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Experimental Infection of Panamanian *Phlebotomus* Sandflies with *Leishmania*[1]

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Over 800 *Phlebotomus* sandflies of five species were fed artificially, by the Hertig pipette method, on a mixture of blood and flagellates from cultures of *Leishmania braziliensis, s. lat.* Flagellate infections of the gut were produced in all five species, with an over-all infection rate of 81%.

As in the case of experimental sandfly infections with Old World species of *Leishmania* causing human disease, growth of the flagellates characteristically took place at the anterior station, i.e., in the cardia and at the proventricular valve, with occasional growth forward into the pharynx. In addition, however, there was usually attachment and growth in the hindgut, particularly in the thin-walled, slightly expanded anterior portion.

Attempts to transmit the infection by feeding infected sandflies on spiny rats (*Proechimys*) and a human volunteer were unsuccessful. The inoculation of suspensions of triturated sandflies into spiny rats, suckling white mice, and hamsters produced an infection only once, in a suckling mouse.

A small number of sandflies of two species was fed with *Leishmania enrietii*. Infections of the cardia were produced in one species; the other was completely negative.

In the early stages of our work on the transmission problem of American cutaneous leishmaniasis in Panama, we were handicapped by the lack of any laboratory animal in which a demonstrable infection could be produced. As a result we had no convenient means of infecting *Phlebotomus* sandflies for use in transmission experiments or for determining differential infection rates which might give a clue to the one or more species concerned in nature. What would seem to be an obvious solution of this difficulty, namely, the use of human patients, was tried. Our colleague, Dr. Phyllis T. Johnson, in 1957 and 1959 fed several batches of laboratory-reared sandflies on patients. The lesion was masked with aluminum foil or plastic film so that sandflies in small feeding cages had access only to the unulcerated border of the lesion. While some sandflies became infected, the method was wholly unsuitable for any extensive series of laboratory experiments. Not only was the feeding of the sandflies erratic and the yield of infections disappointingly low, but it would have been necessary to keep on hand a continuous succession of untreated patients. We therefore resorted to artificial methods of feeding and infecting sandflies.

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Materials and Methods

Membranes. Adler and associates found it possible to feed *Phlebotomus papatasi* through animal membranes, and it was with this method that they infected the sandflies in their series of transmissions of oriental sore

to human volunteers (Adler and Ber, 1941). However, *papatasi* was the only species of the Near East or Mediterranean region which they ever succeeded in feeding through membranes. During 1957 and again in 1959, efforts were made by Dr. Phyllis T. Johnson to develop a satisfactory membrane technique for our Panamanian species. Animal membranes tested included guinea pig mesentery and large bowel, human kidney capsule, human peritoneum, and human skin. Twenty-three trials were made with these membranes, using five different species of sandflies. Of approximately 150 laboratory-reared females offered the chance to feed through membranes, only one *sanguinarius* did take a meal (mixed culture and rabbit blood, through guinea pig mesentery), and it became infected.

Pipette technique. In view of the uncertain prospects for the use of membranes, we turned to the method developed in China by Hertig and Hertig (1927) and used successfully by others (Napier, 1930; Adler and Theodor, 1957).

The apparatus (Figs. 1–3) consists essentially of a capillary tube with an orifice which will admit the piercing stylets but not the labium, which is pushed and bent back to simulate its position in natural feeding. This pipette passes through and is held by a cork sphere which in turn is inserted into a glass or plastic tube, where it serves as a ball-and-socket joint and maintains whatever position to which it may be manipulated. The other end of this tube holds a split cork which is in effect a vise or clamp for holding the sandfly wings. After the orifice of the pipette is slipped over the stylets, the mixture to be fed is introduced at the other end of the pipette so that finally the stylets are immersed in the fluid.

The original design (see Appendix) was followed with certain modifications. In the original apparatus, all models of which were made by A. T. Hertig, the tube was of glass, and the cork parts were turned on an improvised lathe (electric fan with blades removed). Since stocks of glass tubing are variable in diameter, it was necessary to turn the corks to fit individual pieces of tubing. In our current models (made by M. H.) methacrylate plastic tubing was used (1/2 inch outside diameter; 3/8 inch inside;

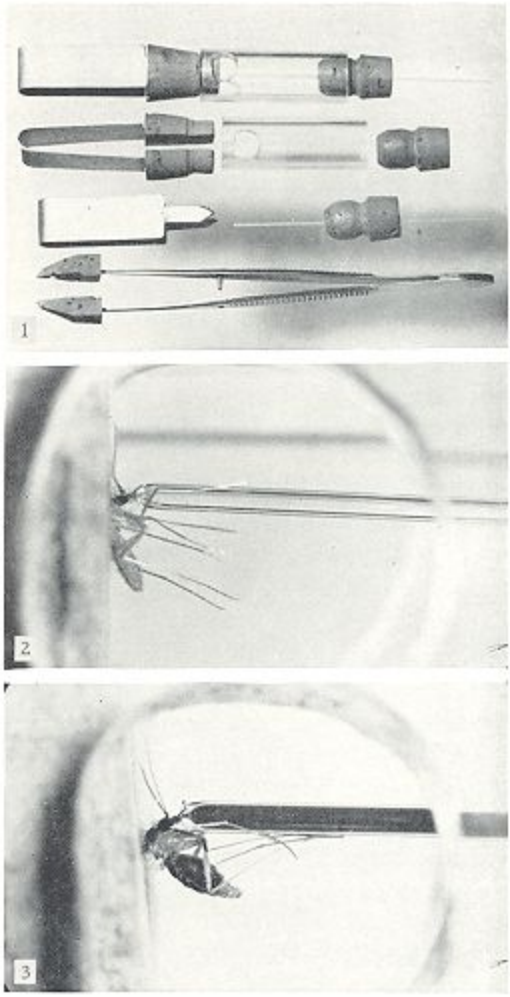


FIG. 1. Apparatus for the artificial feeding of *Phlebotomus* sandflies; cork-tipped forceps for handling sandflies (× 0.5).

FIG. 2. *Phlebotomus gomezi* in position in apparatus before culture-blood mixture has been run into the pipette. The sandfly is held by its wings in the cork vise; the stylets lie within the orifice of the pipette; the labium has been pushed back (× 4.5).

FIG. 3. *Phlebotomus sanguinarius*, engorged (× 4.5).

cut into 35-mm lengths). Since this is manufactured by passing through a die, both outside and inside diameters are accurate and constant, and all parts are therefore interchangeable. The "lathe" on which the corks were turned was a hand drill clamped horizontally in a vise, with the corks inserted directly in the drill's chuck or in plastic tubing held by the chuck. A photographic tripod served as the tool rest. The cutting tools were files, the sharpened edges of triangular files being used to make a series of grooves to the required depth, with flat files and emery cloth used for finishing.

Instead of a sphere for the ball-and-socket feature, one end was turned to form a partial sphere, with the rest of the cork projecting from the plastic tube for greater ease in manipulation. In making the vise the cork was turned to fit part way into the tube; the pointed ends of a spring made of sheet aluminum and bent double were pushed into the cork, which was then split with a razor blade. The opposing edges of the vise were slightly beveled so as not to bend the bases of the wings too sharply and to accommodate part of the width of the sandfly body.

The variable grain of the cork, together with the primitive method of manufacture, at times made it necessary to correct irregularities in the size or shape of the spherical portion. This could be done with emery cloth, or if the fit was too loose the cork could be expanded by rotating or passing it near a small flame so as to cause incipient charring. The sphere was lubricated by coating it with paraffin spread with a hot spatula.

The pipettes were made by drawing out glass tubing, selecting with calipers those portions between 0.4 and 0.5 mm in diameter, and cutting them into 7-cm lengths. The cut, or rather break, should be made with a diamond scratch so that the ends are as even as possible.[3] To form the orifice one end was constricted in a near approach to a micro-flame. Our micro-burner was in effect a minute alcohol lamp made of a bluntly constricted piece of glass tubing set in the cork of a small vial. Threads or a strand of string served as a wick. Absorbent cotton in the glass tube not only held the wick in place but provided a reservoir of alcohol near the orifice. This burner gave a tiny hemispherical blue flame 2 or 3 mm in diameter. With the flame in focus against a dark background under a dissecting microscope tilted so that the column of hot gas would avoid the lenses, the end of the pipette was brought near the side of the flame. Lighting was adjusted so that the inner surface of the pipette was clearly seen. With the beginning of the yellow sodium glow the slow constriction could be observed and stopped at the right point, which was learned by experience. With a hand lens the orifice was compared with a standard known to be satisfactory for a given species of sandfly. Only two sizes are necessary for our principal Panamanian species: the larger one for *sanguinarius* and *gomezi*, and the smaller for *panamensis*, *trapidoi*, and *ylephiletor*.

The hole through the ball-and-socket cork was made by first piercing it with a round needle. The track of this needle was then followed several times with a dissecting needle of the same diameter but with a slanting, chisel-like cutting edge, which made several grooves, the raised edges of which held the pipette without play but permitted easy back-and-forth adjustment.

The striations in the plastic tube made it difficult to see clearly during the maneuvering of the pipette over the stylets. We there-

[3] Most diamond points have irregularities. We have found that for cutting any glass, such as capillary tubing, slides, or cover glasses, it is well to find and mark a position of the diamond which will result in a fine, nearly invisible line. This is actually a sharp, clean groove which may "grow" as a clean crack at right angles to the surface. There should be avoided those positions, so desirable in writing on glass, which cause "tearing" of the surface and initiate many lateral, irregular cracks.

fore ground windows at opposite sides of the tube.

Method of operation. (1) The sandflies, which are free in a releasing cage, are caught in a small vial. About 2 cc of ether vapor is introduced with a medicine dropper. Within 15 seconds there is usually the proper degree of etherization, marked in some species by a characteristic position of the legs. In *sanguinarius* the legs are extended and drawn together; in *gomezi* they are usually spread wide. In any case, the period of immobilization should be just sufficient to permit transfer to the apparatus.

(2) The sandfly is dropped from the vial onto a white background and picked up with fine, straight forceps tipped with cork (Fig. 1). The cork tips are shaped with a razor to form two points which meet in perfect apposition. The forceps should engage legs from both sides of the body, preferably a single pair, so that no subsequent change in position is possible. Under the dissecting microscope the wings, which usually remain in the normal position extended dorsally, are brought into the open jaws of the cork vise, which is then gently closed so that the body lies parallel with and close to the surface of the cork. This is one of the most important steps in the whole technique. If the wings project too much it permits play in the position of the head, and combined with the insect's own movements, this makes all subsequent manipulations difficult or impossible. Care must also be taken not to pull off any legs since this could contribute to early death.

(3) The plastic tube, containing the pipette in its ball-and-socket cork, is then placed over the cork vise, which thus holds it closed, with the windows giving a side view of head and body.

(4) The pipette, maneuvered so that the tip of the proboscis lies in the funnel-like depression, is then gently pushed so that the labium is bent back as the stylets enter the orifice. The sandfly has regained full activity by this time and frequently moves the stylets back and forth within the pipette (Fig. 2).

(5) The culture-blood mixture is then run 10 or 15 mm into the other end of the pipette, which projects 2 or 3 cm. The column of blood is then blown the length of the pipette with a stream of air from a blowpipe. The projecting end of the pipette is passed quickly through an alcohol flame to avoid contamination of the fingers in subsequent procedures.

The microscope light should be arranged so that a white background is illuminated, with the sandfly subjected to as little direct heat as possible. Usually there is some feeding at once, evidenced by the appearance of a red streak in the thorax. Thereafter, feeding may either proceed slowly with intermittent "sips," cease altogether, or proceed to rapid engorgement within a minute or so (Fig. 3). In the latter case it seems as though some particular position or pressure had triggered the rapid feeding response. The first sign is the violent agitation of the red cells as they flow toward the proboscis, accompanied by the rapid and continuous vibration of the pumping structures as seen at the side of the clypeus. We have tried diligently to discover the triggering position. In China, A. T. Hertig thought that moving the stylets forward, as though tilting the insect's head upward, tended to elicit the rapid feeding response. It has been our custom to move the pipette about, gently pushing it on or withdrawing it slightly, and changing the angle of the stylets within the pipette. In this somewhat random manipulation the triggering may occur, but the secret still eludes us. If the proboscis slips out of the pipette it is necessary to use a fresh pipette, since the stylets cannot pierce the surface film of blood which quickly seals the orifice.

During the feeding operation there was observed a number of times the appearance of spherical droplets of a clear fluid at the

tip of the abdomen which were held for a moment, apparently by the setae of the cerci, and were then propelled with some force. At times there was a succession of four or five such droplets. We have assumed that the propulsion was the result of a rapid "squeezing" motion of the cerci, but such motion, if it occurred, was too rapid to detect. The droplets struck the wall of the plastic tube, which permitted estimating the trajectory as dropping 3 mm while traveling a horizontal distance of 2 mm. The deposition of fecal droplets during the act of feeding at once introduces the possibility of transmission by this mechanism, particularly in view of the frequent infection of the hindgut in this experimental series and in the leptomonad infections in wild-caught sandflies described in the following paper (Johnson *et al.*, 1963).

In those cases where engorgement has not occurred immediately, the sandfly is inspected at intervals, with additional manipulation of the pipette, for about 15 minutes. The degree of engorgement is then noted and the sandfly dropped into a moist, plaster-lined vial, its individual history to be followed throughout. Or the whole lot may be pooled (our final method) by dropping it into a porous pot through the entry tube in the bolting cloth cover. This pot, our standard breeding vessel, is also satisfactory for holding adult sandflies (Hertig and Johnson, 1961). A boiled raisin is placed on the bolting cloth. Any sandflies which die or are in distress can be fished out through the entry tube with a needle dipped in alcohol, without disturbing the rest.

In practice it is advantageous to have two operators working together, one carrying the process from etherization to first signs of feeding, and the other taking care of subsequent procedures. With this teamwork sandflies can be fed at the rate of about one every 5 minutes.

Culture-blood mixture. The mixture fed to the sandflies consisted of about 0.5 cc defibrinated rabbit or guinea pig blood, usually the latter, to which was added material from cultures of Panamanian strains of *Leishmania braziliensis* (*sensu latu*). The surface of the culture slant was washed with saline. This fluid, withdrawn with a pipette, was filtered through cotton to remove bits of agar, and spun down. Most of the supernate was removed, the sediment resuspended in a small amount of saline, and then added to the blood. The resulting dilution of the blood was about one part in ten. The mixture was checked for motile flagellates. In the case of several lots salt was added as set forth below.

Strains of Leishmania. All together there were used nine strains isolated from Panamanian patients and two strains from naturally infected, wild-caught spiny rats (*Proechimys semispinosus panamensis*). The spiny rat strains were the 12th and 15th of a series of 21 recovered from a total of 200 of these rodents trapped in 1956 and 1957 (Annual Reports, Gorgas Memorial Laboratory). Since the first strain isolated in 1956 produced a typical lesion in a human volunteer, and in the absence of differential characters, we have felt that we were probably dealing with the same parasite as that causing the human disease.

Regardless of the strain used there were variations in the proportion and intensity of the resulting sandfly infections. We attributed this to various factors, such as the overall success of the feeding operation for a given lot (with the age or physiological state of the sandflies themselves as a possible factor), or to intercurrent infection with bacteria and fungi. In only one case did the sandfly infections reveal a consistent difference attributable to the strain of *Leishmania*. Over a period of 9 months one human strain produced the normal proportion of infections in the four species of sandfly concerned, but the infections were mostly very light. Another human strain produced a number of heavy infections throughout an equal, overlapping

period. In the latter half of our work we tended to use recent human isolations, with no strain used for more than 3 months. The two spiny rat strains, however, had been in culture over 12 months in one case and from 18 to 28 months in the other and still produced sandfly infections, some of which were heavy. The age of individual transfers of any strain ranged mostly from 6 to 16 days, but good infections were produced by cultures as young as 3 days and as old as 19 days.

One small lot was fed with *Leishmania enricttii*. This strain, received through the kindness of Dr. Alfonso Trejos, had been in culture in our laboratory for over 2 years.

It has been the general experience of investigators that in transmission experiments where adult sandflies must be kept alive as long as possible, the mortality tends to be disappointingly high, especially at 4 or 5 days when the first oviposition occurs. In our own work other factors also operated. Sandflies which ingested only minimal amounts of blood tended to die off first, but the principal factor affecting survival was probably the degree of injury or trauma sustained in the feeding operation. The loss of legs, particularly of any two on one side, often caused the sandfly to "fall" to one side, which prevented recovery of normal stance and flight. If the base of the wings had been squeezed too close to the body the wings remained pressed together and flight was impossible. With increasing skill, there was less trauma as the work progressed. Peak mortality of *sanguinarius* and *gomezi* occurred at 3 and 4 days, but three-fourths of these two species survived more than 3 days, by which time the infection, if any, could be well established, and a number survived from 10 to 16 days. The mortality was considerably higher in the case of *panamensis*, *trapidoi*, and *ylephiletor*, with peak mortality at 2 and 3 days and none surviving more than 9 days.

Sandflies which died within 2 days were usually discarded. Those dead or moribund at 3 days or later were either dissected immediately or held in the refrigerator for later disposition. A number of pools of triturated sandflies were injected into spiny rats, suckling white mice, or hamsters. In the attempt to transmit the infection by biting, various sandflies which had been fed at least 4 days previously were transferred to feeding cages (Hertig and Johnson, 1961) and given the opportunity to refeed on the belly of a spiny rat or on the arm of a volunteer.

In preparing a sandfly for dissection the legs and wings were cut off and the body shaken in a vial of saline to remove the setae. The actual dissection was made on a cover glass in a minute drop of saline, following two general methods:

(1) After cutting off the head the entire gut could usually be pulled out by traction at the tip of the abdomen. In the case of gravid females or those with much blood in the stomach, or if the gut broke, the thorax and abdomen were separated and the gut withdrawn from the anterior end of the abdomen. The cover glass was then inverted over a depression slide and the edge sealed with saline, for microscopic examination. Flagellates, especially if motile, could be seen easily at lower powers (125—300 ×) with ordinary illumination. This method of dissection on a cover glass and examination in a very shallow hanging drop avoided drying, crushing, or other disturbance of the specimen and permitted great flexibility in the subsequent handling, ranging from further dissection to storage in the refrigerator. (At times the flagellates in a dissected gut maintained their motility for as much as 2 days in the refrigerator.)

(2) It was soon found that even at 3 or 4 days there tended to be concentration of flagellates in the cardia (anterior portion of the midgut) and particularly at the proventricular valve. In order to make a preliminary examination of the cardia without destroying its connection with the esophagus

and pharynx, the head and thorax were gently pulled apart, which usually resulted in the unbroken cardia lying exposed (an operation which we dubbed "neck-stretch"). If the infection was heavy, or for any other reason, the pharynx and other parts of the foregut lying within the head could then be dissected out. This was most easily done by splitting the head with the cutting edge of the needle slightly to one side of the midline.

Results

From January, 1958 to June, 1960, 73 lots of *Phlebotomus* were fed with cultures of *L. braziliensis* (*s. lat.*), totaling 825 females (410 *sanguinarius*, 265 *gomezi*, 72 *panamensis*, 45 *trapidoi*, and 33 *ylephiletor*). With the exception of four lots of wild-caught sandflies discussed below, all were reared in the laboratory. They were taken from breeding pots in which there had been emergence of adults for at least several days. The ages ranged from 1 to about 7 days.

The great majority of the sandflies fed well, except *ylephiletor*. Excluding that species, the over-all performance of the other four was: 46% fully engorged; 24% half to three-quarters engorged (i.e., 70% well fed); another 20% with at least some blood reaching the stomach, the expanded part of the midgut lying in the abdomen; while in 10% blood did not pass beyond the cardia, the slender part of the midgut in the thorax. *Phlebotomus ylephiletor* was a reluctant feeder: only 16% fed well, another 9% showed blood in the abdomen, and in 75% blood was visible only in the thorax.

Infection rates. The infection rates, based on dissections of sandflies found dead or in distress from the second to 16th days after feeding, were as follows: *sanguinarius*, 73 positive out of 89 dissected, or 82%; *gomezi*, 55/71, 77%; *panamensis*, 19/20, 95%; *trapidoi*, 8/9, 89%; *ylephiletor*, 4/7, 57%; combined, 159 positive out of 196, or 81%. These totals do not include those in which the tissues were decomposed or where the gut had been overgrown with bacteria or fungi.

Growth pattern. The growth and distribution of the flagellates in the sandfly gut followed these general lines in all five species:

At 3 days, obvious multiplication, with long slender forms predominating, had taken place in the blood meal. This was also observed at 2 days in *panamensis* and *trapidoi* in the very few 2-day dissections which were made. The brownish blood remains, enclosed in the peritrophic membrane, usually contained flagellates up to the time of final evacuation at 4–7 days. Gas bubbles commonly appeared in the "empty" stomach (Figs. 8, 10), at times with numerous flagellates pressed between bubble and epithelium or free in the clear liquid.

Beginning at 3 days the hindgut was invaded. Flagellates were typically found singly or in patches and in rosettes adhering to the epithelium of the anterior portion of the hindgut (Fig. 7). This thin-walled section, in outline an elongated triangle with the expanded base just posterior to the opening of the Malpighian tubules, we have called the "hind-triangle." By the fourth or fifth day the wall of this triangle was often completely covered, with very few flagellates attached elsewhere in the hindgut. The attached forms tended to be short and broad. There were usually some, and at times many, long slender forms in the lumen of the gut, either limited to the triangle or extending to the rectal ampulla. On several occasions the lumen of the triangle was packed with flagellates (*ylephiletor* at 3 days; *panamensis*, 4 days; *gomezi*, 4 and 5 days; Fig. 8) and once was actually distended (*gomezi*, 13 days; Fig. 9). The hind-triangle infections tended to persist throughout the life of the sandfly.

In the meantime, at 3 days (2 days in *panamensis*), flagellates were beginning to appear in the cardia anterior to any blood remains, with a tendency to become concen-

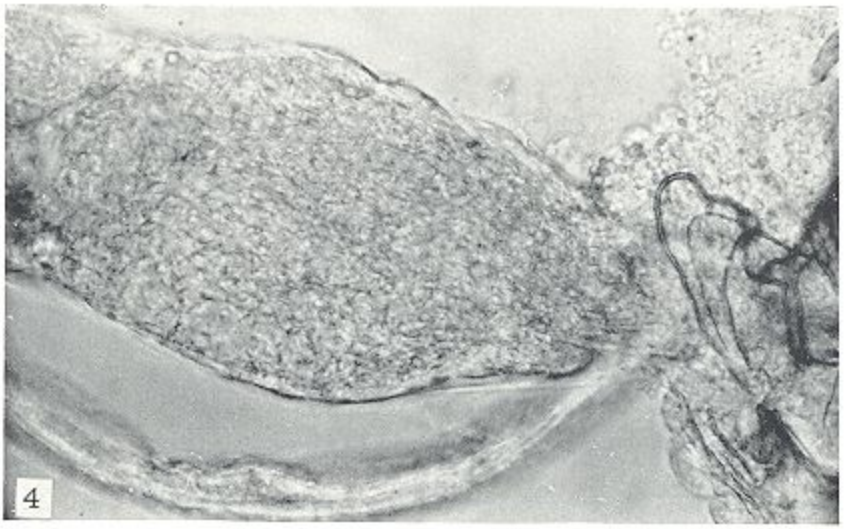
trated near or attached to the proventricular valve. Occasionally at 3 days and often at 4 or 5 days, this region was packed and even distended with flagellates (Fig. 4). At times, however, the cardia was entirely free of flagellates even though they occurred elsewhere. For example, 28 *sanguinarius*, dissected at 6 and 7 days, were all infected, but 7 had no flagellates in the cardia. Infections of the cardia, once established, apparently persisted thereafter. In the case of *gomezi* there was a marked increase in the proportion of cardial infections from 55% for the first 7 days, to 94% (15 out of 16) after 8 days, with some extremely heavy infections in the older sandflies (Fig. 5). The comparable proportions for *sanguinarius* were 73 and 75%. The other three species had too few survivors beyond 8 days to give significant data.

Growth forward from the cardia into the foregut was found in a small proportion of the dissections. This phenomenon was most striking in the case of *gomezi*. Out of a total of 47 of this species, of which dissections of the head were made, six (at from 5 to 14 days) had flagellates in the pharynx, with massive infections in three cases (Fig. 6). Four of the six also had flagellates in the esophagus or esophageal diverticulum or both (Fig. 5). In addition, five other specimens had flagellates in the esophagus or diverticulum, but none in the pharynx. In the case of *sanguinarius*, out of 49 head dissections there was only one moderately heavy infection of the pharynx (at 5 days) with the esophagus also distended with flagellates. In six other specimens (at 3–16 days) flagellates were seen in the pharynx in very small numbers, while in still one other (at 7 days), although the pharynx was negative, two flagellates were seen in the cibarium (buccal cavity) and one motile flagellate was attached to the wall of the food channel in the proboscis. Three additional specimens with negative pharynx had organisms in the esophagus and in one case in the diverticulum as well. Four head dissections of *trapidoi* were made; in one (at 6 days) the esophagus was distended but the diverticulum and pharynx were negative. Of eight *panamensis*, one (at 4 days) had a few organisms in the diverticulum with one flagellate seen in the pharynx. In all the foregoing the infection of the cardia was well established, with the portion just posterior to the proventriculus often a solid mass of flagellates.

At times some of the original culture-blood mixture was taken into the esophageal diverticulum where it remained. On three occasions undigested red cells were found in the diverticulum (*panamensis*, at 3 and 5 days, in the latter case with motile flagellates; *sanguinarius*, at 5 days, with flagellates).

Salt. Adler and Ber (1941) found that the addition of salt to the infective blood meal provided what seemed to be a crucial factor in the transmission of *Leishmania tropica* to human volunteers by the bite of *P. papatasi*. Many previous attempts, with no added salt, had consistently failed, although infections of the sandflies had been easily produced. In the case of the successful transmissions they fed the sandflies through a membrane on a mixture of one part inactivated defibrinated rabbit blood and three parts a suspension of flagellates in 2.7% saline, which would give a concentration of NaCl of about 2.24%, or nearly three times the normal salt content of blood.

To secure whatever benefits might accrue from this technique, ten of our lots received added salt (119 *sanguinarius*, 36 *gomezi*, 11 *trapidoi*: total 166). In the first such lot the flagellates from four culture tubes were spun down and resuspended in defibrinated guinea pig blood to which was then added three volumes of 2.7% saline, giving the same salt concentration used in Palestine. Of four *sanguinarius* dissected at 3–6 days, all had good infections, but the sandfly survival was relatively poor, which we thought might be due to the combination of blood dilution and salt concentration. A second lot of *sanguinarius* was fed a mixture of one part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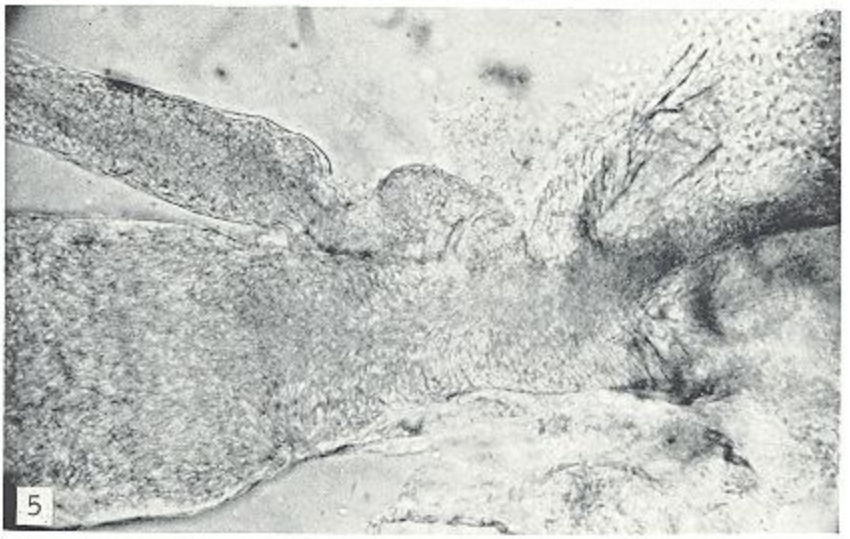
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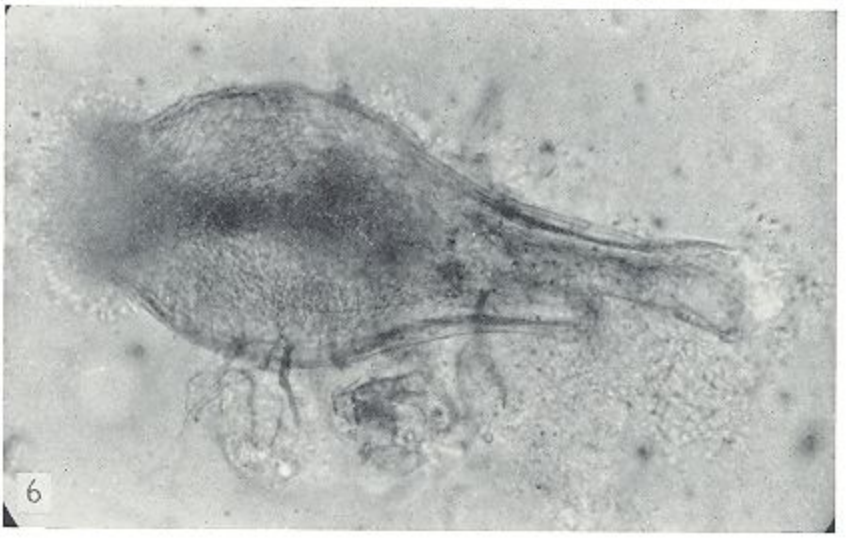
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13.7% saline and nine parts blood with flagellates, giving a salt concentration of 2.14%. None of the 45 sandflies lived more than 4 days, but the two dissected had moderately heavy infections. In the remaining eight lots the salt was further reduced to 1.47% (one part 7.0% saline in nine parts blood). The results were comparable with those of our nonsalt series as to survival (four lots fair to good; four lots indifferent), proportion infected, degree of infection, feeding on and inoculation into animals (all negative), and no further additions of salt were made.

Wild-caught sandflies. The use of wild-caught instead of laboratory-reared sandflies in the four lots mentioned above was undertaken for the following reasons: (1) Wild-caught sandflies which had fed naturally on infected dogs in China (Chung and Feng, 1950-51) transmitted kala azar (*Leishmania donovani*) to hamsters. (2) In the successful experimental transmission of *L. tropica* in Palestine (Adler and Ber, 1941) and of *L. donovani* in India (Smith *et al.*, 1941; Swaminath *et al.*, 1942) there were special features—added salt in the one case and access of the sandflies to raisins in the other—which seemed to represent the principal factors which were lacking in an impressive number of previous failures. (It may be noted that there is as yet no explanation of how these special factors operate to facilitate transmission.) (3) In Peru the common occurrence, in sandflies of both sexes, of an infection of the proboscis with a cultivable, nonpathogenic microorganism (Hertig, 1942) was taken to indicate a source other than a blood meal. (4) If it were a common habit of female sandflies in general to seek "extraneous" sources of liquid and food, then the seemingly crucial substances typified by salt and raisin juice would be acquired in nature apart from an infective blood meal.

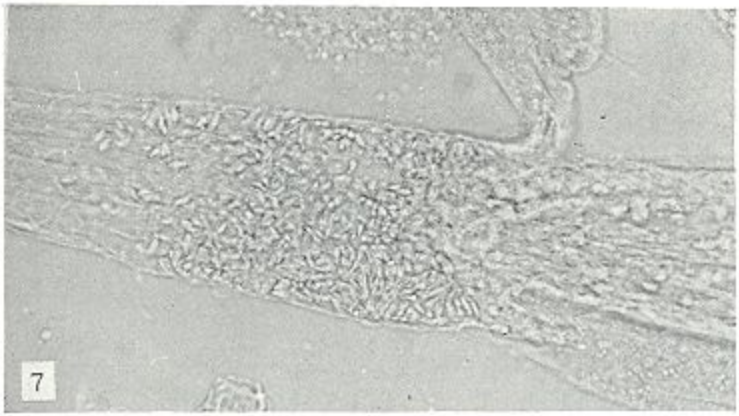
The four lots were all dry-season collections from the same spot, 29 February to 22 March 1960, and totaled 96 (40 *sanguinarius*, 37 *gomezi*, 19 *trapidoi*). They were attracted to horse or human bait and taken mostly before they had an opportunity to feed. When artificially fed, on the first to third day after capture, their response was comparable to that of laboratory-reared sandflies, except that the proportion which fed well was higher for *sanguinarius* and *trapidoi* than the average for those species and lower in the case of *gomezi*. Of the five females which had visible remains of a previous blood meal, one each of *sanguinarius* and *gomezi* imbibed only a small amount, while three *trapidoi* fed well: of four gravid *gomezi*, two fed well, two took only a trace.

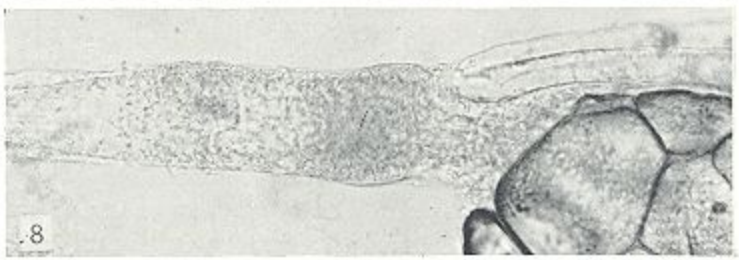
One lot with added salt (final concentration, 1.47%) had no survivors beyond 5 days. Of the seven dissected at 3–5 days, six had mostly light infections of the stomach and hind-triangle; one *trapidoi* had the only cardial infection; and one *sanguinarius* was completely negative. Two other lots with no added salt had somewhat better survival, but of the 14 dissected over half had growth of bacteria or fungi and only five had flagellates. In the remaining lot, without salt, every one of the nine specimens dissected was heavily infected: one *sanguinarius*, at 5 days, cardia and esophagus distended, pharynx also in-

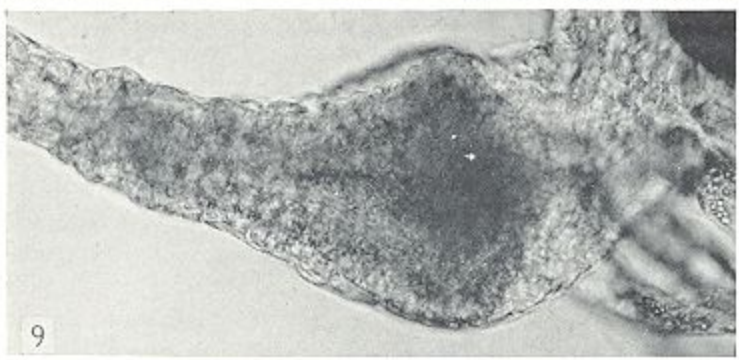
FIG. 4. *Phlebotomus trapidoi*, 6 days after infective meal; cardia distended with mass of flagellates of *L. braziliensis*; parts of head at right, esophageal diverticulum below (× 385).

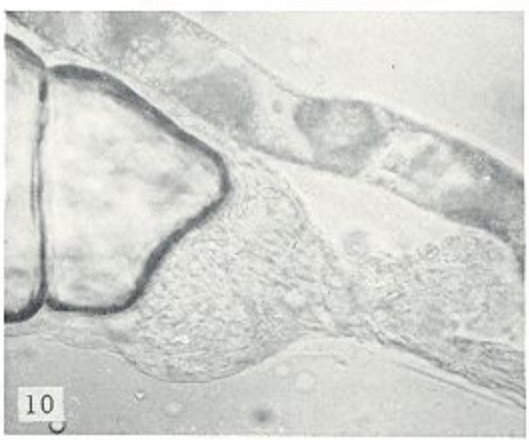
FIG. 5. *Phlebotomus gomezi*, 13 days; cardia (at left), esophagus and basal part of esophageal diverticulum greatly distended with flagellates; proventricular valve obliterated. Note "palisading" of flagellates on walls of esophagus. The pharynx (at right, still attached to the esophagus), when dissected later, was also found to be heavily infected (× 385).

FIG. 6. *Phlebotomus gomezi*, 14 days; same lot as preceding; pharynx heavily infected; mass of flagellates protruding from posterior end (at left) where connection with the esophagus broken in dissection; flagellates also at anterior end. Cardia of this sandfly was also massively infected, as in Fig. 5 (× 385).









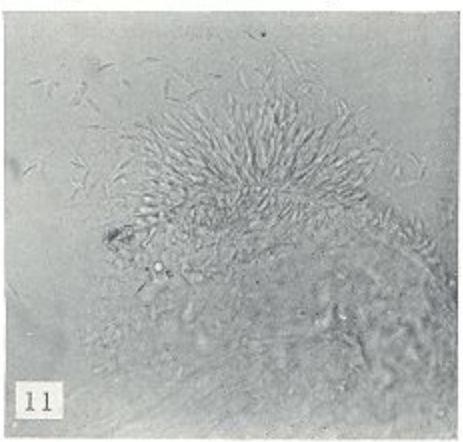


FIG. 7. *Phlebotomus gomezi*, 7 days; flagellates of *L. braziliensis* forming a loose layer attached to the wall of the "hind-triangle" (anterior portion of hindgut) (× 385).

vaded; one *trapidoi*, at 6 days, cardia and esophagus distended, pharynx negative; seven *gomezi*: one, at 8 days, cardia distended, with flagellates also in esophagus and pharynx; on the same day five of the six survivors engorged on a human volunteer; the unfed female died during the feeding operation and was then dissected; cardia distended, pharynx negative. Four of the refed females were dissected 4 and 5 days later, i.e., 12 and 13 days after the original feeding; the cardia was distended in all, as well as the esophagus or diverticulum or both (Fig. 5); the pharynx was moderately to heavily infected in two and negative in two. On the 13th day the last survivor was given a second opportunity to refeed on the same volunteer, but there were no bites felt or other signs of feeding. It was found dead on the following (14th) day; cardia, esophagus, and diverticulum were distended and the pharynx solidly packed with flagellates (Fig. 6). A suspension of this sandfly was injected into a suckling white mouse and a hamster; results were negative. No infection developed in the volunteer.

Natural infections of wild-caught sandflies with leptomonad flagellates (Johnson *et al.*, 1963) were discovered only after the artificial feedings were terminated. Sandflies captured in March 1961 (i.e., a year after the wild-caught feeding series), in the same spot, by the same method and at the same season, had natural leptomonad infections as follows: *sanguinarius*, 3 out of 85, or 3.5%; *gomezi*, 6 out of 146, or 4.1%; and *trapidoi*, none out of 29. These infection rates, together with the fact that such infections rarely showed the massive concentrations in the cardia and pharynx, indicate that there was no significant complication of the artificial feeding results by natural infections.

Transmission experiments. In the attempt to transmit the infection, artificially infected sandflies were fed either on spiny rats (*Proechimys*) or, later in the series, on a human volunteer; to test the infectivity of the flagellates developing in the gut, suspensions of triturated sandflies were inoculated into animals. At the time these transmission experiments were first undertaken we had no really satisfactory experimental animal. Inoculations of hamsters had given only negative results, although later attempts were successful. A number of spiny rats had been found naturally infected with *Leishmania*, one strain of which had produced in a human volunteer a typical course of infection (Annual Reports, G.M.L., 1956, 1957). However, the spiny rat infections could be demonstrated only by culture of the blood drawn from the heart; furthermore, no demonstrable infection was ever produced by the inoculation of *Leishmania* from any source or by any route, even in young animals born in the laboratory. In spite of these discouraging considerations it was thought that since the natural infections were presumably acquired through the bites of insects, the phenomena which take place in the skin during and after an insect bite—resulting in local physiological turbulence, so to speak—might provide the opportunity for the parasites to reach the haven of the cell system in which they could develop.

Sandflies from 10 lots, at 4–9 days after their culture-blood meal, were given the opportunity to feed on a total of 12 spiny rats; 30 of the 69 females were known to have sucked blood (6 out of 20 *sanguinarius*;

FIG. 8. *Phlebotomus gomezi*, 4 days; hind-triangle packed with flagellates, slightly distended; very few posterior to portion shown. (At the right, base of Malpighian tubules, gas bubbles in midgut) (× 230).

FIG. 9. *Phlebotomus gomezi*, 13 days; hind-triangle distended with mass of flagellates (× 360).

FIG. 10. *Phlebotomus gomezi*, 11 days, *L. enrietti*; flagellates in cardia at proventricular valve; esophageal diverticulum extending to right; most of esophages broken off; gas bubbles in cardia; Malpighian tubule above (× 385).

FIG. 11. *Phlebotomus gomezi*, same specimen after further dissection; cardia cut open and everted, showing flagellates attached to epithelium (× 385).

24 out of 49 *gomezi*); a few of the fed females were given a second opportunity and one each of these species refed at 12 and 7 days, respectively. One spiny rat died without examination; the other 11 were bled and cultures made after periods which varied from 1 to 13 months. The results were completely negative.

A volunteer was exposed to the bites of 28 sandflies from 8 lots, 6–15 days after the culture-blood meal:

Phlebotomus sanguinarius: 2 lots; at 6 days, each of 4 females probed and produced wheals, but without visibile signs of having ingested blood; 3 of these females had heavy cardial infections at 6 days, one was negative at 10 days.

Phlebotomus gomezi: 6 lots; at 9 days, one female from one lot probed and produced wheals; one from another lot refused to feed at 9 and 10 days; of 22 sandflies from the remaining 4 lots, 12 fed to engorgement at 7–9 days. Of the 12 which fed, 4 were given additional opportunities at 10–15 days and one probed at 12 days. Ten of the 12 were dissected at 9–16 days and all were infected, 8 of them heavily so (including 5 from the wild-caught lot previously mentioned).

No lesion or other sign of infection developed at any of the carefully charted feeding sites on the volunteer's arm or elsewhere.

Animal inoculations. Sandflies which were found dead or moribund and which were not dissected immediately were stored in moist plaster-lined vials in the refrigerator. The tissues seemed to deteriorate little during such storage. Motility of flagellates was noted after 2–3 days on several occasions and once after 7 days. In the attempt to determine the infectivity of the flagellates in the sandfly gut, pools of dead sandflies, at times including those which had been dissected, were triturated and inoculated into animals:

(1) *Spiny rat*, Proechimys. A total of 16 spiny rats were inoculated with 172 sandflies (26 dissected, 22 positive) of 4 species from 28 lots (*sanguinarius*, 104; *gomezi*, 46; *panamensis*, 16; *trapidoi*, 6). The majority, 104, died or was dissected after 4–6 days, 48 at 2–3 days, 16 at 7–9 days, and 3 from 11 to 13 days. Six of the spiny rats were born in the laboratory; the others, wild-caught, had previously given negative heart-blood cultures. Inoculations were made intradermally at the side of the nose, and in two cases also intraperitoneally. One spiny rat died without being bled; cultures from the others were all negative.

(2) *Suckling white mice*. The inoculation of suckling white mice with cultures of Panamanian human strains had been undertaken at the suggestion of Dr. S. Adler. It was found that intradermal inoculation in the first few days of life produced infection of connective tissue cells with typical L-D bodies at the site of inoculation. The infection could be demonstrated usually within 3 days, but in all cases the parasites disappeared at about the time of weaning. Baby mice were inoculated with individually dissected sandflies from three lots, as follows: (a) *Phlebotomus gomezi*: One female, 14 days, heavy infection of cardia and pharynx; dissected specimen triturated and part inoculated into back of 4-day-old mouse; sacrificed after 13 days; negative. (b) *Phlebotomus sanguinarius*: One female, 16 days, heavy infection of cardia and pharynx; inoculated baby mouse negative when sacrificed after 3 days. A second female, 5 days, moderate cardial infection, inoculated into 4-day-old mouse, which was sacrificed after 7 days; stained smears positive for *Leishmania* (the only demonstrable infection produced in any experimental animal with artificially fed sandflies).

(3) *Hamsters*. Golden hamsters were inoculated intradermally on the tip of the nose with 7 dissected sandflies from 2 lots of *gomezi*. One hamster received part of the same specimen with heavy infection of cardia and pharynx which was inoculated into a suckling mouse. Stained smears were negative on the 50th day and at the death of the hamster on

the 89th day. A second hamster received four separate inoculations with a total of 6 freshly dissected specimens, all with heavy infections of cardia and hindgut; one at 5 days, 3 at 6 days, and one each at 7 and 9 days. Smears were negative on the 29th and 36th days and at death on the 44th day.

Leishmania enriettii. A single lot, consisting of 5 *sanguinarius* and 4 *gomezi* females, was artificially fed with *L. enriettii*. All the *sanguinarius* when dissected (at 5, 8, 11, 13, and 14 days) were negative. Of the 4 *gomezi*, one dead at 2 days was negative, one at 3 days was decomposed, while the remaining 2 at 11 and 12 days had heavy infections of the cardia at the proventricular valve (Figs. 10 and 11), with a few flagellates also in the stomach of one; but the hindgut, esophagus, and pharynx of both were negative. No further experimental work was done with *L. enriettii*.

Discussion

The five species of man-biting *Phlebotomus* which were fed cultures of *L. braziliensis* all developed infections. Since the infection rate was high and the growth pattern of the flagellates was similar in all, there is as yet no basis for singling out any particular species as probable vector. Their ready infectibility, however, combined with the characteristic anterior-station growth, supports the view that sandflies are responsible for transmitting the disease. The frequent infection of the hindgut is a departure from the "classic" growth pattern, but, as set forth in the following paper (Johnson *et al.*, 1963), a similar hindgut infection is an invariable feature of the leptomonad infections of wild-caught Panamanian sandflies.

In the long history of the leishmaniasis problem it has been repeatedly demonstrated that experimental transmission requires more than infected sandflies plus susceptible host. Even in those few cases where valid experimental transmissions by biting have been achieved, the special factors—raisins, salt, or the use of wild-caught sandflies—which seemingly made critical contributions to their success, remain unexplained. The failure to transmit the infection to either spiny rats or the human volunteer repeats for American cutaneous leishmaniasis the early history of comparable failure with Old World kala azar and oriental sore. It may be remarked that all our sandflies throughout adult life had access to boiled raisins and that five of the heavily infected sandflies which bit the volunteer were wild-caught. The special factors which permit or facilitate transmission of *L. braziliensis* are still to be discovered.

Appendix

In the publication of the original article describing this technique (A. T. Hertig and M. Hertig. 1927. *Science* **65**, 328-329), both the plate of drawings and the text references to figures were deleted by the editor. This was at a time when round-trip correspondence between Peking and the United States often took 2 or 3 months, and we knew nothing of this deletion until the arrival of the publication itself. The original text with the plate was then printed by Peking Union Medical College (P.U.M.C.) and circulated privately.

The late Dr. Charles W. Young, who was in charge of the "Kala Azar Field Studies" unit at P.U.M.C., on his return journey to the U. S. attended the 7th Congress, Far Eastern Association of Tropical Medicine, in Calcutta in the fall of 1927. Our chief technician, Mr. Yang Kuo-Hsiang, accompanied Dr. Young and demonstrated this technique at the Congress. Napier (1930) not knowing of the privately reprinted article, redescribed the technique with illustrations.

The original plate is here reproduced. The figures showing the modification of the apparatus for feeding mosquitoes are included for whatever historical or other value they may have. The mosquito apparatus was developed to the workable stage but was actually used little by us. Mosquitoes in general can be fed by simpler methods, but the technique

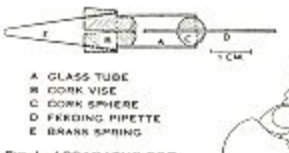


FIG. 1. APPARATUS FOR FEEDING SANDFLIES

FIG. 2. TIP OF BRASS SPRING

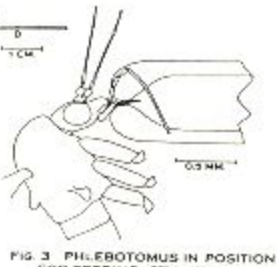


FIG. 3. PHLEBOTOMUS IN POSITION FOR FEEDING; STYLETS WITHIN PIPETTE, LABIUM FOLDED BACK

FIG. 4. APPARATUS FOR FEEDING MOSQUITOES

FIG. 5. MOSQUITO PROBOSCIS WITHIN OUTER PIPETTE

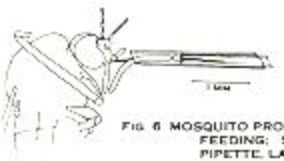


FIG. 6. MOSQUITO PROBOSCIS IN POSITION FOR FEEDING; STYLETS WITHIN INNER PIPETTE, LABIUM FOLDED BACK ON ITSELF

Plate prepared as part of original description of apparatus for feeding sandflies (Hertig and Hertig, 1927) but omitted from publication.

might find application, for example, in the study of substances expelled from the proboscis, not only of mosquitoes but of other insects, before or during feeding.

M.H.

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