The Leishmania-Leptomonad Transformation of *Leishmania donovani*: Nutritional Requirements, Respiration Changes and Antigenic Changes

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The Leishmania-Leptomonad Transformation of *Leishmania donovani*: Nutritional Requirements, Respiration Changes and Antigenic Changes*

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SYNOPSIS. The leishmania-leptomonad transformation of *Leishmania donovani* occurs in 20-40 hr *in vitro* at 27 C in the absence of cell division. The rate and extent of the process depend on the available nutrients. A source of amino acids and glucose (or sucrose) are required. Several basic or acidic amino acids can substitute for all amino acids present in the complete medium. Leishmanial forms freshly isolated from hamster spleen have a QO_2 (μM O_2/min/10^7 cells) of 0.29 $\pm$ 0.01 at 27 C in phosphate buffered saline with glucose. This respiration is sensitive to KCN, antimycin A, and Na amytal, and hence is probably cytochrome dependent. The QO_2 remains constant for 5-6 hr and then increases 5-7 fold by 20-30 hr of incubation at 27 C in the proper medium. The transformation is inhibited in terms of morphogenesis and increase in respiration by Actinomycin D, puromycin and mitomycin C. Appearance of new soluble antigens during the transformation was found by gel diffusion and immunoelectrophoresis.

THE life cycles of the hemoflagellates, especially the African pathogenic trypanosomes, provide interesting examples of cellular differentiations which involve the biogenesis of new mitochondrial material. The hemoflagellates contain a highly specialized mitochondrion, which seems to be especially adapted for the complex parasitic life cycles (15). There is good evidence that in all the hemoflagellates there is only one large, extended, highly convoluted mitochondrion per cell(1,22,25,26). The many electron micrographs of separate mitochondria are probably sections thru the highly ramified mitochondrial tubules. This mitochondrion of the hemoflagellates is unique also because it contains a large amount of DNA concentrated in a fibrous structure situated in the anterior portion of the organelle (2,4,28). The DNA is enclosed by a characteristic disc-shaped structure, which is always located at the base of the flagellum and has mitochondrial tubules extending from its lateral ends(1,5,18,25). This structure is the historical "kinetoplast"—a darkly stained granule at the base of the flagellum, the staining properties of which are due mostly, if not entirely, to the presence of DNA(6,27). I shall use the term, kinetoplast, to refer to the disc-shaped portion of the chondriome even tho it is not a completely separate organelle.

Since the morphogenetic changes that occur in the life cycles of hemoflagellates of the genera *Leishmania* and *Trypanosoma* involve changes in respiratory metabolism, it is not surprising that the kinetoplast plays a major role(19, 30). The most extreme examples of metabolic and morphogenetic changes in the entire family are found in trypanosomes of the "brucei subgroup" of Hoare(11). A varying proportion of these cells during life in the mammalian bloodstream completely lack mitochondrial cytochromes and cris-

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tae and respire by an extra-mitochondrial, flavoprotein oxidase system(9,20). A respiratory switchover to cells with normal mitochondrial cristae and a functional NADH diaphorase(31) occurs in a variable percentage of these blood stream trypanosomes, producing a so-called "polymorphic" population. Only these cells, presumably, are then "competent" to transform into the insect vector or culture form upon infection of an insect or inoculation of a suitable culture(17,31). This morphogenesis involves, among other things, a further increase in the mitochondrial mass of the cell by means of an outgrowth of tubules from the kinetoplast.

Most other trypanosomes do not seem to go thru a stage involving the complete loss of mitochondrial function, but rather undergo a partial decrease in mitochondrial respiratory metabolism. They also have some mitochondrial biogenesis during morphogenesis from the vertebrate to the insect host. There is structural evidence that cells of the genus *Leishmania* undergo a period of mitochondrial biogenesis(19). *L. donovani* lives in the mammalian host as an intracellular parasite of cells of the reticulo-endothelial system. The intracellular forms are small (2-3 μ in diameter), ellipsoid cells (hereafter designated LDs) containing a typical kinetoplast with a very few mitochondrial profiles thruout the cell. Altho it has not been investigated, it is assumed that these profiles are sections thru the single convoluted chondriome. Upon inoculation of a culture at approximately 27 C, the LDs transform within 20-40 hr into the larger (10-15 μ long), flagellated "leptomonads" or culture forms. These forms can no longer survive exposure to 37 C and seem to have simpler nutritional requirements. An apparent biogenesis of mitochondrial material from the kinetoplast was observed to occur very early in the morphogenetic process(19). Also, the leptomonad form had a greater density of mitochondrial profiles containing cristae than LD form.

I have investigated several aspects of this morphogenetic process in *L. donovani* in an effort to quantitate the system in preparation for a study of the process of mitochondrial biogenesis itself.

MATERIALS AND METHODS

Materials

Amino acids—MA Grade, Mann Research Laboratories
Actinomycin D—Merck, Sharp & Dohme
Puromycin-di-HCl—Nutritional Biochemicals Corporation
Mitomycin C—Nutritional Biochemicals Corporation
C^{14}-uridine—New England Nuclear Corporation (30 mc/mM)
H^3-thymidine—New England Nuclear Corporation (6.7 c/mM)

Media

Medium C(29) was prepared without hemin and sterilized by Millipore (HA) filtration. Hemin, dissolved in 0.05 N NaOH and stored frozen, was added just before use.

SBG (saline-buffer-glucose) = 0.15 M NaCl-0.02 PO_4 Buffer (pH 7.9)-0.02 M glucose.

Isolation of LDs

The Sudan strain of *L. donovani* was used for most experiments. A few experiments using the Khartoum strain showed no differences. The infections were maintained in hamsters and passed by intraperitoneal inoculation of infected spleen suspension.

The hamster was decapitated, and the spleen aseptically removed and ground in 0.25 M sucrose-0.02 M Tris-HCl (pH 7.9) or in SBG, in a Ten-Broeck hand homogenizer. The LDs were isolated by differential centrifugation at 0-4 C: The homogenate (10 ml) was centrifuged at 1000 rpm for 10 min in an International PR-2 centrifuge (rotor 269) and the pellet discarded. The supernatant was centrifuged at 3000 rpm for 20 min and the resulting pellet resuspended in 10 ml fresh medium. This cycle was repeated 3 times.

Further purification of the LDs for some experiments was achieved by a 30 min treatment with 0.25% Bactotrypsin (Difco) at 37 C in SBG. The cells were then layered on a 5-27% linear bovine serum albumin (Pentex, Inc.) gradient-2 mM EDTA, and centrifuged in the Spinco SW 39 rotor for 60 min at 20,000 rpm. A whitish band at about 19% albumin consisted mainly of LDs, as seen in Giemsa-staned smears. Such LDs were still capable of transformation.

LS transformation

These experiments were usually done without antibiotics, but occasionally 100 units penicillin/ml and 100 μg streptomycin/ml were used.

The LDs tended to clump during the process; in order to count them in a hemocytometer, it was necessary to dilute 1:1 with 3.7% formalin in 0.15 M NaCl-0.015 M Na citrate (pH 7) and sonicate for 10-30 seconds in a Raytheon 9 KC sonicator.

Oxygen uptake

This was measured with a Clark oxygen electrode (Yellow Springs Instrument Company) in a closed glass chamber with a volume of 2 ml, as described by Kielly & Bronk(13).

Sucrose-gradient centrifugation of normal spleen

The method of Bowers & DeDuve(3) was followed. The band at a density of 1.17 g/ml gave the highest value of succinate-stimulated respiration and was used for the control experiments.

Immunochemistry

"Ago-immunodiffusion plates"—(type B, Mann Research Labs) were used for gel diffusion tests(16).

Immunoelectrophoresis was carried out in 1% Noble agar in barbital buffer of ionic strength 0.05 with Ca lactate (0.038%) and merthiolate (0.0001%), on microscope slides. Electrophoresis was carried out at 48V/slide for 90 min at 4 C. Antiserum was added and the slide let develop at 27 C for 1 week(8).

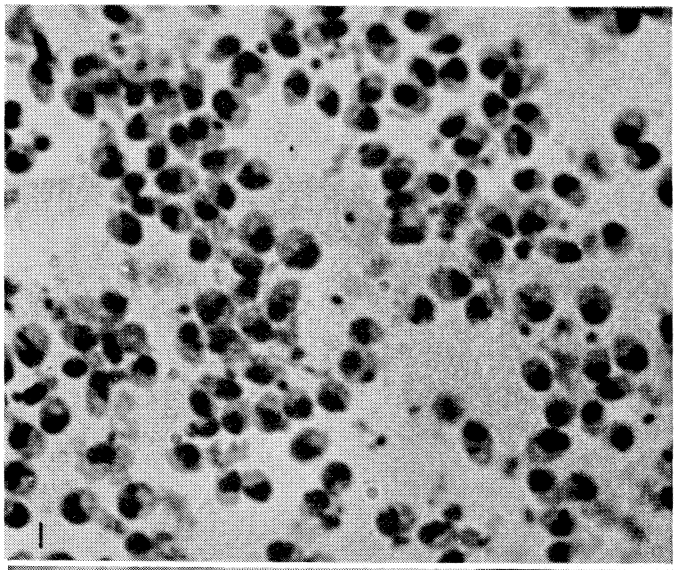
RESULTS

Nutritional requirements of the transformation

The LDs isolated by differential centrifugation (Fig. 1) were incubated at 27 C in the proper culture medium; within 20-40 hr a varying percentage of the cells transformed into the leptomonad or culture form (Fig. 2). The rate and extent of the process depended greatly on the composition of the culture medium. The transformation was more synchronous and occurred more rapidly in a rich medium such as blood agar with Locke's overlay. The cells would also transform in less rich media, in which the culture forms normally would not grow, but the process became less synchronous and took a longer time. However, this ability to transform in simple media permitted a study of the nutritional requirements of the process.

A valid measurement of the transformation was obtained by measuring relative cell size in a Giemsa-stained smear by an eye-piece grid. The length of the cell was measured, not including the flagellum. Time zero (T_0) cells gave a very narrow range, with less than a few percent of the cells larger than 2 grid units (not calibrated in microns). Therefore, any cell larger than 2 grid units was scored as trans-

formed. This was a valid criterion because it was observed that any cell that enlarged usually went on eventually to gain the leptomonad form. Direct cell counts revealed that cell division did not occur until most of the enlarged cells had transformed to the leptomonad form. Once a cell assumed the leptomonad form, it usually began to divide within a short time.



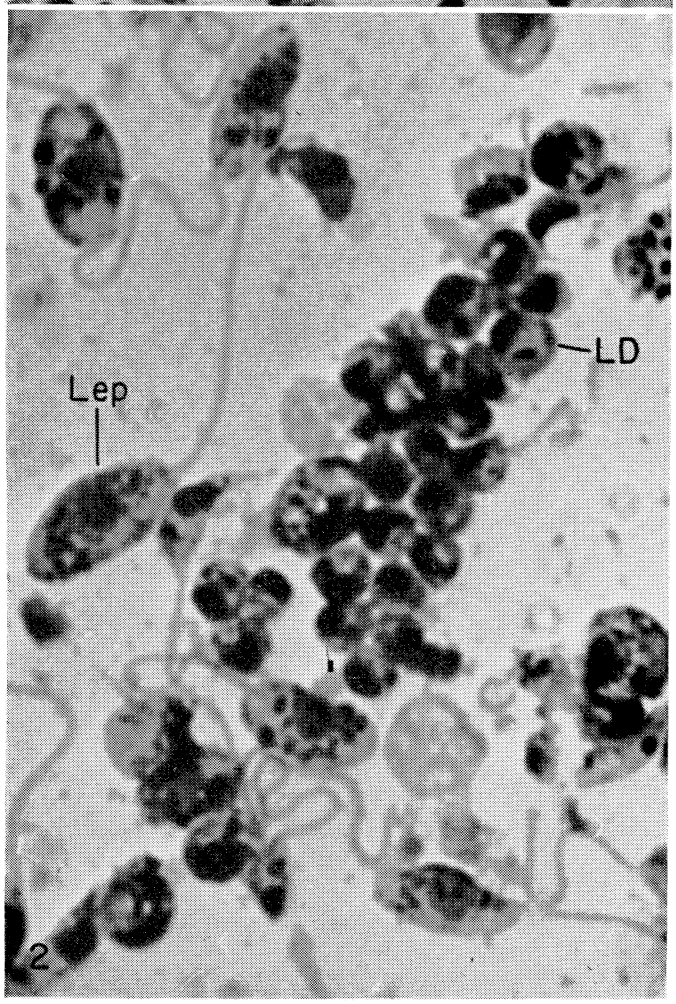


Fig. 1. A freshly isolated preparation of *L. donovani* LDs. Isolated by differential centrifugation in sucrose-Tris. Giemsa-stain, × 1,000.

Fig. 2. Appearance of *L. donovani* cells after 25 hr of transformation in Medium C at 27 C. Untransformed, clumped LDs (LD) and transformed leptomonads (Lep) are seen. Giemsa stain, × 1,250.

The absolute extent of the transformation in any one medium varied somewhat from preparation to preparation (30-60%) but the relative values for cells from any one preparation incubated in different media remained approximately the same. Table 1 shows the variation in percentage of transformed cells after 22 hr incubation in various media at 27 C. Approximately identical percentages were obtained in blood agar, Medium C, SBG + 17 amino acids + hemin, or in simply SBG + 17 amino acids.

TABLE 1. *Effect of incubation media on LD-leptomonad transformation of* L. donovani.

Medium	% transformation of control
Blood agar (control)	100%
Medium C	99
SBG + 17 amino acids + hemin	91
SBG + 17 amino acids	83
SBG + hemin	14
SBG	17

Percent transformation was obtained from increase in cell size. Transformation in the control was taken as 100%.

Very little transformation was obtained upon removal of the amino acids. The cells that failed to develop after 20 hr in SBG at 27 C could still transform if returned to an amino acid-containing medium but the absolute percentage that did so after 20-30 hr incubation had decreased.

Table 2 shows the effect of substituting various amino acid groups for the complete mixture. The cells were preincubated for 10 hr at 27 C in the deficient medium (SBG) to lessen the carry-over of endogenous amino acids, and then incubated for 20 hr in the various media. Either the basic or acidic amino acids supported approximately 70%

TABLE 2. *Effect of amino acid groups on morphogenesis of* L. donovani.

	Medium	% transformation of control	AA moles/l
1.	SBG + 17 amino acids	control	.06
2.	SBG + glycine alanine valine leucine proline (isoleucine)*	10	.03
3.	SBG + serine threonine methionine tryptophan tyrosine	0	.01
4.	SBG + aspartic acid glutamic acid	75	.01
5.	SBG + lysine arginine histidine	70	.01

Percent transformation was obtained as described in Table 1.

* Not used in this experiment, but addition of it does not allow morphogenesis to occur.

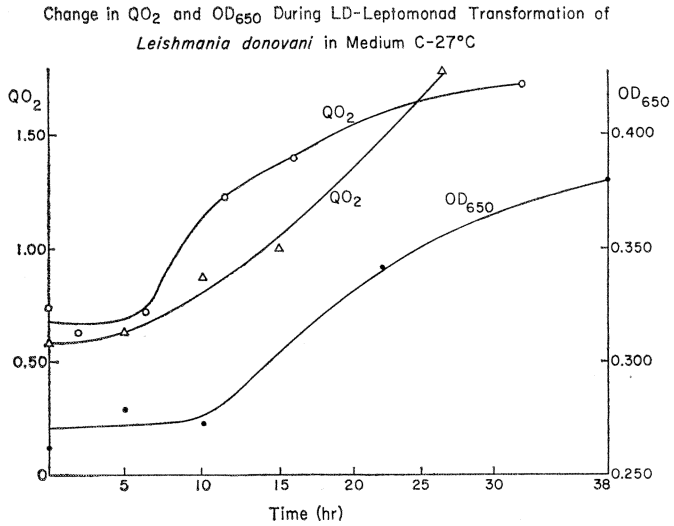


Fig. 3. Kinetics of increase in QO_2 (μM O_2/min/10^7 cells) for 2 preparations of transforming LDs in Medium C at 27 C. The change in OD_{650} of the suspension (after formalin fixation and sonication to eliminate clumps of cells) is also given.

of the control transformation. Very little morphogenesis occurred with the first 2 groups of amino acids.

However, when the first 2 groups of amino acids were supplied at 6× the concentration in the above experiment, some morphogenesis occurred with group #2.

It should be stressed that it is difficult if not impossible to determine absolute nutritional requirements in this system, due to the variable amount of splenic contamination in the final LD preparation. For example, a requirement for hemin could not be found, probably because of the presence of red blood cells.

Also it was observed that recently transformed leptomonads of *L. donovani* could multiply in a simple transformation medium such as Medium C, altho they normally require a blood-based medium. However, subcultures of such cells in fresh Medium C were negative, indicating that the transformation medium contained additional nutrients, contributed either by autolysis of splenic contamination or by the transforming cells themselves.

Table 3 shows the effect of substituting single amino acids at their original concentrations for the basic and acidic groups. No significant stimulation of morphogenesis was observed for any one amino acid. An inhibitory effect of glucose deprivation is also shown in this table. Sucrose could substitute for glucose in this process. The effect was most pronounced when the parasites were isolated in SBG rather than in a sucrose medium.

TABLE 3. *Effect of single amino acids on the LD-leptomonad transformation of* L. donovani.

Medium	% transformation of control
SBG + 17 amino acids (control)	100%
SBG	4
SBG + glutamic acid	12
SBG + aspartic acid	15
SBG + lysine	10
SBG + arginine	10
SBG + histidine	19
17 amino acids + saline-buffer	50

Percent transformation was measured as in Table 1.

Change in rate of respiration during the transformation

Freshly isolated LDs showed a definite rate of oxygen consumption. The QO_2 (μM O_2/min/10^7 cells) in SBG at 27 C was 0.29 ± 0.01 (9 preparations) and the QO_2 in Medium C was 0.42 ± 0.13 (11 preparations). That this oxygen uptake was not a result of contamination by mitochondria from host spleen was indicated by the following observations:

1. Uninfected spleen run thru the same centrifugation procedure in 0.25 M sucrose-0.02 M Tris (pH 7.9) gave very little respiring material; the oxygen uptake of this material decreased 73% after treatment with 0.25% Bactotrypsin at 37 C for 30 min.

2. The respiration of spleen mitochondria isolated by sucrose-gradient centrifugation(3) of uninfected spleen, decreased 50% after 5 hr incubation in Medium C at 27 C, and decreased 83% after treatment with 0.25% Bactotrypsin for 30 min at 37 C.

3. The "LD" respiration was unaffected by such treatments—except when the preparation had much visible contamination—in which case the per cell rate always decreased to the same value.

Exponential log phase culture forms of *L. donovani* had a QO_2 of 2.2, implying a 5-7-fold increase over the QO_2 of LDs at T_0. The actual kinetics of the increase in QO_2 for several preparations are shown in Figs. 3 and 4 for several LD preparations. The rate remains constant for 5-6 hr and then shows a 2-4-fold increase by 30 hr. Assuming that only morphologically transformed or enlarged cells have an increased QO_2, the calculated increase by the transformed cells is again 5-7-fold.

The respiration at T_0 was sensitive to Na amytal, Anti-

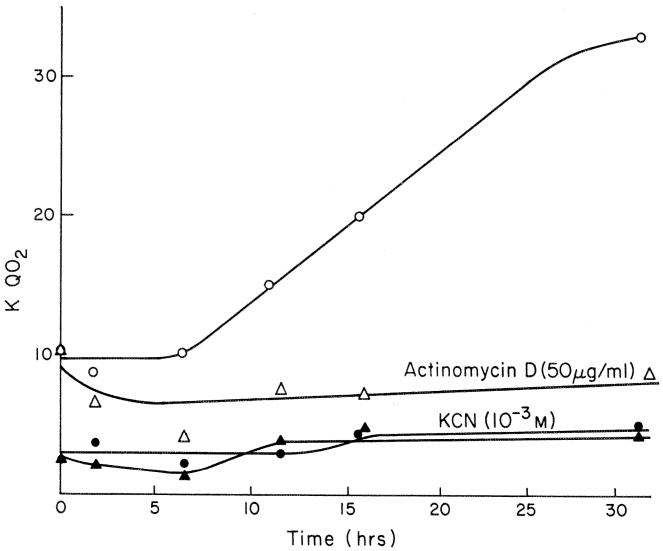


Fig. 4. Increase in QO_2 of transforming LDs and effect of 50 μg Actinomycin D/ml. The cells were incubated at 27 C in Medium C. The darkened dots refer to readings taken immediately after the addition of 10^{-3} M KCN.

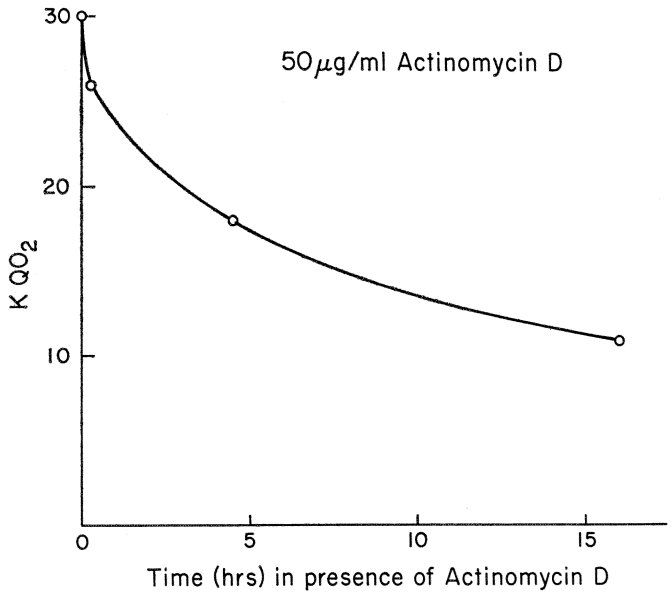


Fig. 5. Effect of Actinomycin D (50 μg/ml) on respiration of recently transformed leptomonads of *L. donovani*.

mycin A and potassium cyanide at about the same levels as the respiration of the culture forms, indicating that the increase in respiratory enzymes probably involved a quantitative increase in all the components rather than a qualitative change in the pattern of cytochromes (Fig. 4).

Also shown in Fig. 3 is the increase in OD_{650} of the suspension during the transformation. The cells were fixed with formalin-saline-citrate and sonicated to eliminate clumping. This increase in optical density reflects the increase in cell mass that occurs without cell division, and is another useful parameter with which to monitor the morphogenetic process.

Effect of inhibitors on the transformation

The transformation was inhibited in terms of increase in size and QO_2 by the following substances: Actinomycin D (50 μg/ml) (Fig. 4), Puromycin (50 μg/ml), mitomycin C (50 μg/ml) and chloramphenicol (2 mg/ml). It was unaffected by acriflavin at 0.3 μg/ml and chloramphenicol at 50 μg/ml (Table 4).

Actinomycin D (50 μg/ml) added at 5, 10 and 15 hr produced the same percentage inhibition of the QO_2 at 25 hr as when it was added to T_0. A direct effect of Actinomycin D on the respiration of the 25 hr forms was also observed. As shown in Fig. 5, the rate of respiration was decreased 50% in 16 hr by Actinomycin D (50 μg/ml). A direct effect of Actinomycin D on respiration has also been found in ascites cells(12) and human leukemic leukocytes(14).

Cells incubated for 20 hr in SBG, and then transferred to Medium C for 28 hr increased their rate of respiration 3.1-fold. Addition of Actinomycin D at 50 μg/ml to the Medium C prior to the final 28 hr incubation completely inhibited the increase in QO_2.

Actinomycin D may inhibit the transformation by virtue of its direct inhibition of respiration rather than as a result of an inhibition of RNA synthesis. However, an effect of Actinomycin D on RNA synthesis was also found. The addition of 50 μg/ml Actinomycin D to T_0 caused a 70% inhibition in the continuous uptake of C^{14}-uridine into TCA-insoluble material by 24 hr, but had no effect on the simultaneous uptake of H^3-thymidine. That these precursors were being incorporated into RNA and DNA respectively was shown by light microscope autoradiography with the appropriate enzyme digestions.

TABLE 4. *Effect of various inhibitors on transformation of* L. donovani *in terms of increase in size and increase in* QO_2.

Substance	Conc.	% inhibition of increase in Size	% inhibition of increase in QO_2	Time (hr)
Actinomycin D	50 μg/ml	80	100	25
Puromycin	50 μg/ml	—	100	23
Mitomycin C	50 μg/ml	—	100	25
Chloramphenicol	2 mg/ml	—	100	30
	50 μg/ml	0	—	25
Acriflavin	0.3 μg/ml	0	0	23

The cells were isolated by differential centrifugation in 0.25 M sucrose–.02 M Tris-HCl (pH 7.9) at 0-4 C, and then incubated at 27 C in Medium C, with or without the inhibitors, for the times noted.

Antigenic changes during the transformation‡

An antiserum was prepared against formalinized *L. donovani* leptomonads, with the use of Freunds' complete adjuvant. A rabbit was injected subcutaneously with 2 ml of a mixture of 1 part of complete Freund's adjuvant and 1 part of a 10% suspension of *L. donovani* leptomonads in 0.2% formalinized saline. The injection was repeated in 2 weeks. A booster of leptomonad homogenate was given after one month. The antiserum was tested against total cell extracts prepared from LDs and leptomonads by freeze-thawing 3 × a 50% suspension in SBG with alcohol-dry ice, and then sonicating the suspension for 30 min in a Raytheon 9 KC sonicator. The final suspension was centrifuged to remove cellular debris (6,300 *g* for 20 min) and the supernatant solutions were used in the gel diffusion tests, as shown in Figs. 6A and 6B. The anti-leptomonad antiserum gave more bands with the leptomonad extract than with the LD extract. Furthermore, all the LD bands appeared to be continuous with leptomonad bands. There appear to be at least 4 common antigens between *L. donovani* LDs and leptomonads and at least 3 leptomonad antigens not in common with any LD antigens.

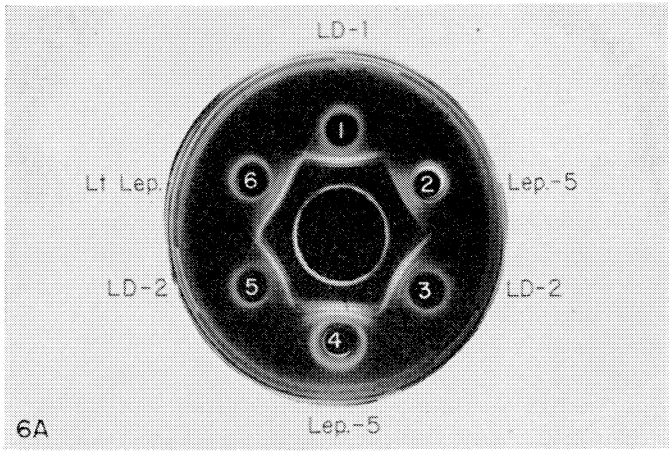
Figs. 6A and 6B also present a cross-species comparison of *L. donovani* LDs and leptomonads with *L. tarentolae* leptomonads. In the gel diffusion test in Fig. 6A one can see 2 bands in common with *L. tarentolae*-leptomonads and *L. donovani*-LDs and one band unique to each. In Fig. 6B one can see 3 bands common to *L. tarentolae* and *L. donovani*-leptomonads, 3 bands unique to *L. donovani*-leptomonads, and only one band unique to *L. tarentolae*. It is interesting that the anti-*L. donovani* leptomonad antiserum can pick up an antigenic component of *L. tarentolae* not found in *L. donovani*. This may be a breakdown product, or it may be an antibody against a component of Freund's

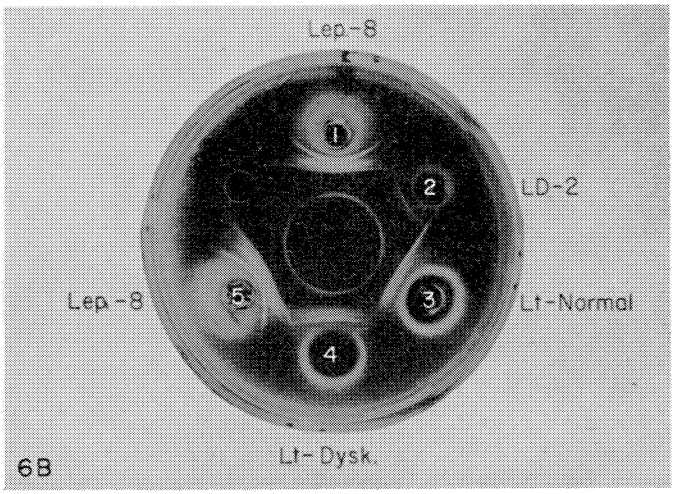
‡ This work was done with the collaboration of Dr. P. D'Alesandro.

complete adjuvant which is found only in *L. tarentolae*.

Immunoelectrophoresis of LD and leptomonad antigens revealed a clear absence of at least 2 bands in the LD extract (Fig. 7).

It is not known where in the cell these new soluble antigens originate, since no cell fractionation studies were performed. It would also be of interest to follow the kinetics of appearance of these antigens during the transformation process.





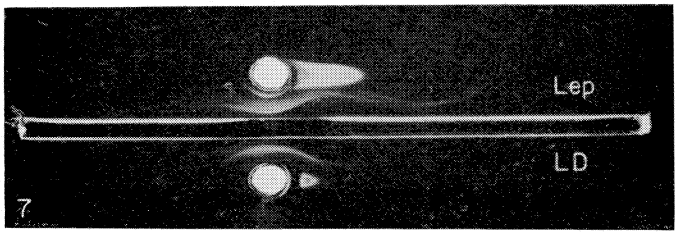
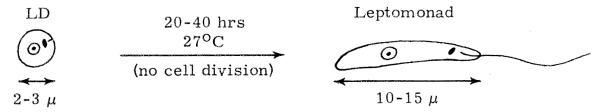


Fig. 6A. An Ouchterlony(16) immunodiffusion comparison of *L. donovani*-LD and -leptomonad antigens and *L. tarentolae*-leptomonad antigens. Well #4 was refilled several times with *L. donovani* leptomonad extract, whereas well #2 was filled one time only. The center well contained anti-*L. donovani*-leptomonad antiserum. Development time, 1 week.

Fig. 6B. Wells #1 and #5 contained *L. donovani*-leptomonad extract; well #2 contained *L. donovani*-LD extract; wells #3 and #4 contained normal and 70% dyskinetoplastic *L. tarentolae*-leptomonad extracts respectively. Center well contained anti-*L. donovani*-leptomonad antiserum. Development time, 1 week.

Fig. 7. Immunoelectrophoresis comparison of *L. donovani* leptomonad and LD antigens, using anti-leptomonad antiserum.

1. Cell size and shape



2. Increase in optical density of suspension.
3. Two nucleoli fuse into one (Rudzinska et al, 1964)
4. Loss of outer cell membrane (Rudzinska et al, 1964)
5. Increase in mitochondrial profiles per cell (Rudzinska et al, 1964)
6. Increase in O_2 uptake per cell (~7x)
7. Absolute nutritional requirements for energy source (glucose, sucrose) and several specific amino acids
8. Antigenic changes:
   i. Differences in agglutination reaction (D'Alesandro, 1954)
   ii. Appearance of new soluble antigens.
9. Inhibited by actinomycin D (50μg/ml), puromycin (50μg/ml), chloramphenicol (2mg/ml), mitomycin C (50μg/ml)

Fig. 8. Summary of changes occurring during the LD-leptomonad transformation of *L. donovani*.

DISCUSSION

The finding that the transformation of intracellular LDs of *L. donovani* to leptomonads involved a 5-7-fold increase in the QO_2, together with the previous finding by Rudzinska et al.(19) of an increase in the number of mitochondrial profiles, provide evidence for an increase in mitochondrial mass. The results coincide in a temporal sense, for no increase in QO_2 is seen until the observed proliferation of mitochondrial membranes, which occurs after approximately 5-6 hr incubation at 27 C. The decrease in temperature is probably the main factor that stimulates this mitochondrial biogenesis.

Since the morphogenesis involves many other biosynthetic reactions in addition to mitochondrial biogenesis, such as growth of the flagellum and increase in cell size, it is not surprising that amino acids and an energy source are necessary for the process to occur. It is surprising, however, that the acidic or basic amino acids in low concentrations can substitute for the complete mixture.

It is not known why this is so but it is suggested that their relationship to Krebs cycle intermediates may be somehow relevant.

It is remarkable that LDs transform in simple media (SBG + amino acids or Medium C), in which neither the LDs nor the culture forms can long survive. However, it should be stressed that many nutrients might be supplied in small amounts by autolysis of the contaminating splenic material in the LD preparation. For example, a clearcut requirement for hemin was not found possibly because of the few red blood cells carried along in the LD preparation. Furthermore, the addition of further nutrients, such as those in Medium C or blood agar, increased the rate of the process and made it more synchronous, altho this was not studied quantitatively.

The fact that the absolute extent of the process varied from preparation to preparation may indicate a variable amount of damage to the LDs during the 2 hr isolation procedure, or it may indicate a heterogeneity among the intracellular LD population. In analogy to the polymorphic

bloodstream trypanosomes, perhaps only a certain percentage of the LDs are competent to transform, and this percentage varies with the cell cycle and the immunologic responses of the host. The various subgroups and genera would then differ mainly in the relative percentage of competent cells in the mammalian host, that is, in the relative percentage of cells which had a functioning mitochondrial-type respiration versus those which had a non-mitochondrial, non-cytochrome respiration.

Three facts do not agree with this hypothesis. The 1st is that freshly isolated LDs from many different preparations have a definite, quantitatively repeatable, cytochrome-type respiration. No indication of a large amount of cyanide-insensitive respiration was noted. Second, the ability to transform into culture forms is not affected by repeated syringe passage in the mammalian host. Third, recent work by Herman(10) indicates that in acriflavin-treated tissue culture cells, a certain percentage of the LD-population becomes dyskinetoplastic and the growth rate of LDs in such cultures is diminished. This problem can be resolved only by further work, such as histochemical staining for mitochondrial enzymes in the intracellular LD population.

The fact that the transformation was inhibited by Actinomycin D, puromycin, and mitomycin C indicates that the process involves RNA and protein synthesis. The synthesis of RNA was verified by direct uptake experiments; it was found that simultaneous DNA synthesis was also occurring, in preparation for the 1st division which takes place directly after the cell assumes the leptomonad form. In terms of rate of respiration, incubation of the cells in the above inhibitors for 20-30 hr not only prevented any increase but brought about a decrease below the initial value.

The antigenic changes occurring during the transformation provide another "handle" to study the process. The appearance of new soluble antigens during the process is not unexpected, but was never clearly found previously, altho there are several reports of gel diffusion tests with various hemoflagellates(7,21,23,24). It would be more meaningful to follow the soluble antigen changes at the organelle level by means of cell fractionation, but this has not been done.

In summary (Fig. 8) the LD-leptomonad transformation of *L. donovani* is a process that can be studied *in vitro* in a simple defined medium and be manipulated by several antibiotics, and that involves (among many other reactions, of course) a biogenesis of mitochondrial material.

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