* and *Lu. gomezi* (Hertig and McConnell 1963; Hertig *et al.* 1969; and the present paper). Equally clearly, the behavior of two strains of *L. mexicana* from Guatemala and British Honduras is strikingly dissimilar from that of the Panamanian strains. With *L. mexicana*, midgut infections are the rule and infections never occur in the hindgut alone. Clinical, epidemiological, and experimental data support the view that *L. mexicana*, the causative agent of "chiclero ulcer" and "bay sore," is a different entity from the agents responsible for human mucocutaneous and lepromatous leishmaniasis.

There is need for a method that will augment or improve the current means of distinguishing species and strains of *Leishmania*. Clinical recognition of the different infections must rest on a comparison of many cases from the same geographic or ecologic area since not all cases manifest classical symptoms. Animal inoculations often provide supportive data. Adler (1963) devised a method of recognizing leishmanial strains or species by their different growth patterns in cultures containing homologous and heterologous immune sera. Immunodiffusion tests, like those performed by Schneider and Hertig (1966), added yet another method of grouping or differentiating strains of *Leishmania*. These immunodiffusion tests indicated that the strains of *L. mexicana* we used for sandfly feeding, from Guatemala and British Honduras, are definitely related to one another but not closely related to any of the Panamanian strains they tested. In an excellent series of studies, Coelho *et al.* (1967a–e) reported that, although both *Lu. longipalpis* and *Lu. renei* were readily infected with *L. donovani*, *L. tropica*, *L. braziliensis s. lat.*, and *L. mexicana*, there were differences in position of the flagellates in the gut according to the species of fly (*longipalpis* developed more anterior infections). In both sandfly species, infections of *L. tropica* often were limited to the stomach. These two species of Brazilian sandflies also differed in relative numbers of hindgut infections according to the strain of *Leishmania* used. Our findings together with those of Coelho *et al.* (1967a–d) indicate that to the methods used for distinguishing the New World human-infecting strains of *Leishmania* may be added the study of the position of flagellates in the sandfly gut.

We lack information on the serological relationships of our Peruvian strain of espundia, so we do not know whether the similarity of growth patterns in the gut of *Lu. gomezi* and *Lu. sanguinaria* also provides evidence of some relationship between Panamanian leishmaniasis and the mucocutaneous leishmaniasis of Peru. In hamster infections, the two strains react differently. Perhaps it is significant that Coelho *et al.* (1967a,c) found hindgut infections (location in the hindgut not specified) in 70% (43/61) of wild-caught *Lu. longipalpis* fed on hamsters infected with a Brazilian strain of espundia as opposed to 7% (2/28) fed on a strain of *L. mexicana* from British Honduras. The last, re-

ceived from P.C.C. Garnham, is perhaps the same as the British Honduran strain we used.

Since natural infections of the foregut were not found in wild-caught sandflies of the two species we used in the hamster feeding series (Johnson *et al.* 1963) and since the latter were laboratory reared, there might be an objection to assuming that our results are comparable to what might occur in nature, even though the growth patterns of natural and experimental infections are closely similar. It should be remembered that, despite frequently heavy anterior infections, laboratory transmissions of any leishmanial species by any species of sandfly have been notably the exception, not the rule. The few successful experimental transmissions involved either the use of wild-caught flies (Chung and Feng, 1950–51; Strangways-Dixon and Lainson 1966; Coelho and Falcão 1962; Biagi *et al.* 1965; Williams 1966; Coelho *et al.* 1967e) or, in the case of laboratory-reared flies, special techniques (Smith *et al.* 1940; Adler and Ber 1941). Thus there is sufficient evidence to indicate that laboratory-reared sandflies lack a factor or factors necessary for efficient bite transmission, a method that assumes foregut and mouthpart infections. We know that even in the man-biting Panamanian sandflies suspected of being vectors of cutaneous leishmaniasis, especially *Lu. trapidoi*, leptomonad infections in wild flies occur mainly in the hind triangle while midgut and posterior hindgut infections are the exception (Johnson *et al.* 1963). The results reported in this paper show that our strains of *Leishmania* from Panama and Peru almost invariably occur in the hindgut, often accompanied by midgut infections but often in the hind triangle alone, while strains from Guatemala and British Honduras do not.

It may be that some species of sandflies would not serve as such sensitive indicators of strain differences in *Leishmania* as do our two Panamanian species. Coelho *et al.* (1967a,c) found that in *Lu. renei*, the Brazilian mucocutaneous strain gave only 24% of hindgut infections (16/67) rather than 70%, as in *Lu. longipalpis*. However, with the "nodular diffusa" and *L. mexicana* strains, hindgut infections in *Lu. renei* [5% (9/173), 6% (4/64) respectively] were comparable to those obtained with *Lu. longipalpis* [9% (11/121), 7% (2/28)].

During our survey of wild-caught sandflies in Panama, the only *Lu. longipalpis* captured and dissected was infected with leptomonad flagellates (Johnson *et al.* 1963). This fly had long, thin or spindle-shaped organisms throughout the midgut and hindgut, extending from the proventriculus to below the rectal ampulla. There were rosettes of flagellates attached to the wall of the hind triangle as well.

In British Honduras Disney (1968) found flagellates in the guts of 11 of 2074 wild sandflies he dissected. Of these 11, 6 had flagellates in the hindgut as well as the midgut: 3 of 8 *Lu. flaviscutellata* (38%), and each of the single specimens of *Lu. beltrani*, *Lu. cruciata*, and *Lu. permira*. The *Lu. beltrani* infection was definitely blastocrithidial, identity of the *Lu. cruciata* flagellates was not certain, and the remaining infections were leptomonad in nature. Considering only his *Lu. flaviscutellata* infections, the percentage of hindgut infections was similar to that occurring in our experimental series using a Guatemalan strain of *L. mexicana*.

Malpighian-tubule infections were found only in sandflies fed on the Guatemalan strain. The absence of such infections with the Panamanian strains is in contrast to the 20% of infected wild-caught Panamanian sandflies that had some flagellates in the Malpighian tubules (Johnson *et al.* 1963). Malpighian tubule infections have been found also in Brazilian *Lu. flaviscutellata* (Lainson and Shaw 1968).

Regarding flagellate structure, we found no marked differences between Panamanian and Guatemalan flagellates except that the Guatemalan flagellates were often larger and had cytoplasmic inclusions. The findings of note concern the occurrence in both strains of a few parasites with a blastocrithidial movement and, in certain Panamanian infections, the presence of flagellates with the flagellum seemingly leaving the side of the organism (Fig. 6c,f). Since the movement of living flagellates is often the only clue as to whether they might be of the blastocrithidial or leptomonad type, these observations suggest that, if not confirmed by examination of stained preparations, one must observe caution in assuming the presence of blastocrithidiform organisms in sandfly infections.

Identity of the *Potos* organism which infected our sandflies is uncertain. Thatcher *et al.* (1965) found *Potos* infected in nature with a species of *Leishmania* that caused an infection in hamsters indistinguishable from Panamanian human strains. Thus, it is possible our *Potos* series, done in 1963, may have involved a similar flagellate. In any event, the location of the *Potos* flagellates in the sandfly gut was similar to that of human and sandfly strains of *Leishmania*.

The series of opossum feedings indicates that some trypanosomes from Panamanian mammals other than bats can establish themselves in small numbers in the hindgut of sandflies, and in fact, may be unable to remain successfully in the midgut or even the upper portion of the hindgut, the hind triangle. "Long-tailed" flagellates such as those found in one stomach infection from an opossum feeding occur elsewhere. They have been found in Panamanian sandfly strains that have infected hamsters as well as in those that have not done so (McConnell 1963; Schneider and Hertig 1966). The culture forms of *Endotrypanum* from sloths include "long-tailed" leptomonads (Shaw 1964; Packchanian and Kelly 1966). Therefore leptomonads with long tails cannot be identified as any particular strain or species of flagellate.

Recently several reviewers and investigators have cautioned that the presence of leptomonads in wild-caught sandflies does not mean that all these infections are associated with human and/or mammalian *Leishmania* (Adler 1964; Shaw 1964; Garnham 1965; Anderson and Ayala 1968; Hanson *et al.* 1968; Disney 1968). Because of the findings reported by ourselves and others, and speculations in the literature regarding flagellates found in wild-caught New-World phlebotomines [especially those reported by Johnson *et al.* (1963)], our information in this area needs to be reassessed.

Sandflies readily ingest microorganisms of many types other than mammal-infecting *Leishmania*, including the microfilariae mentioned in the present paper, trypanosomes, and *Leishmania* of lizards (Adler 1964) and trypanosomes of anurans (Anderson and Ayala 1968), and trypanosomes of mammals (Herrer 1942; McConnell and Correa 1964; Disney 1968; Williams *in* Disney 1968; and the present paper). Sandflies have access to various substances in nature but there is very little information about the feeding habits of either sex other than the blood meal of the female. Sandflies of both sexes have been seen to lower the proboscis to the moist plaster surface of the breeding vessel, presumably for the purpose of drinking. (This "drinking" behavior was the point of departure in the development of the pipet feeding technique in China (Hertig and Hertig 1927; Hertig and McConnell 1963)). Peruvian sandflies have furnished evidence that in nature females may ingest substances other than blood. Hertig (1942) found both sexes of *Lu. verrucarum* frequently infected with a minute, cultivable bacterial organism which coated the tip of the proboscis. It appeared as though the proboscis had been dipped

in a rich suspension of the organisms. The sharply circumscribed masses of organisms at the tip but not elsewhere on the stylets could hardly have been the result of multiplication *in situ*. Plant glands or the excretions of insects or other animals seemed possible sources. Search of the local countryside did yield morphologically similar minute organisms in certain petiolar glands and in the excretions of aphids and scale insects, but cultures showed that none of these were the proboscis organism (unpublished, Hertig). Substances accessible to wild sandflies may provide factors facilitating transmission by bite. In some cases the miscellaneous infections listed above may be doomed to extinction with the disappearance of the blood meal, as may be true of a *Leishmania* of Panamanian porcupines in *Lu. sanguinaria* (G.M.L. Rept. 1967), or end up in a blind alley as with the microfilariae reported in this paper. Others, though they apparently can establish themselves in the gut, do so in such small numbers that the possibility of transmission by bite or posterior-station contamination seems unlikely, as with the *Caluromys* organism. When obvious blastocrithidiae and trypanosomes (leptomonads may be present also) occur in heavy infections, the sandflies concerned might be serving as vectors of the flagellates to bats, other mammals, lizards, and anurans. Especially is this true if the flagellates occur in sandfly species known or presumed to feed on these animals, or to be eaten by them (Herrer 1942; McConnell and Correa 1964; Anderson and Ayala 1968). Except for the trypanosomes of *Caluromys*, which may occur as leptomonads in the sandfly gut, the kinds of infections listed are not apt to be confused, in Panama, with *Leishmania* likely to be transmitted to mammals by phlebotomine sandflies.

Adler and Theodor (1930) could infect *P. papatasi* with several species of insect flagellates, and one might expect that true insect flagellates could occur normally in phlebotomines. This possibility has been suggested by Hanson *et al.* (1968). Nonetheless, in all the years sandflies have been studied, a natural infection of a flagellate probably belonging to an insect-infecting species of *Crithidia* or other invertebrate-infecting genus, has been reported only once from the New World (McConnell 1963, addendum by Johnson and Eisenmann). Flagellates in this infection, from a female *L. sanguinaria*, and limited to the hindgut posterior to the hind triangle, were immediately recognized as different and were found only once in dissections of over 6800 females and 262 males. The ultrastructure of the kinetoplast was of the *Crithidia* type, as opposed to the case of six other sandfly strains studied, in which the kinetoplast was of the *Leishmania* type (Wallace and Hertig 1968). Although one cannot dismiss the possibility that insect-infecting forms may confuse the issue, the possibility seems rather remote, at least in Panama.

It is possible that some mammal-infecting trypanosomes were included in the infections seen in fresh dissections of wild-caught sandflies (Johnson *et al.* 1963). However, flagellates found only in the midgut and/or hind triangle and similar in appearance to the leptomonads of human-infecting strains probably are not trypanosomal in nature. In our experience with Panamanian forms, flagellates of the genus *Trypanosoma* either include blastocrithidial organisms or are not found in the hind triangle but definitely below this area.

Lizard *Leishmania*, to date reported only from Martinique in the New World, might confuse the picture considerably, if present in an area under investigation. Some lizard-infecting *Leishmania* are considered to be closely related to human-infecting species. Not only do they sometimes occur in the anterior as well as the posterior station, but *L. adleri* of African lizards causes cryptic

infections in hamsters and transient infection of man (Manson-Bahr and Heisch 1961; Heisch *et al.* 1962; Adler 1964). Workers at Gorgas Memorial Laboratory (G.M.L. Rept. 1969) examined blood smears of 549 lizards representing 27 species, and further examined 92 of these lizards by blood and visceral culture. *Leishmania* were not found, trypanosomes were present in two lizards and a blastocrithidiform organism in one. If lizard *Leishmania* are present in Panama, they are not common. Sherlock and Pessoa (1966), in Bahia, Brazil, found 12 of 375 *Lu. micropyga* with leptomonad infections. The flagellates were restricted to the hindgut from the insertion of the Malpighian tubules to the rectal ampulla. Other species, including 171 *Lu. whitmani* and 79 *Lu. shannoni*, were not infected. These authors considered the possibility that the leptomonads were developmental phases of a *Leishmania* parasitic in geckos. If this should turn out to be the case, it would be the first reptilian *Leishmania* to be found on the New World mainland.

Adler (1964) believes the leishmanias of the New World have speciated more than those of the Old World, and he points out that New World phlebotomine sandflies also have speciated more. He says, based on the above, that each *Leishmania* species may have its own vectors and host spectrum. Adler's views are not necessarily at variance with Lainson and Shaw's (1968) remarks that *Lu. flaviscutellata* is apparently the vector of *L. mexicana* (mainly to rodents?) (Shaw and Lainson 1968) in Central America and of a leishmaniasis of rodents in Brazil. As yet the above workers have not identified their Brazilian rodent strains with those of human cases. They did state that a strain of *Leishmania* they isolated from an extensive ulcer on a human leg was similar to their rodent strains, but differed markedly from another strain they isolated from "a frank case of nasopharyngeal leishmaniasis" from the same area. It would be interesting to see whether *Lu. flaviscutellata* or another Brazilian sandfly species might develop hindgut infections when fed on hamsters infected with the nasopharyngeal strain of Shaw and Lainson.

There remains the question as to the significance of growth patterns involving the hindgut in terms of method of transmission. There is wide acceptance of the view that growth at the anterior station provides the mechanism for transmission of mammalian leishmaniases by the sandfly bite. The Panamanian and Peruvian strains with their high proportion of hindgut infections may seem to provide contrary evidence, but many of these infections are accompanied by growth in the midgut. In many cases the sandflies did not live long enough under laboratory conditions to provide the full range of final growth patterns. There is no evidence as to what, if anything, growth in the hindgut can contribute to the transmission of these mammalian leishmaniases. Since anterior growth also occurs we see no reason to postulate methods of transmission different from those of other mammalian species of *Leishmania*.

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