PN-ABC-872

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 1/21/89

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VV

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-136
PR - Dec 88

Attachment

FIELD AND LABORATORY STUDIES ON NEW ASPECTS OF EPIDEMIOLOGY OF LEISHMANIASIS

AID-CDR PROJECT C5-136

Progress report - December 1988

by

Yosef Schlein

Department of Parasitology-Hadassah Medical School

Jerusalem, Israel

Rec'd in SCI: JAN 12 1989

Dispersal of the Phlebotomus papatasi in the Jordan Valley

The sandfly Phlebotomus papatasi (Diptera: Psychodidae) is the main vector of cutaneous leishmaniasis in the Jordan Valley, where it enjoyes a close ecological relationship with the main reservoir host of the disease, the burrow dwelling sand rat Psammomys obesus. In recent years intensive agriculture has changed the physical features of the Jordan Valley environment- the natural habitat is receding as more land is cultivated. Most agricultural fields lie fallow during the summer months. Nevertheless, many sandflies are to be found crossing them.

The dispersal of sandflies has been studied in the past, mainly with the mark-release-recapture technique. The overall picture resulting from these studies is that sandflies are generally sedentary but they may travel from 200 to 2000 metres during a night.

Assuming that samples trapped existing burrows consist of both dispersing and non-dispersing flies, whereas samples trapped over the fallow fields would contain mainly dispersing flies, we compared them in order to characterize the dispersing population. The parameters used for comparison were proportions of males and females in the two populations, nutritional status, and in the females - parity, ovarian development, insemination and infection with Leishmania promastigotes.

In all the comparisons between flied caught over the bare

field and those existing burrows a significant difference was found. The ratio of males to fem..les in our laboratory colony and in numerous samples from burrows does not deviate significantly from 1:1. In the bare field the females constituted 76-68.5% of the various samples, a consistently significant departure from the expected 50%, which allows the conclusion that dispersal is mainly a female activity in P. papatasi.

From the data presented in Table 1 it is apparent that gravid, blood fed females do not disperse: 26% of those exiting burrows were in stages III-V of the gonotrophic cycle, as opposed to 11.6% of the dispersing ones. The sugar feeding status of the blood fed females exiting and entering burrows (Table 3) indicates that the main reason for their activity outside burrows is sugar feeding, which explains the short duration and localization of this activity. Blood fed females who test negative to anthrone are young females who take a blood meal in the burrows before taking a sugar meal, and are compelled to exit the burrow during the digestion of the blood meal in order to find a sugar meal.

On average 55.4% of the dispersing females were pareous, as opposed to 39% of those exiting burrows, which indicates that the age of the dispersing flies is higher, and that they have already successfully completed one gonotrophic cycle. The insemination rates of burrow vs. field caught females points to burrows as the mating centres. Leishmania infections were found in 9% of exiting females, and 2.2% had no vestiges of blood in their gut. In the

bare field 2.6% of the females examined were in a similar condition, and none were found who had not completely digested the infective blood meal, so that the proportion of host seeking infective flies in the dispersing population was not significantly different from that of flies exiting burrows. As P. obesus is practically the only source of Leishmania in this region, and these rodents (or their tracks) were never seen in the fallow fields, it is certain that all Leishmania infected females dispersed away from their burrows. It was found that in Turkemenistan the dispersal of P. caucasicus and P. papatasi was limited by the availability of burrows of the great gerbil Rhombomys opimus and in the new world several species of Lutzomyia do not cross bare, arid clearings in the rain forest. It is possible that in the Jordan Valley the abundance of humid, fertilized fallow fields enhances the tendency of P. papatasi females to disperse, therefore increasing the risk of infection. Several workers have suggested the creation of a cordon sanitaire devoid of rodent burrows around human settlements in endemic areas. This may be inadequate even if they are wider than the nocturnal flight range of the vector, if suitable shelter (other than rodent burrows) may be found in them. The blood fed females exiting burrows in need of a sugar meal are a more suitable target for control, in as much as the target population (Leishmania infected females) presents itself in a well defined area (near burrows) with a well defined appetitive behavior-sugar feeding.

le 1. Ovarian development, parity and insemination in female P. papatasi

Trapped exiting burrows and dispersing.

rap ite:	% of total in each physiological category*						
allow ield	I-II;+;P	I-II;-;P	I-II;+;N	I-II;-;N	III-V;+	III-V;-	n
	10.8	54.7	19.5	4.3	2.1	8.6	92
	6	57.7	18.3	6	6	6	66
"	12	32	28	8	12	8	50
"	44.5	18.5	22.2	7.4	0	7.4	64
"	8	32	48	4	4	4	62
±S.D	16.2±2.9	39.2±3.8	27.1±3.5	5.9±1.8	4.8±1.7	6.8±2	334
Burrows	34.2	8.5	31.4	2.8	17.4	5.7	70
"	18.1	18.1	31.8	4.5	27.5	0	44
"	27.2	5.5	38.8	0	19.4	8.3	71
"	40.7	3.7	29.6	0	18.5	7.4	54
±S.D	30.1±4.1	8.9±2.5	33.2±4.2	1.8±1.2	20.7±3.6	5.3±1.9	239

I-V indicate stages of ovarian development; P-parous; N-nulliparous; + or - indicate insemination.

able 2- Leishmania infection in female Phlebotomus papatasi trapped exiting burrows and in a bare field.*

Burrow No:	No. trapped:		Leishmania infection:		
	total	$\bar{x}$ ±S.E	total	$\bar{x}$ ±S.E	%
1	16	5.6±3.1	0	0	0
2	5	1.6±0.3	0	0	0
3	92	30.6±9.9	9	3 ±0.5	9.7
4	108	36 ±12.6	12	4 ±2.5	11.1
5	6	2 ±1.1	0	0	0
6	40	13.3±6.1	7	2.3±1.2	17.5
7	31	10.3±6.1	2	0.6±0.6	6.4
8	13	2.6±1.4	0	0	0
total-	311	102 ±18	28	9 ±0.4	9
Bare field:	147	49 ±14.7	4	1.3±0.3	2.7

* - Pooled from three trapping nights in June, July and August 1986.

le 3- Fructose* in guts of blood fed and non-blood fed female *Phlebotomus papatasi* exiting and entering sand rat burrows.

Behavior	Blood fed	No. examined	% Positive (±S.D)
Exiting Burrows	+	52	38.4±4.2
	-	46	78.2±5.7
Entering Burrows	+	49	93.8±3.9
	-	48	81.2±6.8

- Determined with cold anthrone.

Oviposition diapause and other factors affecting the egg-laying of Phlebotomus papatasi in the laboratory

The maintenance of a sandfly colony is tedious and precarious as is evident from the variety of methods described since the first successful breeding of Phlebotomus papatasi (Scopoli) by Wittingham & Rook (1922). One of the main problems is the death of females before or during oviposition and the ensuing loss of eggs and adults. Our initial intention was to study the oviposition response of P. papatasi to stimuli in the closed environment of the cages, however, during the work we encountered the appearance of oviposition diapause, i.e. the retention of a very large proportion of the eggs by gravid females towards the winter. The phenomenon was relevant, and the study was extended to include the observations on the influence of light on the diapause.

The degree of retention of eggs by females apparently indicates a variable reaction to release and inhibition stimuli, which modulate the process of oviposition. Hence, in addition to the significance for breeding, the oviposition control system is an interesting aspect of behavior.

Therefore, this study was undertaken to elucidate this aspect of the biology of P. papatasi as well as to improve the method of culturing.

In this study we have observed that the rate of oviposition

by P. papatasi females is initially a seasonal phenomen since egg retention occurred at the onset of winter under standard insectary conditions. Exposure of gravid females to various environmental stimuli demonstrated the influence of such factors on the degree of oviposition.

Oviposition promoters

Oviposition of P. papatasi was increased to a highly significant extent above control levels using two kinds of stimuli: availability of furrows or serrations of "egg niches" in the plaster substrate of cages, and the presence of aged cow manure (larval medium) or a combination of both these factors. The greatest number of eggs, around 40/female/week, were obtained in the presence of manure regardless of the structure of the plaster (Table 1). Preference for furrowed surfaces of the oviposition substrate was apparently a reaction of the roughened texture of the plaster of Paris since, smooth depressions made while the plaster was soft, had no such effect (unpublished data). A similar physical distinction between surfaces occurs with mosquitoes; for example Aedes vexans prefers laying among a certain size of sand particles.

Increased oviposition did not cause higher fly mortality. This indicates that the series that laid significantly more eggs

than controls, following exposure to manure or furrowed plaster (Table 1), had a higher survival rate at oviposition.

Oviposition inhibitors

Reduction of oviposition occurred when larvae or pupae were present in the cage and when the flies were confined in a smaller volume. With larvae present oviposition dropped about a half of the control rate of 15.0 eggs/fly/weeks. The effect of larvae apperently diminished to some extent during the experiments, as many died, pupated or were cannibalized. It is interesting that mosquito larvae of many species have the opposite effect and produce oviposition attractants.

The effect of presence of pupae on oviposition was smaller and limited in time, being significantly different from the control 6.2 vs 11.0 eggs only on the 4th day of the experiments (Table 2).

The reaction of flies to confinement in a volume smaller than that of controls was similar whether they had a standard surface area or half of it available for oviposition. In each case most of the oviposition occurred until the third day (Fig. 2) and the weekly total was only about 5 eggs/female whereas controls in larger cages produced 12.5 eggs/female/week. As we have found that reduction of fecundity is an effect of the crowding of gravid females, it is necessary to further investigate the optimum living space required for P. papatasi adults in insectary cages.

In these experiments the reaction of flies to the various test conditions was expressed in the degree of oviposition. Egg laying in nature is preceded by a search for a suitable site. Larval survival and maturation of adults will be achieved in a breeding site that includes larval food, humidity, physical proterction and low larval density. Such factors would be used as cues in the search behaviour guiding the selection. Hence, the experimental factors which stimulated or inhibited oviposition would under natural conditions function primarily as attractants or repellents in the search for a oviposition site.

Seasonal rate of oviposition

Comparison of the control series during the months from April to December demonstrated that oviposition of P. papatasi was subject to seasonal variation. The average number of eggs per female between April and mid-October ranged from 11.5 to 18.6 dropping to only 1.4 eggs/female/week after mid-October. For this extreme reduction in the oviposition we suggest the term oviposition-diapause.

These changes took place in a stock of P. papatasi maintained in constant conditions of 28°C temperature, high humidity and a summer photoperiod of 17:7 L.D. The rate of oviposition of P. papatasi in the laboratory is thus evidently controlled by a circannual rhythm. It is interesting to compare the abundance of P. papatasi in nature with the rate of oviposition in the laboratory. It seems, that the increase of

oviposition in August (Fig. 5) gives rise to the build up of peak populations of adults in September-November (unpublished observations), whereas the decline of oviposition in November precedes the winter disappearance of adult flies in the Jordan Valley at about the end of December. The seasonal rhythm of oviposition therefore seems to be an important factor in determining the seasonal density of P. papatasi adult populations in the field. This phenomenon of oviposition-diapause may be an over-wintering mechanism, possibly comparable to the gonotrophic dissociation which occurs in, for example inseminated female mosquitoes of the Anopheles maculipennis Meigen complex.

An infinite number of variations in timing, intensity and duration of treatment with irradiation are possible and those used in the experiments were chosen randomly. The results do however demonstrate that oviposition diapause can be broken by ultra violet light. UV in the sunlight that reaches the earth is of waves longer than 300 nm. Comparison of the UV maximal intensity during the year in Jerusalem and the monthly averages of oviposition of P. papatasi demonstrate a good correlation (Fig. 5). Furthermore, between October and April, there is no UV in the sunlight until 8.00 hrs or after 16.00 hrs. Thus very little UV irradiation can reach P. papatasi adults when they are active at dawn or dusk at the end of the sandfly season. It is therefore tempting to suggest particular wavelengths of UV light set the cycle of ovisposition-diapause of P. papatasi. This differs from the way that photoperiod, in the form of day-length,

governs winter diapause of many organisms, for example the mosquito Culex pipiens L. in Israel.

Since this is the first observation of oviposition diapause in sandflies, it is not yet known whether it occurs in species other than P. papatasi and whether this phenomenon is correlated to larval diapause. Possibly UV light is the primary cue for both diapauses and this possiblity could be investigated in a species such as P. papatasi in which larval diapause could previously not be interrupted.

Cooperation

The method of marking sandflies by feeding of sucrose bait containing food dyes is employed in Kenya for the study of dispersal and other aspects of sandfly behaviour. This work is carried out by a Ph.D. student guided by Dr. M.J. Mutinga. Further developments in the research indicate the plausibility of using the sugar baits with control agents. I intend to suggest to Dr. Mutinga to carry out field trials in Kenya in the near future. The continuation depends on the possibility to extend the use of the funds allocated for travel beyond the three years period.

Publications:

Schlein, Y., Borut, S. and Jacobson, R.L. 1989. Oviposition diapause and other factors affecting the egg laying of Phlebotomus papatasi in the laboratory. J. Med. Vet. Ent. in press.

Yuval, B., Warburg, A. and Schlein, Y. 1989. Leishmaniasis in the Jordan Valley. V. Dispersal of Phlebotomus papatasi (Diptera: Psychodidae). J. Med. Vet. Ent. in press.

Table 1. Average one-week oviposition of *P. papatasi* females on substrates with different treatments (April-October).

Treatment	Day	Experiment (plaster with treatment) Mean	SE	Control (plain plaster) Mean	SE	P
Furrows present, Cow manure absent	1	1.6	0.87	0.9	0.45	NS
	2	2.5	0.93	1.6	0.72	NS
	3	5.2	1.84	2.9	0.57	NS
	4	8.4	3.39	3.5	0.64	NS
	5	15.1	4.77	5.3	0.38	<0.05
	6	19.8	3.66	6.7	1.08	<0.005
	7	29.6	1.38	10.2	1.96	<0.001
Cow manure present, furrows absent	1	16.9	4.4	7.1	2.58	NS
	2	20.3	3.3	7.7	2.62	<0.02
	3	25.8	4.0	9.5	2.04	<0.01
	4	27.9	3.47	10.2	1.81	<0.002
	5	33.9	4.9	11.4	1.9	<0.005
	6	36.8	5.39	14.2	3.54	<0.01
	7	39.7	3.63	28.6	5.31	<0.02
Cow manure and furrows both present	1	12.0	3.47	7.2	3.00	NS
	2	19.0	4.77	8.0	2.98	NS
	3	25.7	5.1	8.9	2.85	<0.05
	4	29.8	4.31	9.0	2.81	<0.01
	5	34.4	4.37	9.4	2.82	<0.005
	6	39.1	3.04	13.5	4.6	<0.002
	7	40.9	2.44	20.9	6.33	<0.01

SE Standard error
NS Not significant

Table 2. Average one-week oviposition of *P. papatasi* females in presence of 3rd - 4th stage larvae or pupae compared to controls (April - October).

Treatment	Day	Experiment Mean	Experiment SE	Control Mean	Control SE	P
Larvae	1	0.4	0.3	0.2	0.14	NS
	2	0.5	0.3	1.0	0.46	NS
	3	0.6	0.31	3.4	0.74	<0.01
	4	0.9	0.31	6.5	1.8	<0.01
	5	1.4	0.52	9.1	2.11	<0.01
	6	4.3	2.5	12.3	3.37	NS
	7	7.7	4.05	15.0	4.73	NS
Pupae	1	2.4	1.26	5.6	2.55	NS
	2	3.4	1.21	6.0	2.61	NS
	3	5.0	1.19	8.7	1.87	NS
	4	6.1	1.04	11.0	1.56	<0.05
	5	9.2	1.09	12.8	1.72	NS
	6	14.2	1.89	17.0	3.33	NS
	7	16.7	2.29	21.8	5.39	NS

SE standard error
NS not significant

Fig. 1. Oviposition in different months of P. papatasi in standard control cages (see text for specifications); average eggs/fly/day for 7 days.

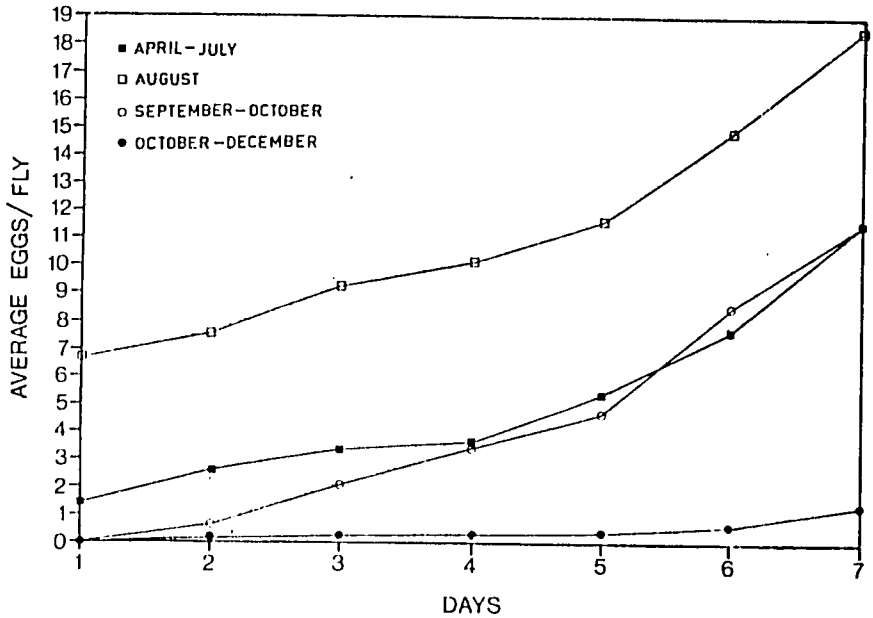


Fig. 2. Oviposition by P. papatasi females in standard control cages, cages with half standard area of plaster substrate or cages of half-volume with half-sized area of plaster; 15 females/cage: average eggs/fly/day for 7 days.

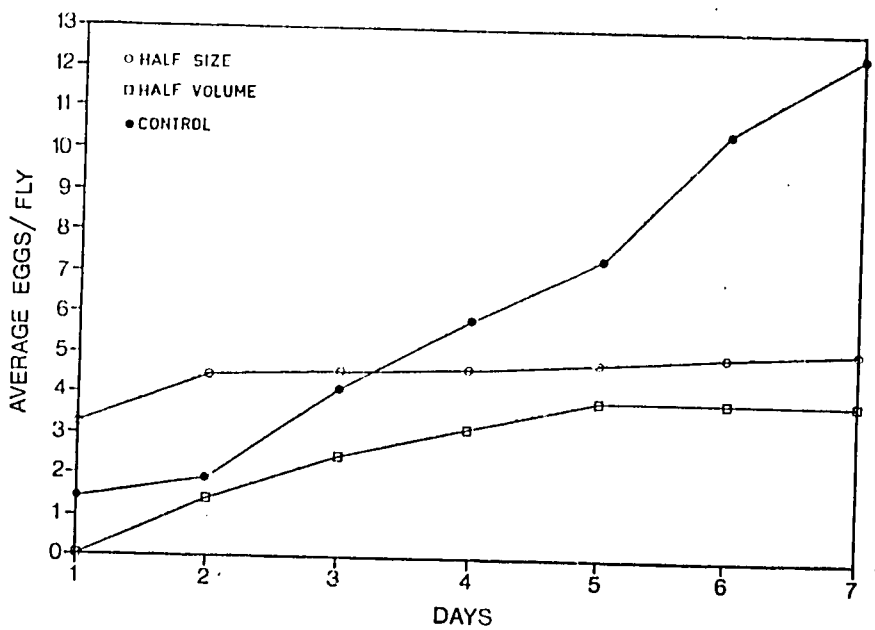


Fig. 3. Oviposition by wintering *P. papatasi* females following 30 mins irradiation with 254 nm ultra violet on the 1st day, 3rd day and untreated controls; 15 females/cage; average eggs/fly/day for 7 days.

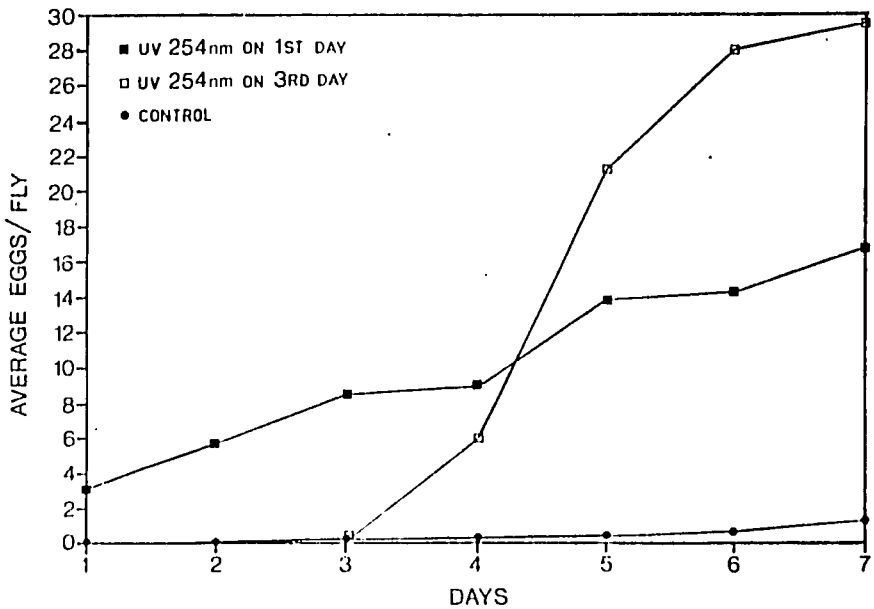


Fig. 4. Oviposition by wintering *P. papatasi* females following irradiation with ultra violet of 312 nm for 90 mins on 1st day, 366 nm for 90 mins on 2nd day or 30 mins on 3 consecutive days (combined results); 15 females/cage; average eggs/fly day for 7 days.

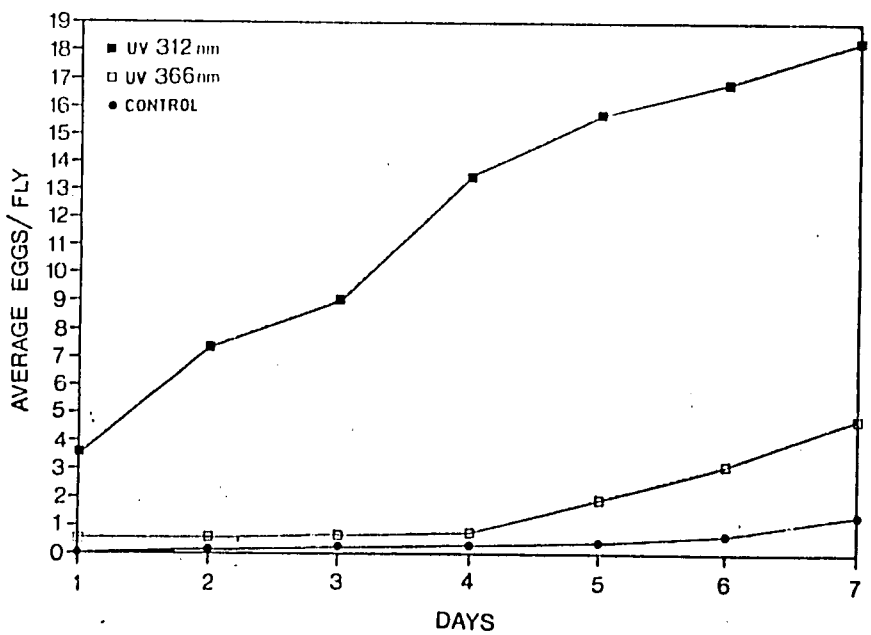
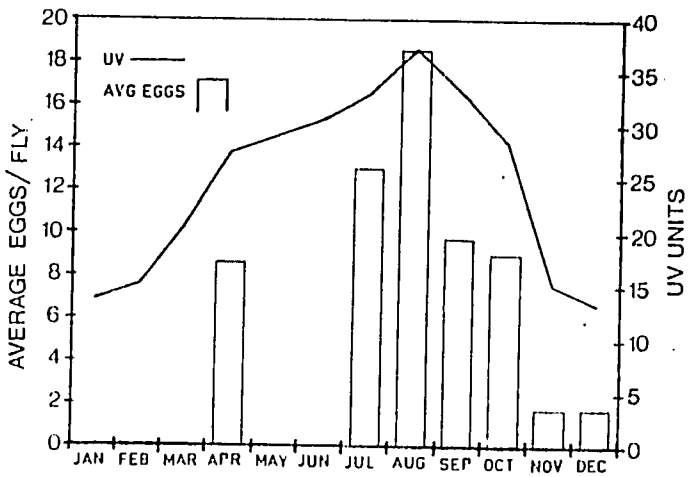


Fig. 5. Monthly levels of fecundity laboratory bred *P. papatasi* (female weekly averages) compared to concurrent maximal sun ultra-violet radiation in Jerusalem (UV in relative units; Ashbel, 1937); values unavailable for January-March and May-June.



C5-136

FIELD AND LABORATORY STUDIES ON NEW ASPECTS OF EPIDEMIOLOGY OF LEISHMANIASIS

AID-CDR Project C5-136

Progress report - April 1988

by

Yosef Schlein

Department of Parasitology

Hadassah Medical School

Jerusalem, Israel

Rec'd In SCI: MAY

The subjects of study are specific and scientists with wide knowledge in parasitology are not necessarily acquainted with the problematics. The summary was therefore extended to include some of the background and previous results which are needed for a rational presentation.

Effects of blood meal on the vector potential of *Phlebotomus papatasi*

The development of *Leishmania* in its sandfly vector is restricted to the alimentary canal. Hence, the subsequent stages of parasites are exposed to a varying medium consisting of the ingested food, induced secretion of enzymes and products of the digestive processes. The interacting factors governing the vector's competence can, thus, be classified into two categories: the innate capability permitting the development of a given *Leishmania* that results from physiological specificities, and the composition of the food taken.

The resistance of vectors unable to support the growth and transmission of *Leishmania* other than the one they transmit naturally has been described several times. However, there has only been one early study (Adler, 1938) which was aimed at investigating the phenomenon and which demonstrated that components of the blood-meal induced vector selectivity. Adler, using artificial feeding, showed that a non-transmitted *Leishmania* could survive in the sandfly following infective meals

of erythrocytes and saline, whereas following meals of serum and saline, the number of flies with parasites was inversely proportional to the amount of serum.

In the sandfly gut, the levels of proteases rise after the ingestion of blood and they could be effectors of Leishmania selection. We compared the effects of the naturally transmitted L. major and of L. donovani, on the gut enzymes of Plebotomus papatasi, using gut homogenates of artificially infected flies and uninfected controls as enzyme preparations. The digestion of C^{14} labelled globin substrate by preparations from flies fed 24 hrs earlier on serum containing L. major was about a third less than that of controls, while that from flies infected with L. donovani was about one third greater. When added to control homogenate, in vitro, promastigotes of other species promoted proteolysis (Schlein and Romano, 1986). Similar preparations of flies fed on rabbit blood were used in further experiments, in which the relative quantity of trypsin-like enzymes was measured at different times after the infective meal (Borovsky and Schlein, 1987).

The overall amount of the enzymes in L. major infections was reduced to 50% and 34% of the controls, 20 and 30 hrs post-feeding and increased to 184% at 52 hrs. The general levels measured for flies infected with L. donovani did not vary considerably from the controls, however, the quantities of different enzymes that were measured from gel-electrophoresis by densitometry were different. The greater effect of L. major leads

to the suggestion that the massive manipulation of gut enzymes by this species represents its defence mechanism against enzymes of its vector. To substantiate this assumption, it was necessary to demonstrate that the trypsin-like enzymes of P. papatasi were harmful to L. donovani. This was confirmed by adding 1% soybean trypsin-inhibitor to infective meals, which resulted, 3 days later, in 63% of the flies harbouring L. donovani compared to the control's 10%.

All the experiments to investigate the fit between vector and pathogen were carried out using the blood of one host, the rabbit. However, there is evidence that the choice of host can also alter vector competence, as shown by the death of L. major in P. papatasi that fed on turkey before or after the infective meal (Schlein et al., 1983). Chicken blood given 24 hrs following an infective meal of parasites in saline had a similar effect and no parasites were observed in flies dissected 3 days later. As with L. donovani, it appeared that trypsin-like enzymes were responsible for the vector's resistance, since the infection succeeded in 63% of the flies, when 1% soybean trypsin-inhibitor was added to the chicken blood-meal (unpublished data). According to these results, it appears that even the adaptation of L. major to P. papatasi is limited and it cannot cope with the composition of trypsin-like enzymes elicited in this sandfly by avian blood. We have previously shown that Phlebotomus papatasi that had been fed on sucrose solution was capable of transmitting L. major. Since with this diet the parasites were able to complete their

cycle it was of interest to observe the effect of sucrose based media on cultured parasites.

In experimental sucrose solution, L. major continued to divide and multiplied by 38-46%. Paramastigotes and aflagellates are forms present in late stages of Leishmania infection in Phlebotomus papatasi. They constituted 79% of the forms in sucrose medium, but a maximum of 15% in NNN. Rate and degree of transformation varied as a function of the life-cycle of the NNN started culture. Motility was lost in sucrose media but was retained in a mixture of sucrose and Ringer's solution. In the latter mixture, the parasites exhibited transformation as well as attachment to the substrate and morphological changes of the flagellum similar to those occurring in the sandfly vector. Parasites from sucrose medium and from P. papatasi reacted similarly, whereas those from NNN reacted differently to a monoclonal antibody. It is suggested that transformation of L.major in sucrose media resembles this process in the vector.

Marking of Phlebotomus papatasi by feeding on sprayed coloured sugar baits

There is no information on the actual sources of natural sugar meal of saandflies, except for the observation that in the laboratory P. papatasi feeds selectively from plants and on honeydew (Schlein and Warburg, 1986). This sandfly - plant interaction suggested the possibility of marking the flies by feeding them on dyed-sugar baits in the field, assuming that the

sprayed baits would compete with naturally available sources of food.

P. papatasi occurs in great numbers in the burrows of the sand-rat Psammomys obesus in the endemic focus of leishmaniasis in the Jordan Valley. We sprayed sugar solution mixed with 1% food dye un plants in a limited area around the rodent colonies and the flies were afterwards caught with CDC light traps. The dye marked an average of 25.9%, 50% and 32.9% of the P. patasi caught respectively after the 1st, 3rd and 6th night following spraying. The usual method for studying sandfly dispersal is based on the application of dyes to captured flies, such marking interrupts the normal behaviour of the sandflies and the recapture is usually low.

The present method can be used for several purposes in the study of sandfly behaviour feeding and dispersal. It could also be used for the assessment of control agents that will be added to the sprayed solution. The influence on caught flies can be observed back in the laboratory, using unmarked flies as controls.

The development of sandfly forms of Leishmania in sucrose solutions

The dominant form of Leishmania in profeinaceous cultures is the promastigote. In the sandfly the ingested amastigotes develop into elongate promastigotes and in P. papatasi these give rise to a great number of infective promastigotes, paramastigotes, and

aflagellates. Paramastigotes and aflagellates appear in small numbers in cultures and were considered degenerate. In the sandfly the parasites develop first in medium of the infective blood meal and then in the subsequent sugar meals. This study was undertaken to see whether development of the specific sandfly-forms of Leishmania can be imitated by exposure of parasites reared in NNN medium to sucrose solution. The medium used was sucrose solution alone or in mixture with Ringer's salt solution. The parasites were initially grown in NNN medium and starters of sucrose cultures were taken at different stages of the development of the NNN mother culture. In sucrose solutions L. major continued to divide and multiply by 38-46%. Paramastigotes and aflagellates constituted 79% in sucrose medium compared to maximum 15% in NNN starter cultures. Motility was lost in sucrose solutions but it was retained in a mixture of sucrose and Ringers' solutions. In the latter, the parasites exhibited transformation as well as attachment to their substrate followed by thickening of the flagellum. The thickening appeared similar to the hemidesmosomes formed by attachment of the flagellum to the cuticle in the gut of the vector. The transformation of L. major in sucrose solution resembles, and is apparently similar to the development in P. papatasi.

It is difficult to study the Leishmania forms in the sandfly as the infection of flies and harvesting of the parasites are complicated and the number of parasites obtained finally would be limited. By growing in sucrose medium it is possible to obtain

greater numbers of parasites and to study their particular traits of the stages occurring in the sandfly vector.

Evaluation of food-dye marking in the study of sandfly behaviour in Baringo district, Kenya

Evaluation of the food-dye marking method for the study of sandfly behaviour was carried out in Baringo district, Kenya. This method was developed by us and tried successfully in the Jordan Valikey (see previous chapter in the report).

Bait solution of sucrose and food-dye was sprayed on the vegetation around an animal burrow which was the source of sandflies. The extent of marking and the distribution of coloured flies within a radius of 200 m was monitored for 4 nights. The species caught wer Sergentomyia clydei, S. schwetzi, S. antennatus, S. squamipleuris, S. bedfordi, S. africanus, S. ingrami and S. affinis. Feeding on the bait marked 68.9% of the flies caught at burrow's entrance. The total number of flies caught at a distance of 50, 100, 150 and 200 m exceeded the theoretical expectation. The varied marking of species in the different locations was discussed in the paper.

The common method for studying sandfly behaviour is based on the application of dyes to capture flies which are then released. Dispersal is determined from the distance od recapture. In the food-dye marking the flies mark themselves by feeding on sucrose and food-dye bait. The advantage of the latter method is that it enables the observation of the normal undisturbed behaviour of

the sandflies. It is also possible to study the behaviour of populations since the marking can continue for a long period. Two major problems in the epidemiology of visceral leishmaniasis in Kenya are the identification of the reservoir animals and the location of the sources of infection. Monitoring the behaviour of sandflies with this method may help to solve these problems and elucidate other aspects in the behaviour of vector sandflies.

PUBLICATIONS

Borovsky D. and Schlein Y. Trypsin and chymotrypsin-like enzymes of Leishmania infected Phlebotomus papatasi (Diptera:Psychodidae) and their possible role in vector competence. Med. Vet. Ent.

Schlein, Y., Borut, S., and Greenblatt, C.L. Development of sandfly forms of Leishmania major in sucrose solutions. J. Parasit. 73: 797-805.

Schlein, Y. Marking of Phlebotomus papatasi (Diptera:Psychodidae) by feeding on sprayed, coloured sugar bait: A possible means for behavioural and control studies. Trans. Roy. Soc. Trop. Med, Hyg. 81: 599 (1987).

Schlein, Y., Mutingo, M.J., Jacobson, R.L. and Kaddu, J.B. Evaluation of food-dye marking in the study of sandfly behaviour in Baringo district, Kenya (sent for publication).

Am. J. Trop. Med. Hyg., 35(5), 1986, pp. 926-930
Copyright © 1986 by The American Society of Tropical Medicine and Hygiene

C5-131

THE EFFECT OF POST-BLOODMEAL NUTRITION OF *PHLEBOTOMUS PAPATASI* ON THE TRANSMISSION OF *LEISHMANIA MAJOR*

ALLON WARBURG AND YOSEF SCHLEIN

Department of Parasitology, The Kwan Centre for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, P.O. Box 1172, Jerusalem 91010, Israel

Abstract. To test the effects of post-bloodmeal nutrition of sand flies on the transmission of *Leishmania major*, groups of infected *P. papatasi* females maintained on diets of sucrose, trehalose, albumin or a mixture of sucrose and albumin, were subjected to forced feeding with capillaries. Transmission was evaluated by counting the parasites egested; numbers ranged from 0 to over 1,000 promastigotes. Infections of the anterior midgut were seen in the majority of flies from all the experimental groups but the percentage of transmitting females was significantly higher in the group maintained on a mixture of sucrose and albumin. There were no attached parasites in the pharynx and cibarium of the flies and the presence of free promastigotes in these parts was not itself indicative of infectivity. However, transmission was positively correlated with apparent inability to engorge. The parasites egested were typical infective form promastigotes and identical to those observed in the esophagus and the anterior thoracic midgut.

A mechanism by which infective stage promastigotes from the esophagus and the stomodeal valve may be transmitted by bite is proposed.

In nature sand flies acquire *Leishmania* infections by feeding on an infected vertebrate host. Transmission occurs several days later when, following oviposition, the next bloodmeal is taken.[1] In the interim period the flies take regular sugarmeals, probably of plant origin.[2,3]

Transmission of *Leishmania* by laboratory-reared sand flies which are routinely maintained on blood and artificial sugar diets is difficult to achieve and unpredictable for reasons which may be attributed to the lack of specific nutritional factors.[1] Under experimental conditions, laboratory-reared *P. papatasi* obtain sugars from plants by probing leaves and stems as well as from honeydew secreted by plant-sucking homopteran insects.[4] The contents of plant sap and honeydew include sugars and significant amounts of amino acids.[5,6] The constituents of sand fly sugarmeals may facilitate the transmission of *Leishmania*.[1]

We assessed the effects of some artificial post-bloodmeal diets on the transmission of *L. major* by infected *P. papatasi* females. Forced feeding of sand flies with microcapillaries simulated normal bloodfeeding and enabled individual testing of a large number of infected flies. Microscopial observations of the mouth parts made during forced feeding provided us with information relevant to the possible mechanism of transmission by bite.

MATERIALS AND METHODS

Sand flies

Phlebotomus papatasi were obtained from a laboratory colony maintained at the Department of Parasitology, Hadassah Medical School.

Leishmania

The *Leishmania major* strain used was a recent isolate from a naturally infected wild-caught *P. papatasi* female.

Artificial infections and post bloodmeal diets

Cultured promastigotes were washed, counted and resuspended in a mixture of defibrinated, inactivated rabbit blood and normal saline (4:1) to a final concentration of 2×10^6 promasti-

Accepted 9 April 1986.

FIGURE 1. Forced feeding of *P. papatasi*. The pre-constricted end of a microcapillary (CP) is slipped over the mouth parts folding back the labium (LM). Sucking is evidenced by flexing of the clypeus (arrow) and fluid is passed to the abdominal midgut. (Bar = 0.5 mm.)

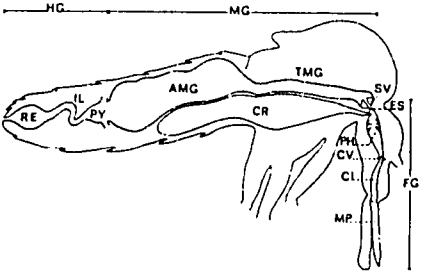


FIGURE 2. Scheme of a sagittal section of a *P. papatasi* female showing the different parts of the alimentary canal: foregut (FG), midgut (MG), hindgut (HG), mouth parts (MP), cibarium (CI), cibarial valve (CV), pharynx (PH), esophagus (FS), stomodeal valve (SV), thoracic midgut (TMG), crop (CR), abdominal midgut (AMG), pylorus (PY), ileum (IL) and rectum (RE).

gotes/ml, i.e., 200 per average sand fly meal. Sand flies were fed on this suspension as previously described.[7] Groups consisting of 30 gorged females each were separated and maintained on one of the following diets: 10% sucrose, 10% trehalose, 10% albumin or a mixture of 2% albumin and 10% sucrose offered in cotton-wool pads beginning 48 hr post-infection.

Forced feeding

Microcapillaries were prepared according to the method described by Hertig and McConnell.[8] The infected flies were lightly etherized and fixed to microscope slides by means of double-stick tape. A capillary prefilled with a mixture of saline and rabbit blood (9:1) was slipped over the mouth parts folding back the labium (Fig. 1) as in the natural bloodfeeding mode.[9] Flies were allowed to feed for 10 min after which time they were dissected and examined. Infections in the different parts of the alimentary canal were scored using a phase contrast microscope. Parasites egested into each capillary were counted under a phase contrast microscope after emptying the fluid onto a glass slide. The results were analyzed using the χ^2 test. Flies egesting parasites into capillaries are hereafter referred to as transmitting flies.

RESULTS

An average infection rate of 78.8% was scored in 241 flies from 10 experimental groups which were dissected following forced feeding 10–12 days after the infective meal. Almost all of the infected flies (97%) had parasites in the thoracic midgut and the stomodeal valve and in 54% of them free promastigotes were observed in the pharynx (Fig. 2). The general development of infections and the distribution of morphological forms of parasites were similar in the different groups, however the percentage of transmitting flies was significantly higher ($P < 0.01$) in the group fed on a mixture of sucrose and albumin (Table 1).

TABLE 1

Effect of post-bloodmeal diets of P. papatasi *on the transmission of* L. major *(10–12 days after infection)*

Diet	1	2	3	4
	10% Sucrose	10% Trehalose	10% Albumin	2% Albumin 10% Sucrose
Number of infected flies*	55	20	35	66
Number of transmitting flies	11	3	4	23
Percent transmitting flies	20.0	15.0	11.4	34.8

* Flies in which no cibarial pumping was observed during forced feeding were discarded.

TABLE 2

Correlation of transmission with feeding and presence of parasites in the pharynx

	Normal engorgement	No engorgement	Parasites in pharynx
Infected flies	162	14	95
Number of transmitting flies	31	10	41
Percent transmitting flies	19.1	71.4	43.2

Microscopial observations made during the forced feeding showed that insertion of the mouth parts into capillaries initiated rhythmic pumping of the cibarial pump in 92.6% of the flies. This was evidenced by the flexing of the clypeus (Fig. 1) at the insertion points of the cibarial dilator muscles and resulted in a flow of fluid into the proboscis. Flies where no pumping was observed were discarded. Most flies were fully gorged within 5–10 min but some ingested only small quantities while in a few, despite vigorous pumping, no fluid passed into the midgut. Many of the flies that did not gorge regurgitated fluid containing parasites into capillaries (Table 2).

Abdominal midgut infections were heavy in all the groups, consisting mainly of elongate, thin promastigotes (nectomonads, 15–25 μm long × 1 μm thick). The thoracic midgut and stomodeal valve were crammed with parasites of different morphological forms including short (4–5 μm), highly active unattached promastigotes which were also found in the esophagus (Fig. 3) and were the only forms seen in fluid from the capillaries. No attached parasites were found anterior to the esophagus, and head infections consisted of free, highly active promastigotes which may have been flushed forward during the feeding. Most of the transmitting flies (75.6%) egested between 7 and 100 parasites ($\bar{x}$ = 33.4), some (19.5%) egested 100–1,000 parasites ($\bar{x}$ = 275.0) and 2 flies (4.9%) egested >1,000 parasites each.

FIGURE 3. Scanning electron micrograph of *L. major* in the esophagus of *P. papatasi*. Note infective stage promastigotes (IS) intertwined between attached paramastigotes (PM). (Bar = 5 μm.)

DISCUSSION

No difference in the development of *Leishmania* parasites was observed among the groups fed on different diets or between transmitting and nontransmitting flies. This observation supports the work of Hertig and McConnell who observed that neither raisins nor sugars fed to infected sand flies had any effect on the development of *Leishmania*.[8] Since there were transmitting flies in all the groups, it seems that there is no single nutritional factor essential for transmission of *Leishmania*. Nevertheless, the group fed on a mixture of sugar and protein yielded the highest proportion of transmitting flies. Hence, the presence of protein in sugarmeals enhanced transmission.

Although unattached promastigotes were observed in the pharynges of all the transmitting flies, they were also seen in many nontransmitting ones (Table 2). Therefore their presence should not be considered indicative of a fly's potential capability to transmit by bite. A high percentage of the flies where cibarial pumping was observed but fluid was not ingested were shown to be transmitters (Table 2). A similar situation was noted by Beach et al. and Killick-Kendrick and Molyneux who suggest that *Leishmania* in the cibarium interfere with cibarial sensillae which control engorgement.[10,11]

This assumption is not supported by our results as only few flies had free promastigotes (in small numbers) in their cibaria and no parasites were observed attached to the cibarial wall in any of them.

Recent scanning electron microscope studies showed that the mass of parasites in the anterior thoracic midgut, the stomodeal valve and the esophagus could be divided into 2 distinct types (Fig. 3): cuticle-attached haptomonads and paramastigotes and unattached highly active short promastigotes with long flagella.[12] The cuticle-attached forms occupied anterior positions in the stomodeal valve and the esophagus but they were never egested into capillaries. These attached forms in the pocket between the gut wall and the stomodeal valve may, however, constrict the lumen of the valve by compressing its surrounding lobes and thus prevent or reduce the passage of blood into the midgut. Furthermore, the attached forms inside the esophagus provide a means of anchorage for the free infective forms seen intertwined between them (Fig. 3). These observations support the "blocked fly" theory of transmission by bite originally suggested by Shortt and Swaminath.[7]

Action of the cibarial and the pharyngeal pumps, triggered by slipping a capillary onto the proboscis, continued as long as the mouth parts were forcibly deployed in the bloodfeeding mode. According to the proposed mechanism, in the case of a blocked or partially blocked stomodeal valve, the rate of flow into the esophagus is faster than the rate with which blood can flow posteriorly into the midgut. Consequently the esophagus expands (a phenomenon known to occur during normal feeding[9]) until the pressure exerted by its walls exceeds that of the pharyngeal pump. This results in a backflow of fluid, flushing with it unattached infective forms of *Leishmania* into the pharynx, cibarium and mouth parts, thereby passively depositing them in the skin. The regurgitation may be further enhanced if, as suggested by Killick-Kendrick and Molyneux, the altered rate of flow impairs the coordinated functioning of the pumps and the valves in the food canal.[11]

A similar distribution of morphological forms of parasites was noted in both transmitting and nontransmitting flies. It would appear that the difference between the two groups is in the degree of restriction of the passage through the stomodeal valve. This could be brought about by the accumulation of attached parasites and infective stage promastigotes in the anterior thoracic midgut and the stomodeal valve. A diet consisting of both protein and sugar, rather than either of these factors alone, seems to be more suitable for sustaining sufficiently heavy infections so as to form at least a partial block of the stomodeal valve.

The presence of infective stage *L. major* in the midguts of infected *P. papatasi* has been demonstrated experimentally.[13] Our observations support this finding and the proposed mechanism explains how large numbers of these infective promastigotes can be egested during bloodfeeding of flies which have no permanent infection of the cibarium or the proboscis.

ACKNOWLEDGMENTS

We thank S. M. LeBlancq for her useful comments on the manuscript.

This work was supported by the project on Epidemiology and Control of Vector Diseases in Israel (REP-NIH-NIAID-AI.12669), AID grant No. DPE-5542-G-SS-4054-00 and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. Killick-Kendrick, R., 1979. Biology of *Leishmania* in phlebotomine sandflies. Pages 395–460 *in* W. H. R. Lumsden and D. A. Evans, eds., *Biology of the Kinetoplastida, Volume 2.* Academic Press, London and New York.
2. Lewis, D. J., and Domoney, C. R., 1966. Sugar meals in Phlebotominae and Simuliidae. *Proc. R. Ent. Soc., Lond. (Series A), 41:* 175–179.
3. Young, C. J., Turner, D. P., Killick-Kendrick, R., Rioux, J. A., and Leaney, A. J., 1980. Fructose in wild-caught *Phlebotomus ariasi* and the possible relevance of sugar taken by sandflies to the transmission of leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg., 74:* 363–366.
4. Schlein, Y., and Warburg, A., 1986. Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. *J. Med. Entomol., 23:* 11–15.
5. Ewart, W. H., and Metcalf, R. L., 1956. Preliminary studies of sugars and amino acids in the honeydews of five species of coccids feeding on citrus in California. *Ann. Ent. Soc. Am., 49:* 441–447.
6. Auclair, J. L., 1963. Aphid feeding and nutrition. *Ann. Rev. Entomol., 8:* 439–491.
7. Schlein, Y., Warburg, A., Schnur, L. F., and Shlomai, J., 1983. Vector compatibility of *Phlebo-*

tomus papatasi dependent on differentially induced digestion. *Acta Trop., 40:* 65–70.

8. Hertig, M., and McConnell, E., 1963. Experimental infection of Panamanian *Phlebotomus* sandflies with *Leishmania. Exp. Parasitol., 14:* 92–106.
9. Shortt, H. E., and Swaminath, C. S., 1928. The method of feeding of *Phlebotomus argentipes* with relation to its bearing on the transmission of Kala-azar. *Indian J. Med. Res., 15:* 827–836.
10. Beach, R., Kiln, G., and Leeuwenburg, J., 1985. Modification of sand fly biting behavior by *Leishmania* leads to increased parasite transmission. *Am. J. Trop. Med. Hyg., 34:* 278–282.
11. Killick-Kendrick, R., and Molyneux, D. H., 1981. Transmission of leishmaniasis by the bite of phlebotomine sandflies: Possible mechanisms. *Trans. R. Soc. Trop. Med. Hyg., 75:* 152–154.
12. Warburg, A., Hamada, G. S., Schlein, Y., and Shire, D., 1986. Scanning electron microscopy of *Leishmania major* in *Phlebotomus papatasi. Z. Parasitenkd., 72:* 423–431.
13. Sacks, D. L., and Perkins, P. V., 1985. Development of infective stage *Leishmania* promastigotes within phlebotomine sand flies. *Am. J. Trop. Med. Hyg., 34:* 456–459.

C5-136

J. Parasit. 73(4), 1987, pp. 797–801
© American Society of Parasitologists 1987

DEVELOPMENT OF SANDFLY FORMS OF *LEISHMANIA MAJOR* IN SUCROSE SOLUTIONS

Yosef Schlein, Shira Borut, and Charles L. Greenblatt
Department of Parasitology, Hadassah Medical School, Jerusalem, Israel

ABSTRACT: Stages of *Leishmania* developing in the vector include different morphs that are exposed first to ingested blood and then to sugar meals. This study sought to determine whether stages occurring in the latter medium could be induced by culturing in sugar-based media. In sucrose solutions, *L. major* continued to divide and multiplied by 38–46%. Paramastigotes and aflagellates are forms present in late stages of *Leishmania* infection in *Phlebotomus papatasi*. They constituted 79% of the forms in sucrose medium, but a maximum of 15% in NNN. Rate and degree of transformation varied as a function of the stage of growth of the NNN starter culture. Motility was lost in sucrose media but was retained in a mixture of sucrose and Ringer's solution. In the latter mixture, the parasites exhibited transformation as well as attachment to the substrate and morphological changes of the flagellum similar to those occurring in the sandfly vector. Parasites from sucrose medium and from *P. papatasi* reacted similarly, whereas those from NNN reacted differently to a monoclonal antibody. It is suggested that transformation of *L. major* in sucrose media resembles this process in the vector.

The development of *Leishmania* of mammals in the phlebotomine vector was followed by light microscopy (Adler and Theodor, 1926, 1927, 1931; Shortt et al., 1926; Hertig and McConnell, 1963; Strangways-Dixon and Lainson, 1966; Johnson and Hertig, 1970) and the morphology of the parasites was described using electron microscopy (Killick-Kendrick et al., 1974, 1977; Molyneux et al., 1975). During the sequence of the development, fairly definite morphological forms follow in a specific order (Adler and Theodor, 1931). These developmental processes were summarized and the nomenclature was discussed by Killick-Kendrick (1979). Amastigotes taken with the blood meal develop into long and slender promastigotes—the so-called nectomonads, in the abdominal midgut. Following migration to the thoracic midgut, they turn into short, broad promastigotes—the haptomonads. These and the paramastigotes with a kinetoplast beside the nucleus, which occur in the foregut, are characteristically attached to the gut lining. The infective forms are small promastigotes that remain free moving.

Female sandflies take 2 types of food: blood meals taken at intervals, usually 1 for each gonotrophic cycle, and regular sugar meals (Lewis and Domoney, 1966; Young et al., 1980; Endris et al., 1984; Yuval and Schlein, 1986). The development of nectomonads takes place in the abdominal midgut, within the infective meal, from which they are released to move forward when the blood is digested. Their transformation occurs in the thoracic midgut and foregut, when they are exposed to the ingested sugar meals. The composition of natural sugar meals is unknown. However, the importance of such meals was first demonstrated by the success in transmitting *L. donovani* to hamsters (Smith et al., 1941) and humans (Shortt, 1945) by the bite of *Phlebotomus argentipes* fed on raisins. In the laboratory *L. major* completed its development in *P. papatasi* that had been maintained on sucrose solution (Killick-Kendrick, 1986; Warburg and Schlein, 1986). It was therefore of interest to study the effect of sugar solutions on the transformation of cultured *Leishmania*.

MATERIALS AND METHODS

The study was carried out with *Leishmania major* LRC-L137 (MHOM IL 67 JERICHO-II), a human isolate from Israel. Cultures were maintained at 28 C. The experiments were started with parasites from NNN cultures grown in 500-ml Erlenmeyer flasks containing 30 ml medium plus 25 ml 0.9% NaCl, inoculated with 2×10^7 parasites from 2-day-old cultures. NNN cultures reached the stationary phase between days 6 and 9 with about 9×10^7 ml parasites. Experimental media were inoculated with parasites at the beginning of culture growth, on the second or the third day; the middle of growth, on the fourth day; end of growth, on the sixth day; and on the 10th day at the stationary phase. Inoculum was prepared from the overlayer, which was centrifuged at 500 *g* for 10 min and the pellet was resuspended in PBS and similarly centrifuged 3 times. The parasites were resuspended in a small quantity of experimental solution made up to about 5×10^6/ml. Experiments with 0.254 M (osmolarity equal to 0.9% NaCl) or 0.127 M sucrose were conducted in 2 or 3 replicates of 1 ml in 13.5-ml plastic tubes (Sterilin, Feltham, Middlesex, England). Another medium was used in 2-ml aliquots of a 4:1 mixture of the above sucrose solutions with Ringer's solution containing 0.65 g NaCl, 0.25 g KCl, 0.03 g $CaCl_2$, and 0.025 g $NaHCO_3$

Received 14 January 1987; revised 3 April 1987; accepted 7 April 1987.

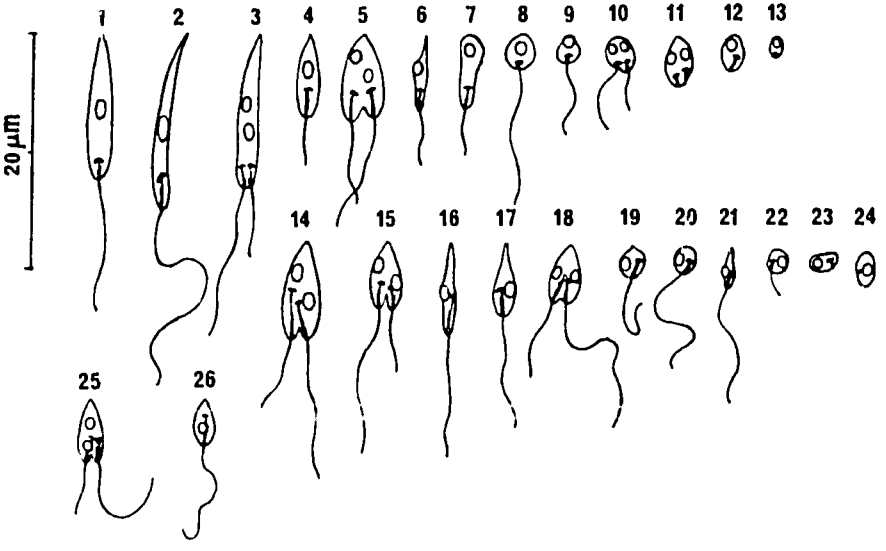


FIGURE 1. Representative forms of the different *L. major* morphs after 24 hr or more in 0.254 M sucrose.

in 100 ml water (Novak, 1966). Media were placed in 35 × 10-mm plastic petri dishes (Falcon, Oxnard, California). Prewetting of the containers for 10 minutes with their respective experimental media was carried out, because without this treatment the promastigotes adhered longitudinally to the surface.

The observation period for cultures in tubes and in petri dishes was 11 and 7 days, respectively. Drop samples from the tubes, taken at different days, were dried on microscopic slides, fixed with absolute methanol, and stained with Giemsa's stain. The *Leishmania* in petri dishes were observed through an inverted microscope. Fluids were removed at 1- or 2-day intervals and parasites attached to the bottom were stained *in situ* while others were sampled on microscopic slides. *Leishmania* forms were determined in 1,000 cells, on each stained preparation and the proportion of each form was calculated. The tendency of parasites to attach was also observed. Glass slides, or strips cut from plastic petri dishes were introduced into cultures for different periods, then examined fresh under a phase microscope or stained. Parasites were defined according to Killick-Kendrick (1979), the criteria being the location of the kinetoplast vs. the nucleus and presence or absence of a flagellum. Flagellates with the kinetoplast anterior to the nucleus were called promastigotes, those with the kinetoplast transverse to the nucleus—paramastigotes, and those with the kinetoplast posterior to the nucleus—opisthomastigotes. Small rounded forms without a flagellum (not mentioned by Killick-Kendrick, 1979) were termed aflagellates to avoid confusion with the vertebrate, intracellular forms—amastigotes.

Accurate distinction between nectomonads (long promastigotes) and haptomonads (short and broad promastigotes) was not possible because there were intermediate forms.

Reaction with monoclonal antibody

Leishmania major from a 3-day-old NNN culture, from 3- or 6-day-old 0.254 M sucrose cultures, and from a 7-day-old infection of *Phlebotomus papatasi* were treated with monoclonal antibody, WIC 79.3 (Ibarra et al., 1982). This monoclonal antibody binds a *Leishmania* surface complement which is believed to function as a ligand to macrophages (Greenblatt et al., 1985; Handman and Goding, 1985). The sandflies were artificially infected by feeding through a membrane on 10^7 ml parasites in rabbit blood (Schlein et al., 1983), and maintained on 20% sucrose solution and water until dissected. The guts were teased apart in the wells of multitest slides (Flow Laboratories, Irvine, Scotland) and fixed in 1.0% formaldehyde in PBS. Parasites from cultures were fixed in the same fixative and stored in fixative solution at 4 C. They were then concentrated by centrifugation at 500 g for 10 min, and brought to a concentration of about 10^7/ml. Twenty microliters was pipetted into each well of multitest slides and dried at 60 C. The slides were exposed for 30 min to a 1:20 dilution of ascitic fluid containing the monoclonal antibody, washed 5 times in PBS, stained with 1:20-diluted fluorescent antimouse globulin antibody (Bio-Yeda, Rehovot, Israel), and examined by fluorescent microscopy.

RESULTS

The apparent transition of *Leishmania* forms

The transformation of cultured *Leishmania* from one form to another, as observed in stained

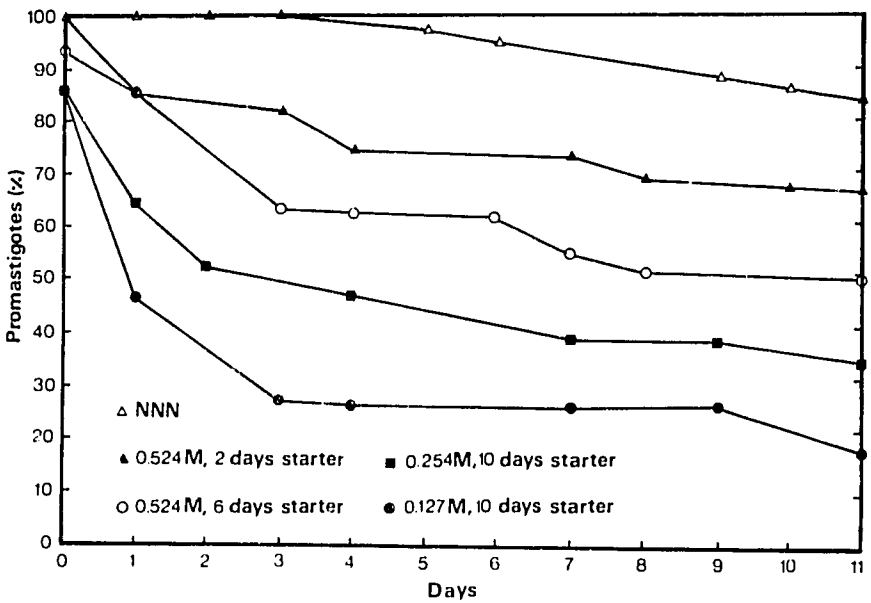


FIGURE 2. Percentage of promastigotes of *L. major* on different days in NNN or sucrose cultures inoculated with starters of different ages in NNN.

preparations, seems to result from cell division. The long promastigotes (Fig. 1, nos. 1, 2) turned into shorter haptomonads (no. 4) and these, by successive divisions (nos. 5, 10, 11), into smaller promastigotes of various shapes and flagellar lengths or even into aflagellates (nos. 12, 13). Divisions of organisms without an external flagellum were rarely observed (no. 11) and such aflagellates as nos. 13 and 24 did not appear to divide.

Paramastigotes and opisthomastigotes seemed to be formed by division of promastigotes, 1 kinetoplast moving to a posterior position and 1 of the nuclei in the opposite direction (nos. 14, 15, 25).

Paramastigotes continued to divide (no. 18) and by repeated divisions may have been diminished both in size, and in the length of the flagellum, to become aflagellates. All these forms were also observed in preparations made from infected *Phlebotomus papatasi*.

The proportions of *Leishmania* forms in sucrose solutions

Promastigotes were the most prevalent form of *L. major* in NNN. Other forms appeared gradually and together constituted about 15% on the 11th day (Figs. 2–4), whereas growth in sucrose solutions accelerated transformation into paramastigotes and aflagellates.

The rate and degree of transformation in sucrose media was correlated with the developmental stage of the NNN starter. Thus, promastigotes constituted 65.8%, 47.9%, and 33.4% of the forms on the 11th day in 0.254 M cultures that had been inoculated with 2-, 6-, and 10-day-old starters, respectively. Low osmolarity of 0.127 M sucrose medium, inoculated with a 10-day-old starter, further decreased the percentage of promastigotes observed to 16.4% on the 11th day (Fig. 2). Most of the observed reduction in promastigotes was replaced by paramastigotes. In 0.254 M sucrose solution, aflagellates constituted 0.9%, 12.3%, and 24.1%, respectively, following 2-, 6-, and 10-day-old starters. In the 0.127 M sucrose solution at that time, the 10-day-old inoculum produced 48.6% aflagellates on the 11th day (Fig. 4). Opisthomastigotes were present in only small numbers and the maximum percentage recorded was 0.2 in NNN and 1.8 in sucrose solutions.

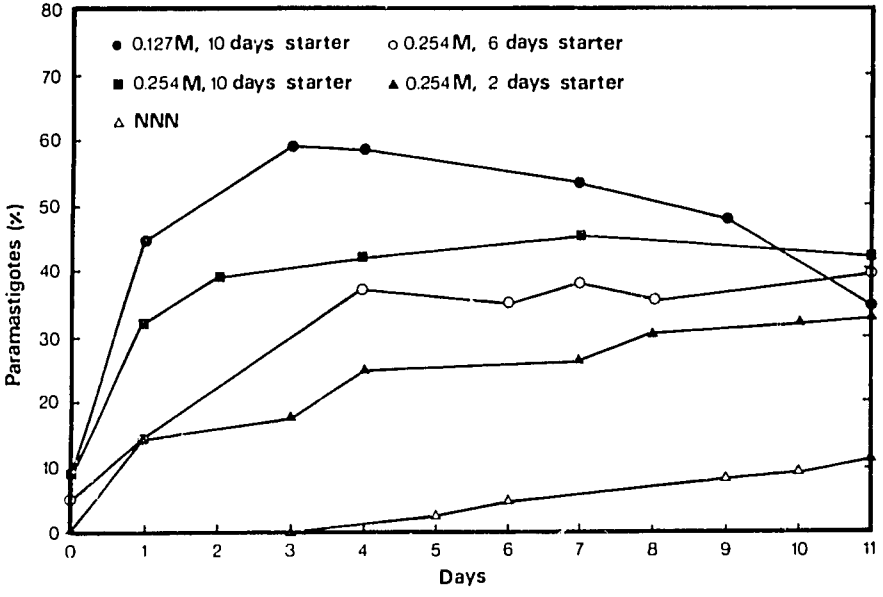


FIGURE 3. Percentage of paramastigotes of *L. major* on different days in NNN or sucrose cultures inoculated with starters of different ages in NNN.

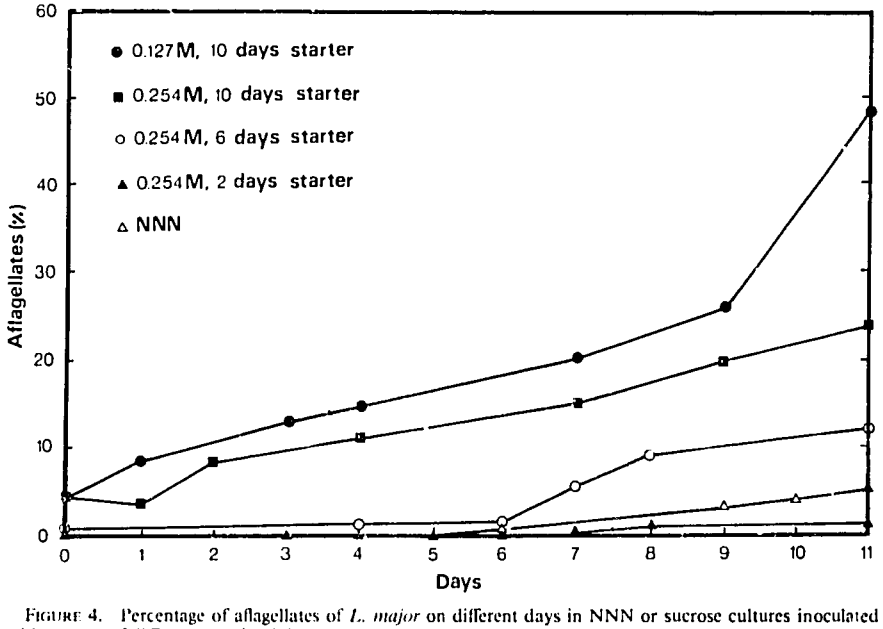


FIGURE 4. Percentage of aflagellates of *L. major* on different days in NNN or sucrose cultures inoculated with starters of different ages in NNN.

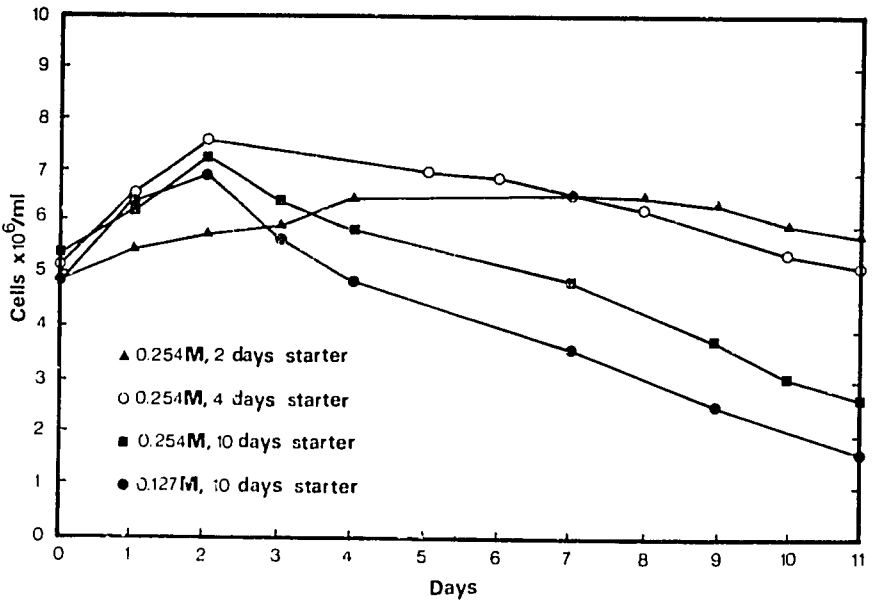


FIGURE 5. Number of *L. major* on different days in sucrose solutions following inoculation with starters of different ages in NNN.

Multiplication in sucrose solutions

Leishmania major continued to multiply in sucrose solutions and reached a level of 38–46% more than in the initial inoculum. In cultures started with a 2-day-old inoculum, both the increase and the decrease in numbers were gradual. When 10-day-old inoculum was used, a steeper increase in numbers occurred within 48 hr whereas reduction to about 50% of the initial inoculum took until the 11th day (Fig. 5).

Dividing promastigotes in cultures of 0.254 M sucrose were counted in stained preparations.

TABLE I. *Percentage of dividing promastigotes and paramastigotes in the starter and in 0.254 M sucrose.*

Age of starter	*Leishmania* forms	Days in 0.254 M sucrose: 0 (starter)	1	3–4	10
2 days	Promastigotes	22.6	11.6	10.7	7.1
	Paramastigotes	0	0.2	1.2	0.9
3 days	Promastigotes	22.3	13.2	11.3	7.0
	Paramastigotes	0	0.4	2.2	0.8
6 days	Promastigotes	9.0	18.8	6.8	2.3
	Paramastigotes	4.6	0.5	1.8	0.2
10 days	Promastigotes	1.5	8.7	1.8	1.2
	Paramastigotes	9.0	1.6	1.3	0.9

Multiplication after 24 hr was found to exceed that of the starters in cultures with 6- and 10-day-old NNN inoculum. The rate of division declined gradually within 10 days, from 11.6 to 7.1% for a 2-day-old inoculum and more rapidly, from 8.7 to 1.2% for a 10-day-old inoculum. The division of paramastigotes was more intensive with a 10-day-old inoculum than with younger ones, and was 1.6% in 0.254 M and 4.5% in 0.127 M sucrose after 24 hr (Table I).

Transformation in mixed sucrose and Ringer's solutions

In media containing a mixture of 0.254 M or 0.127 M sucrose, with Ringer's solution, about 80% of the *Leishmania* quickly sank to the bottom of the petri dishes. Preliminary counts showed that the proportion of the various forms suspended in the fluids or settled at the bottom was similar and counts were based on the latter. On the seventh day, in cultures started with a 10-day-old inoculum, the proportion of the different forms was similar whether grown in sucrose–Ringer's or in sucrose solution alone (Tables II, III; Figs. 2–4).

FIGURE 6. Fluorescent pattern of binding of WIC 79.3 monoclonal antibody by *L. major*. **A**, after 3 days in NNN; **B**, after 3, and **C**, after 6 days in sucrose 0.254 M solution (bar = 10 μm).

Motility and attachments

Most *Leishmania* transferred to sucrose solutions ceased to move after a few hours; only a few were motile after 24 hr. In mixtures of sucrose and Ringer's solutions, parasites remained motile throughout the 7 days of experiments.

Leishmania in NNN medium attached neither to glass nor to plastic. However, immediately after transfer to mixed sucrose–Ringer's mixture, most were seen to attach by their flagellum to both materials. In petri dishes containing this mixture, about 80% of the parasites were always attached to the bottom during the 7 days of experiments. Microscopic examination of stained parasites *in situ* on plastic or glass, after a minimum of 24 hr, showed that some had a short or very short, thick flagellum. In some of the organisms it was possible to discern a thickening of the flagellum at the point of attachment (Fig. 7).

Reaction of *L. major* with a surface-directed monoclonal antibody

Parasites from NNN culture, labeled with WIC 79.3 monoclonal antibody showed a pattern of uniform surface fluorescence when viewed by fluorescence microscopy (Fig. 6A). After 3 days in 0.254 M sucrose, many cells showed patchy and granulated fluorescence (Fig. 6B) which became more pronounced in those that had been in the sucrose solution for 6 days (Fig. 6C). The pattern of fluorescence of the *Leishmania* from *P. papatasi* was similar to that of parasites that had been in sucrose solution for 6 days. However, background fluorescence made photography difficult.

DISCUSSION

By migrating forward in the sandfly gut, *Leishmania* parasites move from a proteinaceous into a sugar-containing medium (Killick-Kendrick,

TABLE II. *Percentage of different forms of* L. major *in 4:1 mixture of 0.254 M sucrose and Ringer's solution following inoculation with 5-day-old NNN starter (parasites counted at the bottom of the petri dishes containing cultures).*

Leishmania forms	Days in solution				
	0 (starter)	1	3	5	7
Promastigotes in division	9.5	5.3	5.0	1.7	1.7
Promastigotes – total	97.1	87.1	64.8	44.0	30.0
Paramastigotes in division	0	0.5	1.4	2.0	1.9
Paramastigotes – total	2.9	12.6	34.0	48.7	56.6
Opisthomastigotes	0	0.3	0.2	0	2.2
Allagellates	0	0	0.9	7.3	12.9

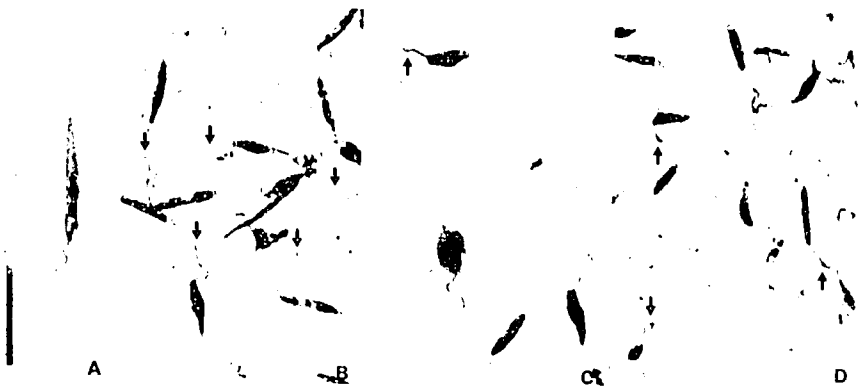


FIGURE 7. **A**, a large *L. major* promastigote, 5-day-old NNN starter culture, Giemsa's staining; **B**, after 24 hr in a 4:1 mixture of 0.254 M sucrose and Ringer's solution, small distension of the flagellar attachment marked by arrows; **C**, thick flagellum and attachment; **D**, 72 hr in sucrose and Ringer's solution, small parasites with a thickened flagellar attachment (bar = 10 μm).

1979); and the organisms that remain in the abdominal midgut are probably exposed to sugars after the blood is digested. Sugar meals in nature are apparently taken directly from plant tissues and from honeydews secreted by homopterans (Killick-Kendrick, 1979; Schlein and Warburg, 1986). The compounds in honeydew are mostly those present in the sap of host plants, which are passed unchanged or slightly modified and among which sucrose is usually one of the major components (Brown, 1975). Furthermore, *L. major* can complete its development and be transmitted by *P. papatasi* maintained on sucrose solution (Killick-Kendrick, 1986; Warburg et al., 1986, Warburg and Schlein, 1986). Hence, we chose sucrose solutions as experimental media and found that the parasites lost their motility in sucrose solutions but regained it with addition of inorganic salts. Such salts are apparently included in natural sugar meals as they are present in various quantities in honeydews (Auclair, 1963) and different plants.

Unlike the sucrose media, natural sugar meals probably contain various nutrients that might be utilized for the metabolic processes of *Leishmania*. Such nutrients are apparently not essential because the parasites retained their vitality and multiplied in the experimental media during the 11 days of observation (Figs. 2, 5; Table I). The resources for these processes must have been innate and their exhaustion was expressed by the size of the parasites, which was diminished from an initial body length of 12 μm to up to 2 μm (Fig. 1). It is possible however that the conditions in the vector are comparable because a similar decrease in size of *L. major* was observed in infected *P. papatasi* (Adler and Theodor, 1926; Warburg et al., 1986).

Sandfly morphs of *Leishmania* appeared in NNN cultures in small numbers. They had been

TABLE III. *Percentage of different forms of* L. major *in 4:1 mixture of 0.127 M sucrose and Ringer's solution following inoculation with 5-day-old NNN starter (parasites counted at the bottom of petri dishes containing cultures).*

Leishmania forms	Days in solution				
	0 (starter)	1	3	5	7
Promastigotes in division	9.5	4.6	1.8	3.2	2.0
Promastigotes—total	97.1	78.3	32.4	28.6	22.1
Paramastigotes in division	0	0.4	2.36	2.1	4.2
Paramastigotes—total	2.9	21.3	48.7	44.4	37.1
Opisthomastigotes	0	0	0	0	0
Aflagellates	0	0.4	18.9	27.0	40.8

observed in early studies and were considered to be dead or degenerate (Wenyon, 1926). The *L. major* cultures in sucrose were viable and their main characteristic was the formation of paramastigotes and aflagellates in greater proportion than in NNN (Figs. 2–5; Tables I–III). It is thus suggested that growth in sucrose medium induces transformation of *Leishmania* in a manner similar to that observed in the sandfly vector. Aflagellates were not mentioned by Killick-Kendrick (1979). However, they were found in the anterior portion of the gut of wild-caught *P. papatasi* infected with *L. major* (=*Herpetomonas tropica*) (Adler and Theodor, 1926) and in preparations made by us from *P. papatasi*. The multiplication and transition to each of the forms in 0.254 M sucrose solutions were dependent on the stage of growth of the NNN starter (Figs. 2–4). In experiments with mixtures of sucrose and Ringer's solutions, the transformation was similar (Tables II, III). Sacks and Perkins (1985) have likewise shown that infective promastigotes of *L. major* and *L. mexicana amazonensis* develop both in culture and in the vector in correlation with the sequence of the multiplication cycles.

Attachment by the flagellum was exhibited by all the forms of *Leishmania* after being transferred to sucrose or to mixtures of sucrose and Ringer's solutions. In the latter medium, some of the parasites that had been attached for 24 hr or more had either thickened short flagella or a thickening at the point of attachment (supposedly hemidesmosomes). Killick-Kendrick (1979) and Molyneux (1983) assumed that association with different parts of the gut, especially contact with the cuticle, and formation of hemidesmosomes determine the morphological features of the *Leishmania*. It is evident from this study that the transition from one form to another in culture is not associated with such factors. However, both the thickening of the flagellum and the formation of attachment–distension, which are considered characteristic of *Leishmania* in the vector, took place in the sucrose-containing media (Fig. 7).

Another similarity between *Leishmania* from sucrose cultures and from the sandfly vector was the pattern of binding of WIC 79.3 monoclonal antibody. Binding was the same for both, but was different for NNN-grown parasites.

The sequence of transformation (Fig. 1) and the gradual reduction in the proportion of promastigotes in sucrose medium (Fig. 2) showed that these were final stages. Infective promastigotes are nondividing organisms (Killick-Kendrick, 1979; Sacks and Perkins, 1984) and they are the only forms transmitted by bite (Killick-Kendrick, 1986). Thus, it appears that development from nectomonads is either into infective promastigotes or into sedentary forms that remain in the sandfly.

It has been suggested several times (recently considered by Beach et al., 1985; Jefferies et al., 1986; Warburg and Schlein, 1986) that the aggregates of sessile parasites in the anterior portion of the gut enhance transmission by restricting the bloodflow during feeding. This interference elicits probing and causes regurgitation of the contents of the foregut which aids in the deposition of infective forms. According to the observations in the present study, this seems to be the only role for these forms of *L. major*.

ACKNOWLEDGMENTS

We gratefully acknowledge grant support from the leishmaniasis component of the UNDP/World Bank WHO Special Programme for Research and Training in Tropical Diseases, the Project on Epidemiology and Control of Vector Borne Diseases in Israel (REP-NIH-NIAID-AL 12669), and AID grant CDR C5-136.

LITERATURE CITED

ADLER, S., AND O. THEODOR. 1926. Further observations on the transmission of cutaneous leishmaniasis to man from *Phlebotomus papatasi*. Annals of Tropical Medicine and Parasitology **20**: 175–194.

———, AND ———. 1927. The transmission of *Leishmania tropica* from artificially infected sandflies to man. Annals of Tropical Medicine and Parasitology **21**: 89–103.

———, AND ———. 1931. Investigations on Mediterranean Kala-azar. I–V. Proceedings of the Royal Society of London, B **108**: 447–502.

AUCLAIR, J. L. 1963. Aphid feeding and nutrition. Annual Review of Entomology **8**: 439–490.

BEACH, R., G. KIEU, AND J. LEWENBURG. 1985. Modification of sandfly biting behavior by *Leishmania* leads to increased parasite transmission. American Journal of Tropical Medicine and Hygiene **34**: 278–282.

BROWN, K. S. 1975. The chemistry of aphids and scale insects. Chemical Society Reviews **4**: 263–288.

ENDRIS, R. G., D. G. YOUNG, AND G. F. BUTLER. 1984. The laboratory biology of the sand fly *Lutzomyia anthophora* (Diptera: Psychodidae). Journal of Medical Entomology **21**: 656–664.

GREENBLATT, C. L., E. HANDMAN, G. F. MITCHELL, F. L. BATTYE, L. F. SCHNUR, AND D. SNARY. 1985. Phenotypic diversity of cloned lines of *Leishman-*

ia major promastigotes. Zeitschrift für Parasitenkunde **71:** 141–157.

HANDMAN, E., AND J. W. GODING. 1985. The *Leishmania* receptor for macrophages is a lipid containing glycoconjugate. EMBO Journal **4:** 329–336.

HERTIG, M., AND E. MCCONNELL. 1963. Experimental infection of Panamanian *Phlebotomus* sandflies with *Leishmania*. Experimental Parasitology **14:** 92–106.

IBARRA, A. A., J. G. HOWARD, AND D. SNARY. 1982. Monoclonal antibodies to *Leishmania tropica major*: Specificities and antigen location. Parasitology **85:** 523–531.

JEFFERIES, D., J. L. LIVESEY, AND D. MOLYNEUX. 1986. Fluid mechanics of bloodmeal uptake by *Leishmania*-infected sandflies. Acta Tropica **43:** 43–53.

JOHNSON, P. T., AND M. HERTIG. 1970. Behavior of *Leishmania* in Panamanian phlebotomine sandflies fed on infected animals. Experimental Parasitology **27:** 281–300.

KILLICK-KENDRICK, R. 1979. Biology of *Leishmania* in phlebotomine sandflies. *In* Biology of the Kinetoplastida, Vol. II, W. H. R. Lumsden and D. A. Evans (eds.). Academic Press, New York, San Francisco, and London, pp. 395–460.

———. 1986. The transmission of leishmaniasis by the bite of the sandfly. Journal of the Royal Army Medical Corps **132:** 134–140.

———, R. LAINSON, A. J. LEANEY, R. D. WARD, AND J. J. SHAW. 1977. Promastigotes of *L. b. braziliensis* in the gut wall of a natural vector, *Psychodopygus wellcomei*. Transactions of the Royal Society of Tropical Medicine and Hygiene **71:** 381.

———, D. H. MOLYNEUX, AND R. W. ASHFORD. 1974. *Leishmania* in phlebotomid sandflies. I: Modifications of the flagellum associated with attachment to the mid-gut and oesophageal valve of the sandfly. Proceedings of the Royal Society of London, B **187:** 409–419.

LEWIS, D. J., AND C. R. DOMONEY. 1966. Sugar meals in Phlebotominae and Simuliidae. Proceedings of the Royal Entomological Society of London, A **41:** 175–179.

MOLYNEUX, D. H. 1983. Host-parasite relationships of Trypanosomatidae. *In* Current topics in vector research, Vol. *1*, K. D. Harris (ed.). Praeger Publications, New York, pp. 118–148.

———, R. KILLICK-KENDRICK, AND R. W. ASHFORD. 1975. *Leishmania* in phlebotomid sandflies. III. The ultrastructure of *Leishmania mexicana amazonensis* in the midgut and pharynx of *Lutzomiya longipalpis*. Proceedings of the Royal Society of London, B **196:** 341–357.

NOVAK, V. J. A. 1966. Insect hormones. Methuen, London, 478 p.

———, AND P. V. PEPKINS. 1984. Identification of an infective stage of *Leishmania* promastigotes. Science **223:** 1417–1419.

———, AND ———. 1985. Development of infective stage *Leishmania* promastigotes within phlebotomine sandflies. American Journal of Tropical Medicine and Hygiene **34:** 456–459.

SCHLEIN, Y., AND A. WARBURG. 1986. Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. Journal of Medical Entomology **23:** 11–15.

———, ———, L. F. SCHNUR, AND J. SHLOMAI. 1983. Vector compatibility of *Phlebotomus papatasi* dependent on differentially induced digestion. Acta Tropica **40:** 65–70.

SHORTT, H. E. 1945. Recent research on kala-azar in India. Transactions of the Royal Society of Tropical Medicine and Hygiene **39:** 13–41.

———, D. J. BARRAUD, AND A. C. CRAIGHEAD. 1926. The life-history and morphology of *Herpetomonas donovani* in the sandfly *Phlebotomus argentipes*. Indian Journal of Medical Research **13:** 947–959.

SMITH, R. O. A., K. C. HALDER, AND I. AHMED. 1941. Further investigation on the transmission of kala-azar. Part VI. A second series of transmission of *L. donovani* by *P. argentipes*. Indian Journal of Medical Research **29:** 799–802.

STRANGWAYS-DIXON, J., AND R. LAINSON. 1966. The epidemiology of dermal leishmaniasis in British Honduras. III. The transmission of *Leishmania mexicana* to man by *Phlebotomus pessoanus*, with observations on the development of the parasite in different species of *Phlebotomus*. Transactions of the Royal Society of Tropical Medicine and Hygiene **60:** 192–201.

WARBURG, A., G. S. HAMADA, Y. SCHLEIN, AND D. SHIRE. 1986. Scanning electron microscopy of *Leishmania major* in *Phlebotomus papatasi*. Zeitschrift für Parasitenkunde **72:** 423–431.

———, AND Y. SCHLEIN. 1986. The effect of post blood-meal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. American Journal of Tropical Medicine and Hygiene **35:** 926–930.

WENYON, C. M. 1926. Protozoology, Vol. I. Bailliere, Tindall and Cox, London, 769 p.

YOUNG, C. J., D. P. TURNER, R. KILLICK-KENDRICK, J. A. RIOUX, AND J. A. LEANEY. 1980. Fructose in *Phlebotomus ariasi* and the possible relevance of sugars taken by sandflies to the transmission of leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene **74:** 363–366.

YUVAL, B., AND Y. SCHLEIN. 1986. Leishmaniasis in the Jordan Valley. III. Nocturnal activity of *Phlebotomus papatasi* (Diptera: Psychodidae). Journal of Medical Entomology **23:** 405–409.

EXPERIMENTAL PARASITOLOGY 62, 376–380 (1986)

C5-136

Leishmania major and *L. donovani:* Effects on Proteolytic Enzymes of *Phlebotomus papatasi* (Diptera, Psychodidae)

YOSEF SCHLEIN AND HELINA ROMANO

Department of Parasitology, The Hebrew University-Hadassah Medical School, P.O. Box 1172, Jerusalem, Israel

(Accepted for publication 6 May 1986)

SCHLEIN, Y., AND ROMANO, H. 1986. *Leishmania major* and *L. donovani:* Effects on proteolytic enzymes of *Phlebotomus papatasi* (Diptera, Psychodidae). *Experimental Parasitology* 62, 376–380. *Phlebotomus papatasi* is susceptible to *Leishmania major* which it transmits in nature, but is resistant to *L. donovani*. The present study compares the effect of *L. major* and *L. donovani* on the proteolytic activity of *P. papatasi* gut enzymes. The experiments measured digestion of C^{14}-labeled globin by gut homogenates of flies. Homogenates were prepared from flies fed on serum only (controls) or from flies fed serum containing promastigotes or their dried culture overlayer. In other experiments, the promastigotes or dried culture overlayers were added *in vitro* to the gut homogenate of control flies. Proteolytic activity of gut homogenate from flies infected with *L. major* was about one-third less than that of controls, while that from flies infected with *L. donovani* was one-third greater. Ingestion of *L. major* dried culture overlayer had an effect on flies similar to that of the promastigotes, while *L. donovani* dried culture overlayer produced no significant effect. When added to gut homogenate *in vitro*, promastigotes of both species promoted proteolysis as did dried culture overlayer of *L. major*. Dried culture overlayer of *L. donovani*, however, had an opposite effect. It is suggested that the observed reduction in proteolytic activity caused by *L. major* infection may result from inhibition of enzyme production. © 1986 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania major; Leishmania donovani;* Trypanosomatidae; Protozoa, parasitic; *Phlebotomus papatasi;* Diptera; Psychodidae; Vector, gut; Enzymes, proteolytic; Dried culture overlayer (DCO); Phosphate buffered saline (PBS).

INTRODUCTION

Leishmania species, the causative agents of leishmaniasis, are transmitted by phlebotomine sandflies. They are ingested with blood meals by female flies, multiply, and migrate forward in the gut from where they are transmitted by bite to another vertebrate host. Various adaptations are required for the parasite to complete a full cycle in the vector (Killick-Kendrick 1979). The basic one seems to be the ability to live in the environment of the fly's gut. In the laboratory, many sandflies are refractory to infection with strains of *Leishmania* spp. other than those they transmit in nature (Killick-Kendrick 1979; Molyneux 1977). This selection by the vector was attributed to factors in the sandfly gut elicited by meals of serum (Adler 1938). In addition, vector competence was found to be an unstable trait when blood meals were taken from an unsuitable host since the balanced conditions, which permit successful infection of the vector by its specific *Leishmania* sp., were upset (Schlein *et al.* 1983). These studies indicate that the digestion of meals is important in determining the survival of a *Leishmania* sp. in the sandfly.

The present study compares the effect of strains of two *Leishmania* species on the proteolytic activity of *Phlebotomus papatasi* gut enzymes. The *L. major* strain used is transmitted by this sandfly in nature and

0014-4894/86 $3.00
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Rec'd in SCI: OCT 14 1986

can produce a high rate of infection (Schlein *et al.* 1983) in contrast to the *L. donovani* strain which rarely survives in *P. papatasi* (Schlein, unpublished data). The assumption was that differences between the two leishmanias, permitting *L. major* but not *L. donovani* to survive in the vector, would find expression in the proteolytic activity of the sandfly gut enzymes.

MATERIALS AND METHODS

The *Phlebotomus papatasi* used in these experiments were from a colony founded (1981) with sandflies caught in the Jordan Valley. The fly colony is maintained at 28 ± 1 C, 80% relative humidity on a diet of 10% sucrose solution and baby mice for blood meals. Experimental groups of flies consisted of 20–50 1–5 day old females that were membrane fed on sterile meals as described by Schlein *et al.* (1983).

Fly groups were allowed to feed on rabbit serum (inactivated at 56 C for 30 min) either alone or mixed with one of the test materials. The midguts of engorged flies were dissected out 24 hr later, homogenized in 0.1 ml Mini Homogenizer (Gencons of Hemel, Hempstead, UK) in a minimal volume of 0.9% NaCl. The homogenate was made up to a volume of 10 µl/sandfly gut with saline, centrifuged for 2 min at 7520*g* (Beckman Microfuge TMB). The supernatant was stored at −20 C and used for the experiments in 50 µl quantities equivalent to five flies. Each repetition of the experiments was carried out using homogenates of experimental and of control groups from the same batch, i.e., fed at the same time.

Leishmania major LRC-L137, a human isolate from the same area in the Jordan Valley as the founders of the sandfly colony, and *L. donovani* LRC-L52 (a human isolate from India) were grown on NNN medium for 3 to 5 days at 28 C. Flagellates were harvested, washed three times in PBS, pH 7.4, and brought to a concentration of 10×10^6/ml.

DCO of 6–9 day old cultures of the two leishmanias was prepared as follows: the fluid was centrifuged at 1000 rpm, filtered through a 0.45-µm filter, brought to pH 5 by adding glacial acetic acid, boiled for 30 min, centrifuged for 10 min at 1000 rpm, dialyzed against distilled water for 48 hr, frozen, and lyophilized. The estimated product of 10×10^6 promastigotes was 0.4 mg DCO. Control material was the overlayer of unused medium that had been incubated for 4 days at 28 C. The protein content of test materials was determined by Lowry's method.

Gut homogenates were prepared from flies fed on meals of 10×10^6/ml promastigotes or 0.4 mg/ml DCO in rabbit serum. Control preparations were made from flies fed on serum only.

Fifty microliter aliquots of gut preparations from flies fed serum only were mixed with 10×10^6 promastigotes in 25 µl PBS or 0.4 mg DCO in 25 µl PBS. The *in vitro* effect was also tested using 11.4 µg globin, the protein equivalent of 10×10^6 promastigotes, or 25 µl unused, incubated culture overlayer. In an additional experiment, substrate digestion by an equivalent number of promastigotes was measured without the addition of the gut enzyme preparation. The routine control for all *in vitro* experiments was a mixture of gut enzyme preparation and 25 µl buffered saline.

Globin substrate: 15.5 mg C^{14}-labeled globin/ml saline with a specific activity of 3×10^5 cpm/mg was prepared according to Roth *et al.* (1971), as modified by Lernau *et al.* (1980). This was used in aliquots of 15 µl mixed 1:1 with 1.25 mg/ml cold globin (NBCO, Cleveland, OH, USA).

Fifty microliters of each test preparation of enzyme was mixed with 30 µl globin substrate and incubated at 28 C for 30 min. The reaction was stopped by the addition of 105 µl cold TCA with a final concentration of 5%. The supernatant was separated following centrifugation at 3000 rpm for 15 min at 4 C and counted in Lumrix Scintillation Liquid (Packard) 40% in toluene. The quantity of digested protein in micrograms was calculated from the cpm in the supernatant.

Statistical analysis was by the Wilcoxon rank test (Bishop 1980).

RESULTS

The effect of *Leishmania donovani* or *L. major* promastigotes in the *in vivo* and the *in vitro* experiments was significantly different from their respective controls. The principal difference between the two species was in the *in vivo* experiments (Table I) where preparations of *Phlebotomus papatasi* infected with *L. major* digested 28.0% less than the controls, whereas flies infected with *L. donovani* digested 32.4% more than the controls. In parallel experiments using *L. major* DCO, there was a 25.0% decrease in proteolysis, similar to the result with promastigotes, while substrate digestion by preparations of flies fed *L. donovani* DCO was 9.6% greater than that of the controls.

In the *in vitro* experiments, where test materials were added to gut enzyme preparations (Table II), the activity measured

TABLE I

Digestion of C^{14}-Labeled Globin by Gut Homogenates of *Phlebotomus papatasi* Females Fed on Serum Containing Either *Leishmania* spp. Promastigotes or Their Dried Culture Overlayer (DCO)

Flies fed on serum containing	No. of repetitions	Mean % digestion by control homogenates	μg Protein digested by homogenates[a]		Difference between control and experiment (%)
			Control flies (average ± SE)	Experimental flies (average ± SE)	
L. major promastigotes	6	22.5	59.29 ± 4.26	42.72 ± 3.40[b]	28.0
L. major DCO	5	21.9	55.38 ± 7.91	41.57 ± 7.91[b]	25.0
L. donovani promastigotes	5	22.5	55.74 ± 4.96	72.96 ± 4.88[b]	+ 32.4
L. donovani DCO	5	20.7	49.68[c]	54.48 ± 8.24 NS	+ 9.6

[a] Preparations equivalent of five guts, ½ hr incubation.
[b] Significantly different from the controls $P < 0.01$ in the Wilcoxon rank test; NS = not significant.
[c] The same control used for all experimental repetition.

was higher than the controls in preparations containing promastigotes of *L. major* (11.3%), *L. major* DCO (11.9%), and *L. donovani* promastigotes (38.0%). Lower activity of −10.2% was observed in the preparations containing *L. donovani* DCO. The results of the *in vitro* experiments were all significantly different from their controls.

In the *in vitro* control experiments, the replacement of flagellates by 11.55 μg globin resulted in cpm counts that were 1% lower than those of controls. Addition of unused culture medium or flagellates without enzyme had a negligible effect. The protein content of 10×10^6 promastigotes of either species was 11.55 μg. Protein weight amounted to 50.8% in the *L. major* DCO and to 51.8% in *L. donovani* DCO.

Discussion

Under natural conditions, only a small number of *Leishmania* species amastigotes are ingested with blood by sandfly vectors. Within this blood meal, the parasites, enclosed in the peritrophic membrane, divide and transform into promastigotes. This development cannot be considered a true es-

TABLE II

The *in Vitro* Effect of *Leishmania* spp. Promastigotes or Their Dried Culture Overlayer (DCO) on the Digestion of C^{14}-Globin by Gut Homogenates of Serum Fed *Phlebotomus papatasi* Females

Experiment	Test material	No. of repetitions	Mean % digested by controls	mg Protein digested[a]		% Difference between control and experiment
				Control (average ± SE)	Average difference control experiment ± SE	
1	*L. major* promastigotes	8	31.7	104.70 ± 16.6	+ 11.86 ± 2.27[b]	+ 11.3
2	*L. major* DCO	6	31.7	104.70 ± 16.6	+ 12.47 ± 2.13[b]	+ 11.9
3	*L. donovani* promastigotes	6	33.4	81.28 ± 12.7	+ 31.25 ± 7.72[b]	+ 38.0
4	*L. donovani* DCO	6	33.4	81.28 ± 12.7	8.25 ± 1.03[b]	− 10.2

[a] Preparations equivalent of five guts, ½ hr incubation.
[b] Significantly different from the controls $P < 0.01$ in the Wilcoxon rank test.

tablishment of infection since early stages of the life cycle are not host specific (Killick-Kendrick 1979) and can occur in the blood meal of many hematophagous arthropods (Wenyon 1926).

In the midgut of unfed or sugar fed mosquitoes or other blood sucking diptera, there is little or no proteolytic activity, and maximal protease level is reached about 18–32 hr following blood intake (Akov 1972; Hardy *et al.* 1983). Thus, early stages of *Leishmania* spp. in the gut of the sandfly would presumably not be exposed to high levels of enzymic activity. High levels of protease activity cause damage to *Plasmodium* spp. (Langley 1975; Yeats and Steiger 1981) and to arboviruses (Hardy *et al.* 1983). These evade digestion by early escape from the gut, whereas *Leishmania* spp. promastigotes remain and multiply. In sandfly species which take a second blood meal before being able to transmit parasites (Killick-Kendrick 1979), *Leishmania* spp. developing in the fly gut are subjected to a second wave of proteolytic activity.

In this study, the principal difference between the two *Leishmania* species was the effect they produced in the vector. *L. major*, which thrives in *P. papatasi*, reduced the activity of gut enzymes by 28.0% while *L. donovani* enhanced enzyme activity by 32.4% in this unsuitable vector (Table I). However, the same *L. major* promastigotes *in vitro*, raised the level of substrate digestion by 11.3% and an increase of 38.0% was caused by *L. donovani*. Since the direct effect of both leishmanias was the enhancement of enzymatic digestion *in vitro*, it appears that the lesser activity of gut proteases of *L. major* infected flies resulted from the presence of smaller quantities of enzymes in the sandfly gut. *L. major* DCO produced an effect similar to that of the promastigotes, i.e., it decreased gut enzyme activity by 25.0% *in vivo* and increased it *in vitro* by 11.9% (Tables I and II), suggesting that the active substances for both functions are produced by cultured promastigotes. Of 150 *P. papatasi* fed serum meals containing 200 promastigotes per fly, only 2% became infected by *L. donovani* (Schlein, unpublished data) in contrast to 85% by *L. major* (Schlein *et al.* 1983).

Adler (1938) noted that infections of *Leishmania* species that are not host specific died out in *P. papatasi* fed infective meals of serum and flagellates. The only strain that could infect the flies successfully was the one they transmit in nature. Since rabbit serum was generally used to grow the promastigotes, it could not be inherently lethal. Moreover, since selection by the vector did not occur when the infective meal consisted of flagellates suspended in saline rather than in serum, Adler speculated that the selective factors were elicited in the gut by the serum. Furthermore, dilution of the serum in the infective meal led to proportional increase in the number of flies infected by a *Leishmania* sp. not specific to the vector (Adler *et al.* 1938). The quantities of protein in the meals have been correlated with the level of activity of gut enzymes in hematophagous insects (Gooding 1974, 1975; Houseman and Downe 1983). Hence, the increased survival of a nonspecific *Leishmania* sp. infection in Adler's (1938) experiments following meals with smaller proportions of serum apparently resulted from the decreased induction of proteases. In the present study, the reduced proteolytic activity was caused by *L. major* infections and not by the dilution of meal proteins. It may, however, have a similar effect and be the reflection of the mechanism which permits the survival of the parasites.

Promotion of proteolysis was caused by *L. major in vitro* and *L. donovani* both *in vivo* and *in vitro*. A possible explanation may be that, *in vivo*, the *L. major* reduce the activity of one protease while promoting that of another, and the observed effect is the net result. Only the latter function may take place under the artificial con-

ditions and short exposure in the *in vitro* experiments with *L. major* whereas the *L. donovani*, a not transmitted species, cannot reduce the gut protease in *Phlebotomus papatasi* either *in vivo* or *in vitro*.

It has been postulated that the susceptibility of mosquito species to arbovirus is influenced by differences in concentration of trypsin and chemotrypsin in the midgut (Hardy *et al.* 1983). Such differences apparently also occur among different species of phlebotomines and contribute to the complex picture of their tolerance to *Leishmania* species.

Vector specificity has been described in Old World sandflies. It is not, however, a general trait of phlebotomines, since neotropical species can harbor infections with various *Leishmania* species (see reviews, Molyneux 1977; Killick-Kendrick 1979). Perhaps, the enzyme composition of the widely susceptible New World vectors is innocuous while in the Old World vectors the induction of harmful enzymes is reduced by the defense system of the specific *Leishmania* species.

ACKNOWLEDGMENTS

We are grateful for support from the Leishmaniasis Component of the UNDP World Bank WHO Special Programme for Research and Training in Tropical Diseases, from the U.S. Agency for International Development (Grant DPE 5542-G-SS-4054-00), and from the U.S. National Institutes of Health (Grant REP-NIH-NIAID-AI-12669).

REFERENCES

ADLER, S. 1938. Factors determining the behaviour of *Leishmania* sp. in sandflies. *Harefuah* 14, 1–5.

ADLER, S., THEODOR, O., AND WILENBERG, G. 1938. Investigations on Mediterranean kala-azar. I. A study of leishmaniasis in Canea. *Proceedings of the Royal Society London, Series B* 125, 491–516.

AKOV, S. 1972. Protein digestion in hematophagous insects. *In* "Insect and Mite Nutrition" (J. G. Rodriguez, ed.), pp. 531–540. North-Holland/American Elsevier, New York.

BISHOP, O. N. 1980. "Statistics for Biology," pp. 1–214. Longman, Burnt Mill, Harlow.

GOODING, R. H. 1974. Digestive processes of haematophogous insects: Control of trypsin secretion in *Glossina morsitans*. *Journal of Insect Physiology* 20, 957–964.

GOODING, R. H. 1975. Digestive enzymes and their control in haematophagous insects. *Acta Tropica* 32, 96–111.

HARDY, I. L., HOUK, E. J., KRAMER, L. D., AND REEVES, W. C. 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology* 28, 229–262.

HOUSEMAN, J. G., AND DOWNE, A. E. R. 1983. Activity cycles and the control of four digestive proteinases in the posterior midgut of *Rhodnius prolixus* Stal. *Journal of Insect Physiology* 29, 141–148.

KILLICK-KENDRICK, R. 1979. Biology of *Leishmania* in phlebotomine sandflies. *In* "Biology of Kinetoplastida" (W. H. R. Lumsden and D. A. Evans, eds.), Vol. 1, pp. 395–449. Academic Press, Orlando, FL London.

LANGLEY, P. A. 1975. Pathogen transmission in relation to feeding and digestion by haematophagous arthropods. *Acta Tropica* 32, 116–124.

LERNAU, O. Z., NISSAN, S., NEUFELD, E. D., AND MAYER, M. 1980. Myofibrillar protease activity in muscle tissue from patients in catabolic condition. *European Journal of Clinical Investigation* 10, 357–361.

MOLYNEUX, D. H. 1977. Vector relationships in Trypanosomatidae. *Advances in Parasitology* 15, 1–82.

ROTH, J. A., LOSTY, K., AND WIERBICKI, E. 1971. Assay of proteolytic enzyme activity using ^{14}C-labelled hemoglobin. *Annals of Biochemistry* 42, 214–221.

SCHLEIN, Y., WARBURG, A., SCHNUR, L. F., AND SHLOMAI, J. 1983. Vector compatibility of *Phlebotomus papatasi* dependent on differently induced digestion. *Acta Tropica* 40, 65–70.

WENYON, C. M. 1926. "Protozoology", Vol. 1, pp. 1–778. Baillière, Tindall & Cox, London.

YEATS, R. A., AND STEIGER, S. 1981. Ultrastructure damage of in vitro cultured ookinetes of *Plasmodium gallinaceum* (Brumpt) by purified proteinases of susceptible *Aedes aegypti* (L.). *Zeitschrift für Parasitenkunde* 66, 93–97.

Short Report C5-136

Marking of *Phlebotomus papatasi* (Diptera: Psychodidae) by feeding on sprayed, coloured sugar bait: a possible means for behavioural and control studies

Y. SCHLEIN

Department of Parasitology, Hadassah Medical School, P.O. Box 1171, Jerusalem 91010, Israel

Phlebotomine sandflies feed on sugars in the field and it has been demonstrated in the laboratory that one of the sugar sources is honeydew taken from the surface of plants (SCHLEIN & WARBURG, 1986). It was further shown that *Phlebotomus papatasi* in the wild is attracted to different plants in various degrees (SCHLEIN & YUVAL, 1987). This sandfly-plant interaction suggested the possibility of marking flies by feeding them on dyed sugar baits, offered on attractive plants in the field, the assumption being that sprayed baits would compete with naturally available sources of food.

The spray solution contained either Indigotine or Brilliant Blue (C.I. Food Blue No. 1 or No. 2; Stern, Natanya, Israel) plus 1:100 sucrose, in 20% aqueous solution. An initial experiment with laboratory-reared *P. papatasi* showed that overnight feeding on the dried solution did not cause mortality within 10 days. Spraying in the field was done with a 7 litre Killaspray (Model 4055, Hozelock-ASL, Birmingham, England) using 5 to 10 litres of solution for each experimental site. The experiments were carried out in September 1986 in the endemic region of cutaneous leishmaniasis in the Jordan Valley, about 30 km north of Jericho. In this semi-arid area *P. papatasi* occurs in great numbers in the burrows of *Psammomys obesus*, the rodent reservoir of the disease (SCHLEIN *et al.*, 1982). The sprayed sites were about 50 × 25 m, around an active colony of *P. obesus*. The vegetation consisted of about 15 bushes of small to middle-size *Atriplex halimus* (maximal thicket length 10 m, width 3 m and height 3·5 m) and *Salsola vermiculata* (Chenopodiaceae). Experiments in this area indicated that some tested plants were attractive to *P. papatasi* but that *A. halimus*, under which *P. obesus* prefers to dig its burrows, was twice as attractive as *S. vermiculata* (see SCHLEIN & YUVAL, 1987). Sugar solution was sprayed on the outer, lower margins of the bushes to a height of 50-70 cm and dried instantly. *P. papatasi* were caught with CDC miniature light traps (Model 512, John W. Hock, Co., Gainesville, Florida, USA), from which the bulbs had been removed. The traps were placed, 4 per site, with their openings about 30 cm above ground. Altogether 3881 flies were caught. The blue dye marked, on average, 25·9% of both male and female *P. papatasi* caught during the first night following spraying. It was readily visible in most and could be revealed in others by pressing them between two sheets of filter paper. The average of marked flies caught on the third night after spraying was 50% and on the sixth night, 32·9%. By that time the labelling of the flies and the blue colour on the plants were less intensive.

The usual method for studying sandfly dispersal is mark-release-recapture, based on the application of dyes to captured flies. Recapture by this method was maximal when blood-gorged *P. ariasi* females were released in cellars, but it was much lower in outdoor studies (KILLICK-KENDRICK *et al.*, 1984). Bait-marking in this study reached 50%, and was shown to label flies for 6 nights after a single application. These results could probably be improved by repeated spraying and by spraying a wider area. Only the proportion of flies captured, and not the number marked by the bait, can be evaluated; hence, the size of the population cannot be assessed. However, this method marks sandflies in their habitat without interrupting their normal behaviour by capture, treatment and release in a new environment. The use of different dyes would permit behavioural studies, such as the assessment of dispersal from more than one location and evaluation of the attractiveness of different plants and of attractants mixed into the baits.

Another application could be to direct the bait-marking method either against the vector, by including insecticides or pathogens in the sprayed solution, or against the *Leishmania*. Decrease in the incidence of *Leishmania* can apparently result from microorganism contamination of the vector's gut (SCHLEIN *et al.*, 1985). Potential gut contaminants could also be added to the sucrose solution and evaluated by examination of isolated, marked flies. The experiments can be carried out on a very small scale and the impact on the environment would be small. Very few control projects have been aimed at vectors of leishmaniasis and most of these have been carried out in human habitations. The spraying of sugary baits on plants seems to be a promising method for control studies during the dry season in zoonotic foci of leishmaniasis.

This study received support from the leishmaniasis component of the UNDP World Bank WHO Special Programme for Research and Training in Tropical Diseases, and the Project on Epidemiology and Control of Vector Borne Diseases in Israel (REP-NIH-NIAID-AI. 12669 and AID-CDR project, C5-136.

References

Killick-Kendrick, R., Rioux, J. A., Bailly, M., Guy, M. W., Wilkes, T. J., Guy, E. M., Davidson, I., Knechtly, R., Ward, R. D., Guilvard, E., Jefferies, J. & Dubois, H. (1984). Ecology of leishmaniasis in the south of France. 20. Dispersal of *Phlebotomus ariasi* Tonnoir, 1921 as a factor in the spread of visceral leishmaniasis in the Cevennes. *Annales de Parasitologie Humaine et Comparée*, **59**, 555-572.

Schlein, Y. & Warburg, A. (1986). Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. *Journal of Medical Entomology*, **23**, 11-15.

Schlein, Y. & Yuval, B. (1987). Leishmaniasis in the Jordan Valley. IV. Attraction of *Phlebotomus papatasi* (Diptera: Psychodidae) to plants in the field. *Journal of Medical Entomology*, **24**, 87-90.

Schlein, Y., Warburg, A., Schnur, L. F. & Gunders, A.E. (1982). Leishmaniasis in the Jordan Valley. II. Sandflies and transmission in the central endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **76**, 581-586.

Schlein, Y., Polacheck, I. & Yuval, B. (1985). Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. *Parasitology*, **90**, 57-66.

Accepted for publication 30 October 1986

Rec'd in SCI: OCT 14 1988

C5-136

J. Med. Entomol. Vol. 24, no. 1: 87–90 January 1987

© 1987 by the Entomological Society of America

LEISHMANIASIS IN THE JORDAN VALLEY
IV. Attraction of *Phlebotomus papatasi* (Diptera: Psychodidae) to plants in the field[1]

Yosef Schlein and Boaz Yuval[2]

Abstract. The attraction of *Phlebotomus papatasi* to 12 plant species was examined in the field using plant-baited traps. Of the species tested, 8 attracted significantly more flies than unbaited controls, with *Capparis spinosa* var. *aravensis*, *Solanum nigrum*, *Prosopis farcta*, and *Atriplex halimus* giving the highest catches. Traps provided with a source of humidity also gave significantly higher catches than controls. Laboratory tests showed that 86.4, 75.6, 71, 52.1, and 43% of the *P. papatasi* fed on *Prosopis farcta*, *Capparis spinosa*, *Rumex commutatus*, *Solanum nigrum*, and *Kochia indica*, respectively; these plants were also attractive to sand flies in the field. None of the flies fed in the laboratory on *Atriplex halimus*, *Tamarix nilotica*, or *Chenopodium ambrosioides*, which were attractive in the field, and 2.3, 4.7, 10, and 14.2%, respectively, fed in the laboratory on the nonattractive *Salsola vermiculata*, *Suaeda aegyptiaca*, *Solanum incanum*, and *Amaranthus retroflexus*.

The sand fly *Phlebotomus papatasi* Scopoli is widely distributed in arid, tropical, and subtropical regions of the Old World, where it is a principal vector of the pathogen causing zoonotic cutaneous leishmaniasis (Lewis 1974; Le Blancq et al. 1986). As do many other groups of blood-sucking flies, phlebotomines regularly feed on sugars (Chaniotis 1974; Friend & Smith 1977; Killick-Kendrick 1979; Endris et al. 1984). Different sugars have been identified in wild-caught sand flies (Lewis & Domonev 1966). The presence of fructose was recorded in abut 80% of *P. ariasi* Tonnoir from southern France (Young et al. 1980) and *P. papatasi* from the Jordan Valley (Yuval & Schlein 1986).

Although undoubtedly of plant origin, the actual sources of sugar meals in nature are unknown. Killick-Kendrick (1979) suggested honeydew as the principal source, while Schlein & Warburg (1986) demonstrated in the laboratory that *P. papatasi* obtains sugar selectively, directly from plant tissues as well as from honeydew. Sugar meals affect the transmission of leishmaniasis, since they play a role in the development of *Leishmania* in the vector (Killick-Kendrick 1979). Sugar feeding is also important in the biology of sand flies, since their search for sugars is a major factor influencing their behavior in the field (Yuval & Schlein 1986).

The purpose of this study was to find out whether *P. papatasi* was attracted to specific plants under field conditions and whether this attraction could be correlated to its feeding preferences in the laboratory.

MATERIALS AND METHODS

Study area

The study was carried out in the Jordan Valley, about 30 km north of Jericho, in an area endemic for cutaneous leishmaniasis and in which *P. papatasi* is the only *Phlebotomus* species. The experimental area was in a ploughed and leveled field, 900 × 600 m, and was devoid of vegetation, since comparative experiments could not be carried out where plants of various densities and sizes were present. Previous captures in this field had shown that sand flies randomly occurred there in great numbers (Yuval & Schlein, unpubl. data).

Experimental design

Sand flies were caught with CDC miniature light traps (model 512, John W. Hock Co., Gainesville, FL) from which the bulbs were removed. Traps were operated overnight between 1900 and 0700 h, separated by a distance of 30 m, with the trap opening ca. 30 cm above the ground. All experiments included 1 control trap and several others, each baited with branches of 1 plant or with wet filter paper. The position of the control trap in relation to

1. This work was supported by the Leishmaniasis component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and the Project on Epidemiology and Control of Vector-borne Diseases in Israel (REP-NIH-NIAID-AL-12669).

2. Department of Parasitology, The Hebrew University—Hadassah Medical School, P.O. Box 1172, Jerusalem 91010, Israel.

Rec'd in SCI: OCT 14 1988

TABLE 1. Captures of *Phlebotomus papatasi* in plant-baited vs. unbaited traps.

	Bait weight (g)	Bait evaporation (ml)*	Control traps: No. flies captured	Control traps: $\bar{x}$	Control traps: SE	Baited traps: No. flies captured	Baited traps: $\bar{x}$	Baited traps: SE	P**	Attraction index***
Capparis spinosa var. *aravensis*	139	78.6	195	65	11.7	573	251	57	0.001	3.9
Capparis spinosa	146	81.4	125	31.7	7.7	263	65.7	11.5	0.001	2.1
Solanum nigrum	191	55.8	265	53	4.5	602	113	9.1	0.001	2.1
Ricinus communis	101	49.3	360	72	7	519	103	10.1	0.001	1.4
Prosopis farcta	109	47.5	39	13	1.1	79	26.3	2.0	0.01	2.1
Atriplex halimus	188	39.7	40	13.3	1.3	85	28.3	3.8	0.01	2.1
Chenopodium ambrosioides	143.7	71.7	53	13.2	3.4	96	24	4.5	0.01	1.8
Kochia indica	200	101.6	256	42.6	4.9	346	57.6	6.8	0.01	1.3
Tamarix nilotica	90	40	47	15.6	1.3	88	29.3	3.0	0.05	1.8
Solanum incanum	126.5	61.9	53	13.2	3.4	77	19.2	3.8	NS	1.4
Suaeda aegyptiaca	250	36	164	54.6	15.2	192	64	17.1	NS	1.1
Salsola vermiculata	117.4	24.4	130	32.7	7.4	121	30.2	5.8	NS	0.9
Amaranthus retroflexus	161	74.5	146	73	9.2	123	61.5	12.2	NS	0.8
Wet filter paper	—	90	140	28	3.2	314	62.8	5.5	0.001	2.2

* Quantity of water evaporated by plant samples or by filter paper.

** Chi-square test.

*** $\bar{x}$ baited traps/$\bar{x}$ controls. The proportion of ♂♂ and ♀♀ in the controls and experimental captures was similar.

the test traps was changed each night to avoid spatial bias. Tests with each of the baits were repeated 3 to 5 times on 17 nights between June and August 1985.

Branches of each test plant were of similar size and weighed between 100 and 250 g. Their stems were placed in a 100-ml Erlenmeyer flask containing 100 ml water; the opening of the flask was then sealed with Parafilm and the stems were secured with cellotape. Each flask was placed beside one of the traps, so that the branches were under the metal covering of the trap, near its opening. Flags of folded filter paper were inserted into other flasks containing water; these were similarly placed beside traps to assess the effect of humidity alone. Evaporation was calculated for each plant sample by adding the overnight loss of weight of the branches to the decrease in water quantity in the flask. Evaporation by the filter papers was measured by adding remaining water in the flask to weight of water soaked in the paper.

Sand flies were collected in the morning and catches were compared only to their respective controls, since the number of sand flies varied considerably from one night to another due to differing environmental conditions. The differences between control and baited traps and between plant-baited traps and evaporation traps were analyzed using the chi-square test. The comparative attraction index was calculated following Bradbury & Bennett (1974).

Plant species tested

The following plants, common in the biotope in the summer, were used in the experiments: *Solanum nigrum* (Solanaceae); *Capparis spinosa*, *C. spinosa* var. *aravensis* (Capparaceae); *Ricinus communis* (Euphorbiaceae); *Chenopodium ambrosioides*, *Kochia indica*, *Atriplex halimus*, *Suaeda aegyptiaca*, *Salsola vermiculata* (Chenopodiaceae); *Tamarix nilotica* (Tamaricaceae); *Prosopis farcta* (Mimosaceae); and *Amaranthus retroflexus* (Amaranthaceae).

Laboratory experiments

Feeding of *P. papatasi* on the test plants was studied in the laboratory. Mixed groups of unfed males and females were offered a branch of 1 plant overnight, as described by Schlein & Warburg (1986). Feeding was determined by testing with anthrone for the presence of fructose (Van Handel 1972) using the procedure described by Young et al. (1980).

TABLE 2. Overnight feeding of *P. papatasi* on plant branches in the laboratory.*

	No. flies exposed			Anthrone-positive flies			Total
	♀	♂	Total	♀	♂	Total	%
Capparis spinosa	37	37	74	29	27	56	75.6
Solanum nigrum	28	18	46	13	11	24	52.1
Ricinus communis	41	35	76	28	26	54	71.0
Prosopis farcta	26	24	50	22	21	43	86
Atriplex halimus	43	44	87	0	0	0	0
Chenopodium ambrosioides	25	25	50	0	0	0	0
Kochia indica	25	21	46	8	12	20	43
Tamarix nilotica	25	25	50	0	0	0	0
Solanum incanum	25	25	50	3	2	5	10
Suaeda aegyptiaca	30	33	63	1	2	3	4.7
Salsola vermiculata	42	43	85	2	0	2	2.3
Amaranthus retroflexus	18	24	42	4	2	6	14.2

* Feeding determined by the presence of fructose in the gut.

RESULTS

Of the 12 plants tested in the field, 9 attracted significantly more *P. papatasi* than did the unbaited controls (Table 1). *Capparis spinosa* var. *aravensis* was the most attractive, with a 3.9 attraction index (mean number captured in baited traps ÷ mean number captured in control traps). *Solanum nigrum*, *Prosopis farcta*, and *Atriplex halimus* attracted 2.1 times as many flies as their respective controls. The yield of traps provided with wet filter paper was also significantly higher than the dry controls ($P < 0.001$), with an attraction index of 2.2.

A comparison between the humidified and the plant-baited traps showed that *C. spinosa* var. *aravensis*, *S. nigrum* ($P < 0.001$), and *C. spinosa* ($P < 0.02$) attracted more flies, while *S. aegyptiaca*, *S. vermiculata*, *A. retroflexus*, *R. communis*, and *K. indica* (at the $P < 0.01$ level) attracted fewer flies. Evaporation by different plant samples varied from 24.4 ml per night (*S. vermiculata*) to 101.6 ml (*K. indica*), whereas the wet filter paper evaporated 90 ml (Table 1).

In laboratory tests 5 plants, *P. farcta*, *C. spinosa*, *R. communis*, *S. nigrum*, and *K. indica*, were fed upon by 43 to 86% of the flies. *Amaranthus retroflexus*, *S. aegyptiaca*, and *S. vermiculata* were fed upon by 14.2, 4.7, and 2.3%, respectively, while no feeding was recorded on *A. halimus*, *C. ambrosioides*, or *T. nilotica* (Table 2).

DISCUSSION

Phlebotomus papatasi was attracted to 8 of the 12 plant species tested in the field (Table 1). Of the plants offered to sand flies in the laboratory, 5 species that were attractive in the field (*P. farcta*, *C. spinosa*, *S. nigrum*, *R. communis*, and *K. indica*) were fed upon by 43 to 86% of the flies (Table 2). *Atriplex halimus*, *T. nilotica*, and *C. ambrosioides*, though attractive to the flies in the field (Table 1), induced no feeding in the laboratory, and other species tested induced less than 14.2% feeding and were not attractive in the field.

Results of the field trials show that *P. papatasi* is attracted to specific plants that are potential sources of sugar meals, but it was also attracted to plants not fed on in the laboratory. Sugar feeding is therefore not the only reason for attraction to plants, and other factors such as the search for shelter or breeding sites may also be involved. It is noteworthy in this context that the attractive but inedible *A. halimus* is one of the dominant plant species in the habitat of *P. papatasi* in the Jordan Valley. Burrows of the sand rat *Psammomys obesus*, in which sand flies breed and rest (Schlein et al. 1982), frequently are located between its roots.

Endris et al. (1984) demonstrated that *Lutzomyia anthophora* (Addis) takes several sugar meals in the laboratory, completely digesting one before taking another. Yuval & Schlein (1986) showed that the need for such meals is an important factor in the nocturnal activity of *P. papatasi* in the field. Thus, although it is not clear what proportion of meals are derived from plant vs. indirect sources, such as homopteran-exuded honeydews, it seems that the search for

sugar meals is an important component of sand fly behavior.

Plant attraction at night involves 2 general factors, released water vapor and CO_2, in addition to plant-specific emissions. Our results demonstrated that the yield of traps provided with wet filter paper was significantly higher than that of controls (Table 1). Hence, sources of evaporation were evidently attractive to the sand flies in the arid areas of the Jordan Valley. We also found that CO_2 is attractive to *P. papatasi* under similar conditions (Yuval & Schlein, unpubl.). The role of these factors would apparently diminish in a humid environment with dense vegetation, and the question is whether the specific plant emissions alone may affect the behavior of sand flies. Evaporation by plant samples varied considerably between species and was not in direct correlation to their attraction (Table 1); emission of CO_2 was not considered in the present study. However, 2 examples seem to demonstrate attraction and repellence by plant odors. The catches using *A. retroflexus* were significantly lower than those of humidified traps ($P < 0.001$) even though the plant sample evaporation, 74.5 ml, was among the highest. *Capparis spinosa* var. *aravensis*, which transpired less than the filter papers, attracted significantly more flies ($P < 0.001$) than traps with filter paper.

The results in this study represent a simplified situation, and the artificial presentation of the plants may have altered attractivity. It is conceivable, however, that plant preferences, such as described in the present study, normally influence sand fly distribution. Confirmation of this assumption will add a useful tool for the investigation of the epidemiology of leishmaniasis.

Acknowledgment. We thank Mrs M. Boaz of the Department of Botany, The Hebrew University, for her help. We also acknowledge grant support from the leishmaniasis component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, the Project on Epidemiology and Control of Vector Borne Diseases in Israel (REP-NIH-NIAID-AI. 12669) and AID grant CDR C5-136.

LITERATURE CITED

Bradbury, W.C. & G.F. Bennett. 1974. Behavior of adult Simuliidae (Diptera). II. Vision and olfaction in near orientation and landing. *Can. J. Zool.* **52:** 1355–64.

Chaniotis, B.N. 1974. Sugar-feeding behavior of *Lutzomyia trapidoi* (Diptera: Psychodidae) under experimental conditions. *J. Med. Entomol.* **11:** 73–79.

Endris, R.G., D.G. Young & J.F. Butler. 1984. The laboratory biology of the sand fly *Lutzomyia anthophora* (Diptera: Psychodidae). *J. Med. Entomol.* **21:** 656–64.

Friend, W.G. & J.J.B. Smith. 1977. Factors affecting feeding by blood sucking insects. *Annu. Rev. Entomol.* **22:** 309–31.

Killick-Kendrick, R. 1979. Biology of *Leishmania* in phlebotomine sandflies. p. 395–449. *In:* W.H.R. Lumsden & D.A. Evans, eds., *Biology of the Kinetoplastida.* Vol. 2. Academic Press, London.

Le Blancq, S. M., L. F. Schnur & W. Peters. 1986. *Leishmania* in the Old World: I. The geographical and hostal distribution of *L. major* zymodemes. *Trans. R. Soc. Trop. Med. Hyg.* **80:** 99–112.

Lewis, D.J. 1974. The biology of Phlebotomidae in relation to leishmaniasis. *Annu. Rev. Entomol.* **19:** 363–84.

Lewis, D.J. & C.R. Domoney. 1966. Sugar meals in Phlebotominae and Simuliidae. *Proc. R. Entomol. Soc. Lond. Ser. A. Gen. Entomol.* **41:** 175–79.

Schlein, Y. & A. Warburg. 1986. Phytophagy and the feeding cycle of *Phlebotomus papatasi* (Diptera: Psychodidae) under experimental conditions. *J. Med. Entomol.* **23:** 11–15.

Schlein, Y., A. Warburg, L.F. Schnur & A.E. Gunders. 1982. Leishmaniasis in the Jordan Valley. II. Sandflies and transmission in the central endemic area. *Trans. R. Soc. Trop. Med. Hyg.* **76:** 582–86.

Van Handel, E. 1972. The detection of nectar in mosquitoes. *Mosq. News* **32:** 458.

Young, C.J., D.P. Turner, R. Killick-Kendrick, J.A. Rioux & J.A. Leaney. 1980. Fructose in *Phlebotomus ariasi* and the possible relevance of sugars taken by sandflies to the transmission of leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* **74:** 363–66.

Yuval, B. & Y. Schlein. 1986. Leishmaniasis in the Jordan Valley. III. Nocturnal activity of *Phlebotomus papatasi* (Diptera: Psychodidae) in relation to nutrition and ovarian development. *J. Med. Entomol.* **23:** 411–15.

EXPERIMENTAL PARASITOLOGY 62, 376–380 (1986)

C 5-130

Leishmania major and *L. donovani:* Effects on Proteolytic Enzymes of *Phlebotomus papatasi* (Diptera, Psychodidae)

YOSEF SCHLEIN AND HELINA ROMANO

Department of Parasitology, The Hebrew University-Hadassah Medical School, P.O. Box 1172, Jerusalem, Israel

(Accepted for publication 6 May 1986)

SCHLEIN, Y., AND ROMANO, H. 1986. *Leishmania major* and *L. donovani:* Effects on proteolytic enzymes of *Phlebotomus papatasi* (Diptera, Psychodidae). *Experimental Parasitology* 62, 376–380. *Phlebotomus papatasi* is susceptible to *Leishmania major* which it transmits in nature, but is resistant to *L. donovani*. The present study compares the effect of *L. major* and *L. donovani* on the proteolytic activity of *P. papatasi* gut enzymes. The experiments measured digestion of C^{14}-labeled globin by gut homogenates of flies. Homogenates were prepared from flies fed on serum only (controls) or from flies fed serum containing promastigotes or their dried culture overlayer. In other experiments, the promastigotes or dried culture overlayers were added *in vitro* to the gut homogenate of control flies. Proteolytic activity of gut homogenate from flies infected with *L. major* was about one-third less than that of controls, while that from flies infected with *L. donovani* was one-third greater. Ingestion of *L. major* dried culture overlayer had an effect on flies similar to that of the promastigotes, while *L. donovani* dried culture overlayer produced no significant effect. When added to gut homogenate *in vitro*, promastigotes of both species promoted proteolysis as did dried culture overlayer of *L. major*. Dried culture overlayer of *L. donovani*, however, had an opposite effect. It is suggested that the observed reduction in proteolytic activity caused by *L. major* infection may result from inhibition of enzyme production. © 1986 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania major; Leishmania donovani;* Trypanosomatidae; Protozoa, parasitic; *Phlebotomus papatasi;* Diptera; Psychodidae; Vector, gut; Enzymes, proteolytic; Dried culture overlayer (DCO); Phosphate buffered saline (PBS).

INTRODUCTION

Leishmania species, the causative agents of leishmaniasis, are transmitted by phlebotomine sandflies. They are ingested with blood meals by female flies, multiply, and migrate forward in the gut from where they are transmitted by bite to another vertebrate host. Various adaptations are required for the parasite to complete a full cycle in the vector (Killick-Kendrick 1979). The basic one seems to be the ability to live in the environment of the fly's gut. In the laboratory, many sandflies are refractory to infection with strains of *Leishmania* spp. other than those they transmit in nature (Killick-Kendrick 1979; Molyneux 1977). This selection by the vector was attributed to factors in the sandfly gut elicited by meals of serum (Adler 1938). In addition, vector competence was found to be an unstable trait when blood meals were taken from an unsuitable host since the balanced conditions, which permit successful infection of the vector by its specific *Leishmania* sp., were upset (Schlein *et al.* 1983). These studies indicate that the digestion of meals is important in determining the survival of a *Leishmania* sp. in the sandfly.

The present study compares the effect of strains of two *Leishmania* species on the proteolytic activity of *Phlebotomus papatasi* gut enzymes. The *L. major* strain used is transmitted by this sandfly in nature and

Rec'd in SCI: OCT 14 1986

0014-4894/86 $3.00
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved

Rec'd in SCI: OCT 1 ---

can produce a high rate of infection (Schlein *et al.* 1983) in contrast to the *L. donovani* strain which rarely survives in *P. papatasi* (Schlein, unpublished data). The assumption was that differences between the two leishmanias, permitting *L. major* but not *L. donovani* to survive in the vector, would find expression in the proteolytic activity of the sandfly gut enzymes.

MATERIALS AND METHODS

The *Phlebotomus papatasi* used in these experiments were from a colony founded (1981) with sandflies caught in the Jordan Valley. The fly colony is maintained at 28 ± 1 C, 80% relative humidity on a diet of 10% sucrose solution and baby mice for blood meals. Experimental groups of flies consisted of 20–50 1–5 day old females that were membrane fed on sterile meals as described by Schlein *et al.* (1983).

Fly groups were allowed to feed on rabbit serum (inactivated at 56 C for 30 min) either alone or mixed with one of the test materials. The midguts of engorged flies were dissected out 24 hr later, homogenized in 0.1 ml Mini Homogenizer (Gencons of Hemel, Hempstead, UK) in a minimal volume of 0.9% NaCl. The homogenate was made up to a volume of 10 µl/sandfly gut with saline, centrifuged for 2 min at 7520g (Beckman Microfuge TMB). The supernatant was stored at −20 C and used for the experiments in 50 µl quantities equivalent to five flies. Each repetition of the experiments was carried out using homogenates of experimental and of control groups from the same batch, i.e., fed at the same time.

Leishmania major LRC-L137, a human isolate from the same area in the Jordan Valley as the founders of the sandfly colony, and *L. donovani* LRC-L52 (a human isolate from India) were grown on NNN medium for 3 to 5 days at 28 C. Flagellates were harvested, washed three times in PBS, pH 7.4, and brought to a concentration of 10×10^6/ml.

DCO of 6–9 day old cultures of the two leishmanias was prepared as follows: the fluid was centrifuged at 1000 rpm, filtered through a 0.45-µm filter, brought to pH 5 by adding glacial acetic acid, boiled for 30 min, centrifuged for 10 min at 1000 rpm, dialyzed against distilled water for 48 hr, frozen, and lyophilized. The estimated product of 10×10^6 promastigotes was 0.4 mg DCO. Control material was the overlayer of unused medium that had been incubated for 4 days at 28 C. The protein content of test materials was determined by Lowry's method.

Gut homogenates were prepared from flies fed on meals of 10×10^6/ml promastigotes or 0.4 mg/ml DCO in rabbit serum. Control preparations were made from flies fed on serum only.

Fifty microliter aliquots of gut preparations from flies fed serum only were mixed with 10×10^6 promastigotes in 25 µl PBS or 0.4 mg DCO in 25 µl PBS. The *in vitro* effect was also tested using 11.4 µg globin, the protein equivalent of 10×10^6 promastigotes, or 25 µl unused, incubated culture overlayer. In an additional experiment, substrate digestion by an equivalent number of promastigotes was measured without the addition of the gut enzyme preparation. The routine control for all *in vitro* experiments was a mixture of gut enzyme preparation and 25 µl buffered saline.

Globin substrate: 15.5 mg C^{14}-labeled globin/ml saline with a specific activity of 3×10^5 cpm/mg was prepared according to Roth *et al.* (1971), as modified by Lerman *et al.* (1980). This was used in aliquots of 15 µl mixed 1:1 with 1.25 mg/ml cold globin (NBCO, Cleveland, OH, USA).

Fifty microliters of each test preparation of enzyme was mixed with 30 µl globin substrate and incubated at 28 C for 30 min. The reaction was stopped by the addition of 105 µl cold TCA with a final concentration of 5%. The supernatant was separated following centrifugation at 3000 rpm for 15 min at 4 C and counted in Lumax Scintillation Liquid (Packard) 40% in toluene. The quantity of digested protein in micrograms was calculated from the cpm in the supernatant.

Statistical analysis was by the Wilcoxon rank test (Bishop 1980).

RESULTS

The effect of *Leishmania donovani* or *L. major* promastigotes in the *in vivo* and the *in vitro* experiments was significantly different from their respective controls. The principal difference between the two species was in the *in vivo* experiments (Table I) where preparations of *Phlebotomus papatasi* infected with *L. major* digested 28.0% less than the controls, whereas flies infected with *L. donovani* digested 32.4% more than the controls. In parallel experiments using *L. major* DCO, there was a 25.0% decrease in proteolysis, similar to the result with promastigotes, while substrate digestion by preparations of flies fed *L. donovani* DCO was 9.6% greater than that of the controls.

In the *in vitro* experiments, where test materials were added to gut enzyme preparations (Table II), the activity measured

TABLE I

Digestion of C^{14}-Labeled Globin by Gut Homogenates of *Phlebotomus papatasi* Females Fed on Serum Containing Either *Leishmania* spp. Promastigotes or Their Dried Culture Overlayer (DCO)

Flies fed on serum containing	No. of repetitions	Mean % digestion by control homogenates	μg Protein digested by homogenates[a]		Difference between control and experiment (%)
			Control flies (average ± SE)	Experimental flies (average ± SE)	
L. major promastigotes	6	22.5	59.29 ± 4.26	42.72 ± 3.40[b]	28.0
L. major DCO	5	21.9	55.38 ± 7.91	41.57 ± 7.91[b]	25.0
L. donovani promastigotes	5	22.5	55.74 ± 4.96	72.96 ± 4.88[b]	+ 32.4
L. donovani DCO	5	20.7	49.68[c]	54.48 ± 8.24 NS	+ 9.6

[a] Preparations equivalent of five guts, ½ hr incubation.
[b] Significantly different from the controls $P < 0.01$ in the Wilcoxon rank test; NS—not significant.
[c] The same control used for all experimental repetition.

was higher than the controls in preparations containing promastigotes of *L. major* (11.3%), *L. major* DCO (11.9%), and *L. donovani* promastigotes (38.0%). Lower activity of − 10.2% was observed in the preparations containing *L. donovani* DCO. The results of the *in vitro* experiments were all significantly different from their controls.

In the *in vitro* control experiments, the replacement of flagellates by 11.55 μg globin resulted in cpm counts that were 1% lower than those of controls. Addition of unused culture medium or flagellates without enzyme had a negligible effect. The protein content of 10×10^6 promastigotes of either species was 11.55 μg. Protein weight amounted to 50.8% in the *L. major* DCO and to 51.8% in *L. donovani* DCO.

Discussion

Under natural conditions, only a small number of *Leishmania* species amastigotes are ingested with blood by sandfly vectors. Within this blood meal, the parasites, enclosed in the peritrophic membrane, divide and transform into promastigotes. This development cannot be considered a true es-

TABLE II

The *in Vitro* Effect of *Leishmania* spp. Promastigotes or Their Dried Culture Overlayer (DCO) on the Digestion of C^{14}-Globin by Gut Homogenates of Serum Fed *Phlebotomus papatasi* Females

Experiment	Test material	No. of repetitions	Mean % digested by controls	mg Protein digested[a]		% Difference between control and experiment
				Control (average ± SE)	Average difference control experiment ± SE	
1	*L. major* promastigotes	8	31.7	104.70 ± 16.6	+ 11.86 ± 2.2[b]	+ 11.3
2	*L. major* DCO	6	31.7	104.70 ± 16.6	+ 12.47 ± 2.13[b]	+ 11.9
3	*L. donovani* promastigotes	6	33.4	81.28 ± 12.7	+ 31.25 ± 7.72[b]	+ 38.0
4	*L. donovani* DCO	6	33.4	81.28 ± 12.7	8.25 ± 1.03[b]	− 10.2

[a] Preparations equivalent of five guts, ½ hr incubation.
[b] Significantly different from the controls $P < 0.01$ in the Wilcoxon rank test.

tablishment of infection since early stages of the life cycle are not host specific (Killick-Kendrick 1979) and can occur in the blood meal of many hematophagous arthropods (Wenyon 1926).

In the midgut of unfed or sugar fed mosquitoes or other blood sucking diptera, there is little or no proteolytic activity, and maximal protease level is reached about 18–32 hr following blood intake (Akov 1972; Hardy *et al.* 1983). Thus, early stages of *Leishmania* spp. in the gut of the sandfly would presumably not be exposed to high levels of enzymic activity. High levels of protease activity cause damage to *Plasmodium* spp. (Langley 1975; Yeats and Steiger 1981) and to arboviruses (Hardy *et al.* 1983). These evade digestion by early escape from the gut, whereas *Leishmania* spp. promastigotes remain and multiply. In sandfly species which take a second blood meal before being able to transmit parasites (Killick-Kendrick 1979), *Leishmania* spp. developing in the fly gut are subjected to a second wave of proteolytic activity.

In this study, the principal difference between the two *Leishmania* species was the effect they produced in the vector. *L. major*, which thrives in *P. papatasi*, reduced the activity of gut enzymes by 28.0% while *L. donovani* enhanced enzyme activity by 32.4% in this unsuitable vector (Table I). However, the same *L. major* promastigotes *in vitro*, raised the level of substrate digestion by 11.3% and an increase of 38.0% was caused by *L. donovani*. Since the direct effect of both leishmanias was the enhancement of enzymatic digestion *in vitro*, it appears that the lesser activity of gut proteases of *L. major* infected flies resulted from the presence of smaller quantities of enzymes in the sandfly gut. *L. major* DCO produced an effect similar to that of the promastigotes, i.e., it decreased gut enzyme activity by 25.0% *in vivo* and increased it *in vitro* by 11.9% (Tables I and II), suggesting that the active substances for both functions are produced by cultured promastigotes. Of 150 *P. papatasi* fed serum meals containing 200 promastigotes per fly, only 2% became infected by *L. donovani* (Schlein, unpublished data) in contrast to 85% by *L. major* (Schlein *et al.* 1983).

Adler (1938) noted that infections of *Leishmania* species that are not host specific died out in *P. papatasi* fed infective meals of serum and flagellates. The only strain that could infect the flies successfully was the one they transmit in nature. Since rabbit serum was generally used to grow the promastigotes, it could not be inherently lethal. Moreover, since selection by the vector did not occur when the infective meal consisted of flagellates suspended in saline rather than in serum, Adler speculated that the selective factors were elicited in the gut by the serum. Furthermore, dilution of the serum in the infective meal led to proportional increase in the number of flies infected by a *Leishmania* sp. not specific to the vector (Adler *et al.* 1938). The quantities of protein in the meals have been correlated with the level of activity of gut enzymes in hematophagous insects (Gooding 1974, 1975; Houseman and Downe 1983). Hence, the increased survival of a nonspecific *Leishmania* sp. infection in Adler's (1938) experiments following meals with smaller proportions of serum apparently resulted from the decreased induction of proteases. In the present study, the reduced proteolytic activity was caused by *L. major* infections and not by the dilution of meal proteins. It may, however, have a similar effect and be the reflection of the mechanism which permits the survival of the parasites.

Promotion of proteolysis was caused by *L. major in vitro* and *L. donovani* both *in vivo* and *in vitro*. A possible explanation may be that, *in vivo*, the *L. major* reduce the activity of one protease while promoting that of another, and the observed effect is the net result. Only the latter function may take place under the artificial con-

ditions and short exposure in the *in vitro* experiments with *L. major* whereas the *L. donovani*, a not transmitted species, cannot reduce the gut protease in *Phlebotomus papatasi* either *in vivo* or *in vitro*.

It has been postulated that the susceptibility of mosquito species to arbovirus is influenced by differences in concentration of trypsin and chemotrypsin in the midgut (Hardy *et al.* 1983). Such differences apparently also occur among different species of phlebotomines and contribute to the complex picture of their tolerance to *Leishmania* species.

Vector specificity has been described in Old World sandflies. It is not, however, a general trait of phlebotomines, since neotropical species can harbor infections with various *Leishmania* species (see reviews, Molyneux 1977; Killick-Kendrick 1979). Perhaps, the enzyme composition of the widely susceptible New World vectors is innocuous while in the Old World vectors the induction of harmful enzymes is reduced by the defense system of the specific *Leishmania* species.

Acknowledgments

We are grateful for support from the Leishmaniasis Component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, from the U.S. Agency for International Development (Grant DPE 5542-G-SS-4054-00), and from the U.S. National Institutes of Health (Grant REP-NIH-NIAID-AI-12669).

References

Adler, S. 1938. Factors determining the behaviour of *Leishmania* sp. in sandflies. *Harefuah* 14, 1–5.

Adler, S., Theodor, O., and Witenberg, G. 1938. Investigations on Mediterranean kala-azar. 1. A study of leishmaniasis in Canea. *Proceedings of the Royal Society London, Series B* 125, 491–516.

Akov, S. 1972. Protein digestion in hematophagous insects. *In* "Insect and Mite Nutrition" (J. G. Rodriguez, ed.), pp. 531–540. North-Holland/American Elsevier, New York.

Bishop, O. N. 1980. "Statistics for Biology," pp. 1–214. Longman, Burnt Mill, Harlow.

Gooding, R. H. 1974. Digestive processes of haematophogous insects: Control of trypsin secretion in *Glossina morsitans*. *Journal of Insect Physiology* 20, 957–964.

Gooding, R. H. 1975. Digestive enzymes and their control in haematophagous insects. *Acta Tropica* 32, 96–111.

Hardy, I. L., Houk, E. J., Kramer, L. D., and Reeves, W. C. 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology* 28, 229–262.

Houseman, J. G., and Downe, A. E. R. 1983. Activity cycles and the control of four digestive proteinases in the posterior midgut of *Rhodnius prolixus* Stal. *Journal of Insect Physiology* 29, 141–148.

Killick-Kendrick, R. 1979. Biology of *Leishmania* in phlebotomine sandflies. *In* "Biology of Kinetoplastida" (W. H. R. Lumsden and D. A. Evans, eds.), Vol. 1, pp. 395–449. Academic Press, Orlando, FL/London.

Langley, P. A. 1975. Pathogen transmission in relation to feeding and digestion by haematophagous arthropods. *Acta Tropica* 32, 116–124.

Lernau, O. Z., Nissan, S., Neufeld, B., and Mayer, M. 1980. Myofibrilla. protease activity in muscle tissue from patients in catabolic conditions. *European Journal of Clinical Investigation* 10, 357–361.

Molyneux, D. H. 1977. Vector relationships in Trypanosomatidae. *Advances in Parasitology* 15, 1–82.

Roth, J. A., Losty, K., and Wierbicki, E. 1971. Assay of proteolytic enzyme activity using ^{14}C-labelled hemoglobin. *Annals of Biochemistry* 42, 214–221.

Schlein, Y., Warburg, A., Schnur, L. F., and Shlomai, J. 1983. Vector compatibility of *Phlebotomus papatasi* dependent on differently induced digestion. *Acta Tropica* 40, 65–70.

Wenyon, C. M. 1926. "Protozoology", Vol. 1, pp. 1–778. Baillière, Tindall & Cox, London.

Yeats, R. A., and Steiger, S. 1981. Utrastructure damage of in vitro cultured ookinetes of *Plasmodium gallinaceum* (Brumpt) by purified proteinases of susceptible *Aedes aegypti* (L.). *Zeitschrift für Parasitenkunde* 66, 93–97.

Medical and Veterinary Entomology (1987) 1, 235–242

C 5 - 131

Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence

D. BOROVSKY and Y. SCHLEIN* University of Florida, IFAS, Florida Medical Entomology Laboratory, 200 9th St., S.E. Vero Beach, Florida 32962, U.S.A, and *Department of Parasitology, The Hebrew University–Hadassah Medical School, Jerusalem, Israel

ABSTRACT. *Phlebotomus papatasi* (Scopoli) is susceptible to infection with *Leishmania major* Yakimov & Schokov and resistant to *L.donovani* Laveran & Mesnil. The possibility that susceptibility depends on midgut levels of trypsin and chymotrypsin-like (esterolytic) enzymes was investigated. Infection with *L.major* reduced the trypsin-like activity to 93.5% and 86% of the control value at 20 and 30 h post feeding and increased it to 106% at 52 h. Infection with *L.donovani* reduced trypsin-like activity to 64% and 73% of the control value at 30 and 52 h post feeding. The overall amount of trypsin and chymotrypsin-like enzymes in *L.major* infections was reduced to 50% and 34% of the control value at 20 and 30 h post feeding and increased to 184% at 52 h. Only one of the enzymes separated by gel electrophoresis was lower throughout, i.e. peak D. Overall, the midgut enzyme level with *L.donovani* infection was 86% of the control value at 30 h post feeding and 105% at 52 h; their relative amounts changed throughout. Soybean trypsin inhibitor enabled *L.donovani* to survive and multiply in *P.papatasi*. It is suggested that a specific component of the trypsin-like activity prevents the survival of *L.donovani* in *P.papatasi* and that modulation of this factor enables *L.major* to survive.

Key words. *Plebotomus papatasi*, proteolytic enzymes, vector-competence, *Leishmania major*, *Leishmania donovani*.

Introduction

Leishmania parasites are ingested by the sandfly vector with a blood meal taken from an infected host. In the midgut the parasites are exposed to proteolytic enzymes which are elicited, as in other haematophagous insects, by the intake of the meal (Gooding, 1974, 1975). In the Old World sandfly vectors only retain infections with particular *Leishmania* species, other species do not survive (Patton & Hindle, 1927; Adler & Theodor, 1930, 1931; Hindle, 1931; Adler *et al.*, 1938; Adler, 1938, 1947; Heyneman, 1963). Aspects of *Leishmania* vector specificity were discussed in Molyneux (1977) and Killick-Kendrick (1979, 1985).

Adler (1938) demonstrated that the factors which cause the death of *Leishmania* in

Correspondence: Dr Y. Schlein, Department of Parasitology, The Hebrew University–Hadassah Medical School, P.O.B. 1172, Jerusalem, Israel.

Rec'd in SCI OCT 14 1988

55

Phlebotomus papatasi (Scopoli) were elicited in the gut by ingestion of serum. Schlein & Romano (1986) studied the effects of two *Leishmania* species on the activity of proteolytic enzymes in *P.papatasi*. Infections with *L.donovani* Laveran & Mesnil, which does not normally survive in this sandfly, induced a 32.4% increase, whereas *L.major* Yakimov & Schokov, the natural parasite, caused 28% decrease in proteolytic activity.

In the present study the effects of *L.major* and *L.donovani* on the activity and amounts of trypsin and chymotrypsin-like enzymes elicited in *P.papatasi* and their possible role in parasite survival were investigated.

Materials and Methods

Sandflies. The *Phlebotomus papatasi* colony originated from sandflies caught in the Jordan Valley. Adults were kept at 28±1°C with 80% relative humidity and fed on 20% sucrose and baby mice. Experimental series consisted of twenty to forty females, 1–5 days old; they were maintained on 20% sucrose.

Proteolytic enzyme preparation. Groups of ten or more sandflies were separated at different times after feeding and homogenized with a mini-glass homogenizer (Gencons of Hemel Hempstead, U.K.) in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.9, at 4°C. Homogenates were centrifuged for 2 min at 7000 g (Beckman Microfuge TMB), and supernatants were stored at −20°C until used. Each experiment consisted of experimental and control groups that were reared together and fed at the same time.

Leishmania preparation. L.major LRC-L137, a Jordan Valley isolate, and *L.donovani* LRC-L52, a human isolate from India, were grown at 28°C on NNN medium for 3–5 days. Promastigotes were harvested, washed three times in phosphate-buffered saline, pH 7.4, and brought to a concentration of 10^7/ml. Sandflies were infected via membrane feeding on meals of inactivated rabbit blood or serum (incubated at 56°C for 30 min) containing 1×10^6/ml promastigotes (Schlein *et al.*, 1983). Controls were given meals without promastigotes.

Treatment with soybean trypsin inhibitor. Sandflies were fed meals of inactivated rabbit blood with 1% soybean trypsin inhibitor and 10^6/ml *L.donovani* promastigotes. Control flies were given similar meals without inhibitor. Following infection the flies were maintained on 20% sucrose and water. The dissected guts were examined 3 days later under a phase contrast microscope to determine the proportion of infected flies in each group. The degree of meal digestion, number and vitality of parasites were assessed. The experiment was repeated twice.

Assay of proteolytic activity. General proteolytic activity of homogenates was determined using hide-powder azure (an insoluble protein-dye complex) as substrate according to Borovsky (1985). Hide-powder azure (15 mg) was incubated for 1 h at 37°C in a 6 ml reaction mixture containing 0.08 M borate buffer, pH 8.5. Protease activity was followed at 600 nm.

The esterolytic activity of trypsin was measured by the increase in absorbance at 410 nm in 1.0 ml reaction mixture using BApNA as a substrate (Erlanger *et al.*, 1961). One unit of trypsin activity is defined as the amount of enzyme which hydrolyses 1.0 μmol of BApNA per minute at 30°C. Each sample of 10 μl was equivalent to one fly homogenate.

Relative quantitation of trypsin and chymotrypsin. [1,3-^{3}H]diisopropylphosphorofluoridate (specific activity 3.5 Ci/mmol) (DFP) (Amersham) (1 μl, 5 μCi) was incubated with several dilutions of sandfly homogenates for 18 h at 4°C in 0.1 ml of 50 mM Tris-HCl buffer pH 7.9. Following incubation the [1,3-^{3}H]diisopropylphosphoryl-trypsin and chymotrypsin derivatives were assayed by adsorbing aliquots of two fly equivalent (20–50 μl) onto 2×2 cm pieces of Whatman no. 3 filter paper, washing at 4°C in 10% trichloroacetic acid for 15 min, then twice in 5% trichloroacetic acid for 15 min and a 5 min wash in absolute ethanol. Samples were dried at 50°C and counted in a liquid scintillation counter (Minaxi & TRI CRAB 4000).

Polyacrylamide gel electrophoresis. [1,3-^{3}H]DIP-trypsin and chymotrypsin were subjected to modified Laemmli (1970) gel electrophoresis on native gels 1 mm thick, 15 cm long. The stacking gel was 4% (w/v) polyacrylamide and 0.125 M Tris-HCl, pH 6.8, and the separating gel was 10% (w/v) polyacrylamide and 0.375 M Tris-HCl, pH 8.8. Samples applied to the gel contained 20–50 μg protein, the homogenate of two fly-equivalents. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in a 1:7:6 mixture of acetic acid, methanol and water, respectively, for 30–60 min. Gels were

destained for 24 h in 7% acetic acid containing 5% methanol.

For fluorography the gels were soaked in 100 ml dimethyl sulfoxide (DMSO) for 30 min, fresh DMSO for 30 min and 22% 2,5-diphenyloxazole (PPO) in DMSO for 3 h. They were rinsed and soaked in water for 30 min, dried under a vacuum at 60°C in a gel drier (Bio-Rad, Richmond, Calif., U.S.A.) and exposed to X-ray film for 5–7 days at −70°C. Quantitation was by densitometry (Quick Scan R&D, Helena Laboratories). The sum of the area under the peaks in the control, for each experimental time, was defined as 100 densitometric units (d).

Results

Maximum proteolytic activity in blood fed flies, measured using hide-powder azure, was seen 30 h after the meal (Fig. 1). 90–92% of the total esterolytic activity was accounted for by trypsin-like activity, as measured with BApNA, and 8–10% by chymotrypsin-like activity.

Activity of trypsin-like enzymes determined with BApNA in control flies was 6.2, 7.5 and 7.0 units per fly at 20, 30 and 52 h post feeding. In *L.major*-infected flies trypsin-like activity was 93.5%, 86% and 106% of the respective controls at 20, 30 and 52 h. In *L.donovani*-infected flies

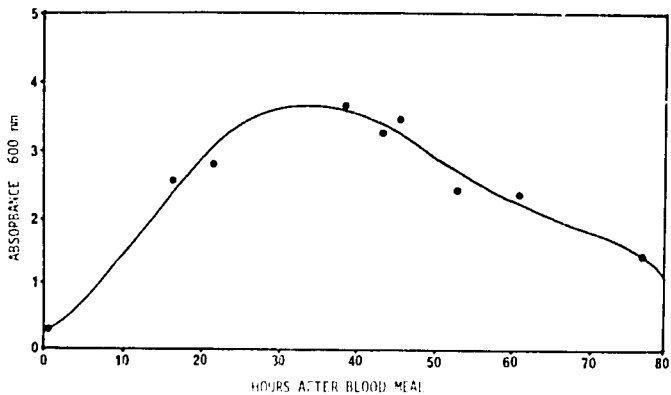


FIG. 1. Alkaline protease activity in *P.papatasi* during digestion of blood meal: 10 μl samples of fly homogenates (one fly equivalent) were incubated at 37°C for 1 h with hide-powder azur. Activity was followed at 600 nm. Each point represents the average of two determinations.

TABLE 1. Trypsin-like activity and levels of trypsin and chymotrypsin-like enzymes of *P.papatasi* at different times following artificial infection with *L.donovani* and *L.major*. Trypsin-like activity was measured following 20 min incubation of 10 μl fly homogenate aliquots (1 fly equivalent) with BApNA. Relative amounts of esterolytic enzymes were evaluated by the bound [1,3-^{3}H]DIP in 20 μl fly homogenate aliquots.

Meal	Time post feeding					
	20 h		30 h		52 h	
	DIP level (cpm)	Activity	DIP level (cpm)	Activity	DIP level (cpm)	Activity
Rabbit blood	27,740	6.2	31,350	7.5	20,468	7.0
Rabbit blood+*L.major*	13,912	5.8	10,614	6.45	37,720	7.4
Rabbit blood+*L.donovani*	ND*	ND	27,008	4.8	21,489	5.1
Rabbit serum	ND	ND	26,981	2.3	ND	ND
Rabbit serum+*L.major*	ND	ND	20,956	1.8	ND	ND
Rabbit serum+*L.donovani*	ND	ND	20,580	4.6	ND	ND

* ND=not done.

TABLE 2. Relative quantities of [1,3-^{3}H]DIP trypsin and chymotrypsin-like derivatives from *P.papatasi* fed on blood with or without *Leishmania* at different times post infection. Analysis as in Fig. 2.*

Peak	Time post feeding							
	20 h		30 h			52 h		
	Control	*L. major*	Control	*L.major*	*L.donovani*	Control	*L.major*	*L.donovani*
A	5.1†	2.2	1.8	1.8	0	0	0	0
B	0	0.6	3.7	1.8	3.8	0	0	0
C	40.0	11.4	40.0	20.6	20.6	31.5	86.2	43.4
C_1	0.8	0.6	12.9	0	0	8.0	12.0	19.3
D	16.4	1.8	19.4	3.6	18.0	29.4	16.0	24.1
D_1	10.2	3.0	8.0	2.7	0	18.1	24.0	9.6
E	4.8	0.4	2.1	0.9	19.5	7.6	12.0	8.4
E_1	0.2	0.2	1.8	0	3.7	5.4	14.0	1.5
F	14.3	7.8	7.5	0.9	16.7	0	4.3	0
F_1	4.1	3.0	2.2	0	0.9	0	12.0	0
G	4.1	16.6	0.6	0	0	0	0	0
Total	100.0	47.6	100.0	32.3	83.2	100.0	180.5	106.3

* The sum of the area under the peaks in the control at each experimental time was defined as 100 densitometric (d) units

† Densitometric units.

trypsin-like activity was 64% and 73% of the control values at 30 and 52 h. Trypsin-like activity in serum fed control flies was 2.3 units at 30 h; in *L.major*-infected flies it was 78% of the control value; in *L.donovani* it was 200% of the control value (Table 1).

The quantitation of trypsin-like and chymotrypsin-like enzymes by the conversion of [1,3-^{3}H]DFP to DIP derivatives is based on the specific covalent binding of radiolabelled diisopropyl by phosphorylation of the serine residues of trypsin and chymotrypsin (Jansen *et al.*, 1949; Dixon *et al.*, 1958). Trypsin and chymotrypsin-like enzymes were considered together in this study.

The total binding of DFP in blood fed controls was 27,744, 31,350 and 20,468 cpm at 20, 30 and 52 h post feeding (Table 1). The total binding in *L.major* infected flies was 50% of the uninfected control value at 20 h and 34% at 30 h post infection. The binding in *L.donovani* infections was 86% of the control value 30 h post infection. At 52 h post feeding these proportions were reversed such that in *L.major*-infected flies binding was 84% greater than in the controls; whilst in *L.donovani*-infected flies binding was 5% higher than in the controls.

The total binding in serum-fed controls was 86% of the uninfected blood-fed controls 30 h

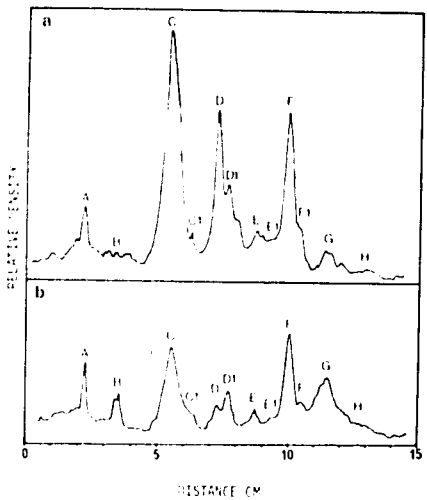


FIG. 2. Densitogram of trypsin and chymotrypsin-like enzymes of *P.papatasi*, 20 h after artificial blood feeding, with or without *Leishmania*: (a) control; (b) infected with *L.major*. Fly homogenates were incubated with [1,3-^{3}H]DFP. 20 μl aliquots (two fly equivalents) of each homogenate containing the [1,3-^{3}H]DIP enzyme derivatives were separated via polyacrylamide gel electrophoresis. Autoradiographs of the gels were analysed by densitometry.

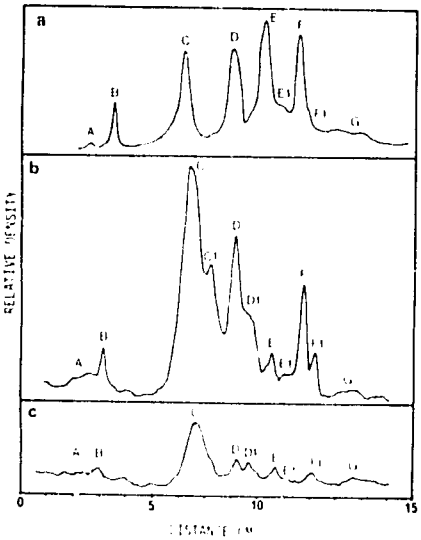


FIG. 3. Densitogram of trypsin and chymotrypsin-like enzymes of *P.papatasi* 30 h after artificial blood feeding, with or without *Leishmania*: (a) infected with *L.donovani*; (b) control; (c) infected with *L.major*. Legend as in Fig. 2.

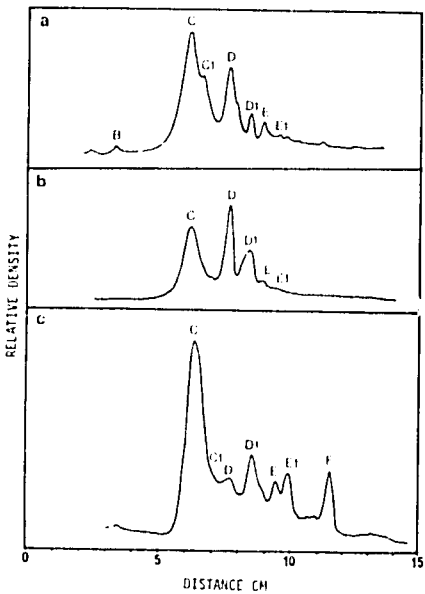


FIG. 4. Densitogram of trypsin and chymotrypsin-like enzymes of *P.papatasi* 52 h after artificial blood feeding, with or without *Leishmania*: (a) infected with *L.donovani*; (b) control; (c) infected with *L.major*. Legend as in Fig. 2.

post feeding. The binding in flies given either meals containing *L.major* or *L.donovani* with serum were 67% and 65% of the blood-fed control value, respectively.

Densitometry of the radiolabelled enzymes (DIP derivatives of trypsin and chymotrypsin-like enzymes) of *L.major* infected flies showed: at 20 h post infection a reduction in all the peaks (total=47.6 units) compared to the control (100 units), except B and G; at 30 h post infection further reduction in all peaks (total=32.3 units); at 52 h post infection an increase in all peaks (total=180.5 units), except for D (Table 2; Figs. 2–4).

Densitometry of the DIP derivatives of *L.donovani*-infected flies showed: at 30 h post infection a decrease in peaks A, C, C_1, D_1, E_1, G and an increase in the remaining peaks (total=83.2 units); at 52 h post infection peaks C, C_1, E_1 were greater and peaks D, D_1, E smaller, giving an overall value of 106.3 units (Table 2; Figs. 2–4).

Twenty-four of the thirty-eight flies fed 1% soybean inhibitor in blood meals with 10^6/ml *L.donovani* promastigotes contained both *L.donovani* promastigotes and undigested blood. Numbers of parasites per fly ranged from several hundreds to about 2000. Eleven of these flies contained only dead parasites. Only six of the forty control flies retained some of the blood meal and four had live, active infections. The number of parasites did not exceed several hundreds. The presence of parasites in the experimental versus the control group was significantly different ($P<0.001$) using the χ^2 test.

L.donovani multiplied in NNN cultures containing 1% soybean trypsin inhibitor to 2.5×10^6/ml after 2 days and decreased to 1.7×10^6 by day 4. Control cultures were 21×10^6/ml on day 4.

Discussion

As in other haematophagous Diptera the blood meal of *P.papatasi* induces proteolytic activity (Champlain & Fisk, 1956; Gooding, 1973; Briegel & Lea, 1975; Graf & Briegel, 1985;

Borovsky, 1985, 1986). The peak of protease activity at 30 h post feeding in *P.papatasi* was similar to that of *Culex nigripalpus* Theobald (35 h) (Borovsky, 1986). Further similarities were seen in the proportion of esterolytic activity contributed by trypsin-like enzymes, 90–92% in *P.papatasi* and 80% in *C.nigripalpus* (Gooding, 1973; Borovsky, 1986) and 75% in *Aedes aegypti* (L.) (Briegel & Lea, 1975).

Both the type of blood meal ingested by the fly and the presence of *Leishmania* elicited different levels of proteolytic enzymes and activities in *P.papatasi*. The three parameters examined were trypsin-like activity, the total quantity of trypsin and chymotrypsin-like enzymes and their relative amounts over time. The effects of feeding with serum are clearly different from those obtained by feeding with natural meals of whole blood. The level of trypsin-like activity was markedly reduced in serum-fed flies (20% of the blood fed control value) with a further reduction to 15% in the presence of *L.major* and an increase to 70% with *L.donovani* (Table 1). Similar results were reported previously (Schlein & Romano, 1986). However, the amount of trypsin and chymotrypsin-like enzymes was 86% of that seen in blood-fed flies, whilst infection with either *L.major* or *L.donovani* reduced this further to 65% and 67% of the control value (Table 1).

Infection with *L.major* in the presence of a blood meal reduced the level of activity to 93.5% and 86% of the control value at 20 and 30 h post feeding, and increased it to 105.7% at 52 h. The overall amount of trypsin and chymotrypsin-like enzymes was reduced to 50% and 34% of the control values at 20 and 30 h post feeding, but increased to 184% at 52 h (Table 1). The relative amounts of these enzymes as separated by gel electrophoresis changed with time paralleling the overall values, but specific components showed contrary changes: peaks B and G were greater at 20 h post infection and peak D smaller at 52 h post infection (Table 2; Figs. 2 and 4).

Infection with *L.donovani* in the presence of a blood meal produced a different effect from that elicited by *L.major*. There was a larger reduction in trypsin-like activity at 30 h (64% of the control value) and a smaller increase at 52 h (73% of the control value). The overall amount of trypsin and chymotrypsin-like enzymes was only reduced to 86% of the control value 30 h post feeding, rising to 105% at 52 h (Table 1). Similarly the composition of these enzymes changed, those represented by peaks A, C, D, F_1, G decreased, whilst the remainder increased 30 h post infection. At 52 h post infection peaks C, C_1, E increased and D, D_1, E decreased (Table 2; Figs. 3 and 4).

L.major is transmitted by *P.papatasi* in nature. Small numbers of parasites are sufficient to establish high rates of infection. The ability of *L.major* to survive and thrive in *P.papatasi* does not involve an overall reduction in protease activity or more specifically trypsin-like activity. However, there is a modification in the amounts of trypsin and chymotrypsin-like enzymes elicited: early on there is a marked decrease followed by a marked increase in all the components, with the exception of peak D. Thus, generally high levels of proteolytic enzymes can be tolerated by *L.major*. This suggests it is the modulation of a specific component, e.g. peak D, which facilitates survival.

L.donovani is not transmitted by *P.papatasi* in nature and seldom survives in artificial infections with small numbers of parasites (Adler, 1947; Schlein, unpublished observations). The effect of *L.donovani* on the proteases of *P.papatasi* differs in several respects from that of *L.major*. Although there is a greater reduction in overall trypsin activity, the amount of trypsin and chymotrypsin-like enzymes is much higher early on in infection, but is not followed by the increase seen in *L.major*. The presence of soybean trypsin inhibitor in infective meals of *L.donovani* enables the parasites to survive and multiply. This indicates that trypsin-like enzymes are instrumental in preventing the establishment of *L.donovani* infections. The data suggest that a specific component of the trypsin-like activity prevents the survival of *L.donovani* in *P.papatasi*.

There is evidence that insusceptibility of Old World vectors to different *Leishmania* species may be overcome by ingestion of much larger numbers than are ingested in nature (Adler, 1938, 1947; Adler & Theodor, 1927, 1929). Since small numbers of *L.major* can lower the amounts of trypsin and chymotrypsin-like enzymes, albeit to a much lesser extent than *L.donovani*, much larger numbers of the latter might induce a sufficient reduction, either overall or in a specific component such as peak D, thereby facilitating survival.

Leishmaniae are not the only pathogens which are affected by gut enzymes of the vector. High levels of mosquito proteases cause damage to *Plasmodium* (Langley, 1975; Yeats & Steiger, 1981) and arboviruses (Hardy *et al.*, 1983). However, they evade the enzymes by early escape whereas *Leishmania* remain in the gut. As reported by Schlein & Romano (1986) and confirmed in this paper, *Leishmania* is able to manipulate the levels and activities of proteolytic enzymes in the vector. We suggest that a specific component of the trypsin-like activity prevents the survival of *L.donovani* in *P.papatasi* and modulation of this factor by *L.major* enables it to thrive. A hypothetical candidate is the enzyme represented by peak D, which is the only component reduced in the midgut of flies infected with *L.major*.

Acknowledgments

We gratefully acknowledge the Lady Davis foundation for supporting Dr D. Borovsky as a Visiting Professor. This work was also supported by the project on Epidemiology and Control of Vector Borne Diseases in Israel (REP-NIH-NIAID-AI-12066), AID-CDR project C5-136 and the UNDP World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

References

Adler, S. (1938) Factors determining the behaviour of *Leishmania* sp. in sandflies. *Haretuah*, **14**, 1–5.

Adler, S. (1947) The behaviour of a Sudan strain of *Leishmania donovani* in *Phlebotomus papatasi*. A comparison of strains of *Leishmania*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **40**, 701–712.

Adler, S. & Theodor, O. (1927) The behaviour of cultures of *Leishmania* sp. in *Phlebotomus papatasi*. *Annals of Tropical Medicine and Parasitology*, **21**, 111–134.

Adler, S. & Theodor, O. (1929) Attempts to transmit *Leishmania tropica* by bite: the transmission of *L.tropica* by *Phlebotomus sergenti*. *Annals of Tropical Medicine and Parasitology*, **23**, 1–16.

Adler, S. & Theodor, O. (1930) The behaviour of insect flagellates and leishmanias in *Phlebotomus papatasi*. *Annals of Tropical Medicine and Parasitology*, **24**, 193–196.

Adler, S. & Theodor, O. (1931) Investigation on Mediterranean kala azar. I–V. *Proceedings of the Royal Society*, B, **108**, 447–502.

Adler, S., Theodor, O. & Wittenberg, G. (1938) Investigation on Mediterranean kala azar. XI. *Proceedings of the Royal Society*, B, **125**, 491–516.

Borovsky, D. (1985) Characterization of proteolytic enzymes of the midgut and excreta of the biting fly *Stomoxys calcitrans*. *Archives of Insect Biochemistry and Physiology*, **2**, 145–159.

Borovsky, D. (1986) Proteolytic enzymes and blood digestion in the mosquito *Culex nigripalpus*. *Archives of Insect Biochemistry and Physiology*, **3**, 147–160.

Briegel, H. & Lea, A.O. (1975) Relationship between protein and proteolytic activity in the midgut of mosquitoes. *Journal of Insect Physiology*, **21**, 1597–1604.

Champlain, R.A. & Fisk, F.W. (1956) The digestive enzymes of the stable fly *Stomoxys calcitrans* (L.). *Ohio Journal of Science*, **56**, 52–62.

Dixon, G.H., Kaufman, D.L. & Neurath, H. (1958) Amino acid sequence in the region of diisopropylphosphoryl binding in diisopropylphosphoryl-trypsin. *Journal of Biological Chemistry*, **233**, 1373–1381.

Erlanger, B.F., Kokowsky, N. & Cohen, W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics*, **95**, 271–278.

Gooding, R.H. (1973) The digestive processes of haematophagous insects. IV. Secretion of trypsin by *Aedes aegypti* (Diptera: Culicidae). *Canadian Entomologist*, **105**, 599–603.

Gooding, R.H. (1974) Digestive processes of haematophagous insects: control of trypsin secretion in *Glossina morsitans*. *Journal of Insect Physiology*, **20**, 957–964.

Gooding, R.H. (1975) Digestive enzymes and their control in haematophagous insects. In: Blood digestion in haematophagous insects. *Acta Tropica*, **32**, 96–111.

Graf, R. & Briegel, H. (1985) Isolation of trypsin isoenzymes from the mosquito *Aedes aegypti* (L.). *Insect Biochemistry*, **15**, 611–618.

Hardy, J.L., Houk, E.J., Kramer, L.D. & Reeves, W.C. (1983) Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annual Review of Entomology*, **64**, 229–262.

Heyneman, D. (1963) Leishmaniasis in the Sudan Republic. 12. Comparison of experimental *Leishmania donovani* infections in *Phlebotomus papatasi* (Diptera: Psychodidae) with natural infections found in man baited *P.orientalis* captured in a kala-azar endemic region of Sudan. *American Journal of Tropical Medicine and Hygiene*, **12**, 725–740.

Hindle, E. (1931) The development of various strains of *Leishmania* in Chinese sandflies. *Proceedings of the Royal Society*, B, **108**, 366–383.

Jansen, E.F., Nutting, M.D.F., Jang, R. & Balls, A.K. (1949) Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropylfluorophosphate: crystalization of inhibited chymotrypsin. *Journal of Biological Chemistry*, **179**, 189–199.

Killick-Kendrik, R. (1979) Biology of *Leishmania* in phlebotomine sandflies. *Biology of the Kinetoplastida* (ed. by W. H. R. Lumsden and D. A. Evans), Vol. 2, pp. 395–544. Academic Press, London.

Killick-Kendrik, R. (1985) Some epidemiological consequences of the evolutionary fit between leishmaniae and their phlebotomine vectors. *Bulletin de la Société de Pathologie Exotique*, **78**, 747–755.

Laemmli, U.K. (1970) Cleavage and structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Langley, P.A. (1975) Pathogen transmission in relation to feeding and digestion by haematophagous arthropods. *Acta Tropica*, **32**, 116–124.

Molyneux, D.H. (1977) Vector relationships in Trypanosomatida. *Advances in Parasitology*, **15**, 1–82.

Patton, W.S. & Hindle, E. (1927) The development of Chinese *Leishmania* in *Phlebotomus major* var. *chinensis* and *P. sergenti*. *Proceedings of the Royal Society*, B, **101**, 369–390.

Schlein, Y. & Romano, H. (1986) *Leishmania major* and *L. donovani*: effects on proteolytic enzymes of *Phlebotomus papatasi* (Diptera: Psychodidae) and their possible role in adaptation to the vector. *Experimental Parasitology*, **62**, 376–380.

Schlein, Y., Warburg, A., Schnur, L.F. & Shlomai, J. (1983) Vector compatibility in *Phlebotomus papatasi* dependent on differentially induced digestion. *Acta Tropica*, **40**, 65–70.

Yeats, R.A. & Steiger, S. (1981) Ultra structure damage of *in vitro* cultured ookynetes of *Plasmodium gallinaceum* (Brumpt) by purified proteinases of susceptible *Aedes aegypti* (L.). *Zeitschrift für Parasitenkunde*, **66**, 93–97.

Accepted 15 February 1987