Leishmania donovani is the parasite responsible for the causation of kala-azar, a visceral disease, and post-kala-azar dermal leishmaniasis or dermal leishmanoid of Brahmachari, a sequel that develops in about 5—10 per cent of cases cured of kala-azar. Leishmania form of the parasite or the Leishman-Donovan body is found in the infected individual and the leptomonal form in culture or in the sand-fly Phlebotomus argentipes Ann. & Brun., its insect vector. Both the leishmania and the leptomonal forms were subjected to cytochemical study but the minute size of the former prevented certain methods being applied and results studied accurately.

Morphology of the leishmania and the leptomonal forms is briefly described below.

Leishmania form.

The Leishman-Donovan body is an intracellular parasite growing in the cells of reticulo-endothelial system. In dry smears stained by Leishman, Giemsa or similar Romanowsky stains, it appears as a rounded, oval or torpedo shaped body which is often found extracellularly due to rupture of the host cell during the...
process of making the smear. It measures 2 to 5 microns in length and about 1 to 3 microns in breadth in the oval form. In this form the parasite is biconvex in outline with one side more convex than the other. The nucleus which is oval or rounded measures a little over half the diameter of the parasite. It lies apposed to the more convex border. Close to the less convex border and usually lying tangentially to the nucleus is a rod shaped structure, the kinetoplast, which shows similar staining reaction as the nucleus but is usually stained a darker shade. The kinetoplast consists of the para-basal body and the rhizoplast, the rudimentary flagellum. Between the nucleus and the kinetoplast and closely related to the latter a vacuole is often visible. The cytoplasm of the leishmania form stains a pale blue colour.

In tissue sections, the intracellular nature of the leishmania form of the parasite is quite obvious. Both in tissue sections and in wet-fixed smears, stained with iron-haematoxylin, the nucleus appears as a ring and the kinetoplast is either attached to its margin, is superimposed by or on it, or lies as a rod shaped structure close to it. The nucleus shows a more or less central karyosome in wet fixed smears, but this structure

Please see next page.
can hardly be made out in tissue sections. Unless correctly fixed and properly differentiated after iron haematoxylin stain, the ring like appearance of the nucleus cannot be made out and the nucleus appears as dot and the karyosome if it can be made out is seen as a small rod.

The nucleus appears as a more or less uniformly stained oval structure in average smears stained with Leishman stain. But in properly stained smears a central karyosome and the nuclear membrane can be distinguished.

**Leptomonad form.**

The free flagellate form or the leptomonad is usually cigar shaped, oval, pear shaped or long and slender. It measures from 10 to 20 microns depending on its shape and stage of development. In films stained with Leishman or Giemsa stain (after exposure to osmic acid vapour, fixation with methyl alcohol and washing to remove the acid) the cytoplasm stains pale blue and the nucleus which is more or less central is oval in shape and stains reddish purple. The kinetoplast lies near the anterior end of the body and the vacuole is seen in most specimens. The flagellum arises close to the kinetoplast, passes over the flagellar vacuole and extends freely forwards outside the body; it measures
about as long or slightly more than the length of the body. With ordinary microscope it appears as a single filamentous structure. With the electron microscope, flagellates that had been subjected to acid hydrolysis, show that the flagella are made up of nine fibrils held together by cementing substance (Das Gupta et al, 1954).

The cytoplasm of the leptomonad at times shows reddish purple granules, the volutin granules; these are more commonly seen in old cultures.

In wet fixed smears stained with iron-haematoxylin, the nucleus shows its vesicular character, with a central dot shaped or elongated karyosome. The nuclear membrane is said to be free from chromatin (Knowles, 1927), but with correct degree of differentiation, it is possible to demonstrate the presence of chromatin in the nuclear membrane. It may occur all around or dots of chromatin may appear in areas of the nuclear membrane or the membrane may be free from any chromatin dots. The kinetoplast is stained with iron haematoxylin.

In dry smears stained by the Romanowsky stains, the nucleus often appears as a dark homogenous mass, or as a series of dots. But in properly stained specimens, the nuclear membrane and the karyosome can be distinguished in the oval reddish purple stained nucleus.
The material studied for cytochemical investigation consisted of smears from spleen, bone marrow or lesions of dermal leishmanoid and histological sections of liver, bone marrow, spleen, lesions of dermal leishmanoid, for the leishmania form of *L. donovani*. The leptomonads were obtained by culture of spleen or bone marrow material obtained by puncture, in N.N.N. medium.

**Cytochemical Observations.**

The cytochemical pattern of *L. donovani* was first studied during 1949-52 and the results published in 1953 (Sen Gupta, Bhattacharyya and Ray, 1953). The mitochondria, the Golgi complex, nucleic acids, and the number of chromosomes were described in this paper. Subsequently localisation of alkaline and acid phosphatase (Sen Gupta and Ray, 1954) and the nuclear structure of the parasite were described (Sen Gupta and Ray, 1954a). More recently the presence of cytochrome oxidase and succinic dehydrogenase have been described in the mitochondria of the leptomonad form of *L. donovani* (Ray, Guha, Mukerjee and Sen Gupta, 1955). The results
of the above investigations have been included in the account given below.

Mitochondria. (Plate II, Figs. 1-3)

On supravital staining with equal parts of about 1:100,000 dilution of janus green B it was possible to stain the mitochondria which appeared as dots scattered irregularly in the cytoplasm. If the concentration of janus green was higher, the kinetoplast and at times the karyosome were stained with janus green, and the leptomonads died within a short time. The leishmania form could not be studied satisfactorily for mitochondria on account of their minute size, but in at least one occasion two granules of janus green B stained dots that gradually changed to pink colour could be seen.

The mitochondria of the leptomonad form were found to oxidise the Nadi reagent and they strongly reduced neotetrazolium into formazan bodies (Ray, et al. loc. cit.).

The Golgi complex. (Plate II, Fig. 4 & 5)

The Golgi complex could be visualised as small vacuoles in which minute grains of neutral red had been included when examined under the phase contrast microscope. The Golgi complex were distributed more in the portion of the flagellate behind the nucleus than in the
anterior part. The areas of the nucleus and the flagellar vacuole were free from neutral red.

**Feulgen's nuclear reaction.** (Plate I, 2 & 7; Plate II, 8-12; Plate III, 1-10, 19 & 20)

With the Feulgen reaction, the nucleus of the leishmania form was found to contain DNA in the nuclear membrane and the more or less central karyosome. The latter appeared as a small sphere or as oval or elongated structure in wet fixed smears. It could be visualised only in rare occasions in tissue sections. The kinetodot plast appeared as a Feulgen-positive rod or according to the aspect visualised.

In the leptomonad form the following types of distribution of DNA could be demonstrated in the nucleus:

(a) Along the nuclear membrane and in the globular karyosome which in some cases appeared to stain somewhat lighter in the centre.

(b) Similar distribution in the nuclear membrane but with an elongated karyosome which was either rod shaped, dumb-bell shaped, beaded or curved rod shaped.

(c) Grains of DNA on the nuclear membrane with rounded or rod shaped karyosome.
(d) Karyosome not seen; dots or small rods of DNA arranged: (i) at intervals all around the nuclear membrane, (ii) in two groups at either pole of the nucleus, (iii) mainly on one side of the nucleus, or (iv) all along the nuclear membrane.

(e) No DNA noted in the nuclear membrane; (i) chromosomes arranged in a metaphase pattern about the centre, (ii) thick rod shaped or (iii) globular karyosome showing DNA, or (iv) chromosomes irregularly arranged in a group of small rods in the middle of very thin elongated leptomonads.

The kinetoplast was invariably found to be Feulgen-positive.

Staining with methyl green and pyronin.

Methyl green stained the nucleus and the kinetoplast of the leishmania form in tissue sections. The former appeared as a ring. Further details could not be satisfactorily made out.

The nucleus of the leptomonad with the karyosome and the kinetoplast were stained with methyl green. Methyl green stained material was found in the nuclear membrane. Finer details of structure could not be determined as was possible with the Feulgen reaction.
Cytoplasm of the leptomonads was stained pink by pyronin.

The effect of ribonuclease digestion.

The cytoplasm of the leishmania form was somewhat less basophilic and the nucleus did not appear to show any marked change in dry fixed smears subjected to ribonuclease digestion. Similar results were obtained with smears of leptomonads. The results obtained were not convincing enough, and it could be concluded that the method described by Snapper et al. (1947) for ribonuclease digestion of leishmania though it was good enough for plasma cells.

By subjecting living culture of Leishmania donovani to the action of ribonuclease, cytoplasmic basophilia and the volutin granules were digested away. The nucleus showed a paler colour and in some of the flagellates the darker staining karyosome and the peripheral ring of the nuclear membrane were replaced by a number of short dot or rod like structures. The specimens in which the number of these structures could be counted, showed six such bodies against the pale background of the rest of the nucleus, arranged along the margin of the nucleus or in two groups of three each at its either pole. In a few specimens each of the six rod-like structures was seen to be breaking up into pairs and the parabasal
body (kinetoplast) was also found divided into two parts. In other specimens the darker staining peripheral ring and the dot-like or elongated karyosome could be readily made out against the rest of the nucleus that was stained paler. In control specimens though the nucleus at times showed similar rod or dot-like structures, the picture was never so clear as in the ribonuclease treated smears. (Plate I, 10-12; Plate II, 13-19 and 20-23)

The kinetoplast was unaffected by ribonuclease digestion.

Periodic acid-Schiff and Bauer-Feulgen reactions.

Polysaccharides and glycogen could not be detected with certainty in the leptomonad and the leishmania forms of *L. donovani* by the above techniques, wet fixed smears being used for purpose. In sections of liver biopsy material obtained from cases of kala-azar, the cytoplasm of the L.D. body showed red coloration.

Sudan Black stain for lipids.

Sudanophil granules could be detected in the cytoplasm of the leptomonad form of *L. donovani* stained with Sudan Black B. These granules corresponded to the distribution of the Golgi complex.
**Coupled tetra-azonium reaction for protein.**

Both the *L. donovani* and the leptomonad forms of *L. donovani* showed the presence of proteins in the protoplasm. In the leptomonad the distribution could be studied more satisfactorily. It was found that the areas occupied by the nucleus and the volutin granules were relatively lighter than rest of the cytoplasm; the area of the flagellar vacuole was unstained. The flagella reacted positively.

**Localisation of alkaline phosphatase.** (Plate I, 3, 8, 9; Plate III, 11-18)

In the *leishmania form*, the nucleus gave a marked reaction for the enzyme, the nuclear membrane appeared as a dark ring with the nucleoplasm showing a faint reaction. The karyosome could often be visualised in bone marrow smears as a dark small dot or rod shaped structure. In bone marrow smears, it was usually not possible to be definite about the reaction of karyosome in the *leishmania* in tissue sections. Border of the parasite showed distinct reaction for the enzyme; the cytoplasm showed weak reaction and the kinetoplast usually showed a distinct reaction. The vacuole did not appear to contain any alkaline phosphatase.

In the *leptomonad form*, the enzyme was found to be present in the karyosomes and the chromosomes. The
pattern of distribution of alkaline phosphatase corresponded to that of DNA described previously.

**Localisation of acid phosphatase.**

The L.D. body showed marked reaction for the enzyme in the nuclear membrane and at times a diffuse reaction could be made out in the nucleoplasm as well. The karyosome was stained only occasionally. The cytoplasm and the margin of the parasite showed weak reaction for the enzyme and the kinetoplast was often found to give a positive reaction. Occasionally a few granules could be seen in the cytoplasm of the L.D. body.

The **leptomonad form** did not show any diffuse reaction in the cytoplasm or the nucleus; but a variable number of black granules of lead sulphide could be seen in different parts of the leptomonads indicating the presence of acid phosphatase. These granules seemed to correspond to the distribution of neutral red vacuoles in the flagellate.

**Conclusions.**

The following conclusions regarding the chemical composition of the various structures in the parasite *Leishmania donovani* may be based on the results of cytochemical study described above.
The nucleus. The nucleus of both the leishmania and the leptomonad forms contains DNA in the nuclear membrane and the karyosome, rest of the nucleus apparently contains RNA being stained green by light green in the Feulgen reaction, and showing dissociation of chromosomes after ribonuclease digestion. Besides protein, it contains alkaline phosphatase and at least in the leishmania form it contains acid phosphatase also. The nucleus does not contain polysaccharides or lipids.

It has been found that the number of chromosomes present in L. donovani in six. Each of these divide into two so that on division, the two daughter leptomonads share equal number of chromosomes.

The chromosomes show marked variation in shape, and distribution in the leptomonad form and the karyosome shows variation in size and shape in both the L.D. body and the flagellate form. Some of the patterns of distribution of chromosomes noted are unrelated to nuclear division.

Cytoplasm. The body of the parasite is made up of protein and the flagellum also shows the presence of protein by the coupled tetraazonium reaction. The cytoplasm also contains RNA as indicated by staining with
pyronin and specific loss of cytoplasmic basophilia through the action of ribonuclease. The volutin granules are apparently rich stores of RNA with small amount of protein so that the sites occupied by the granules show relatively weaker reaction for protein than rest of the cytoplasm.

Mitochondria are present in the form of small granules in the cytoplasm, more in the posterior half of the body than in the anterior. Two mitochondria could be visualised in the leishmania form. The mitochondria in the latter case showed reduction of Janus Green B to diethyl saffranine.

The staining reactions indicate that cytochrome oxidase and succinic dehydrogenase are present in the mitochondria of L. donovani.

The Golgi complex are present in the cytoplasm of the flagellate and they are apparently of the nature of spheroids which take up granules of neutral red. The distribution of lipids as determined by Sudan black B appear to correspond to that of the Golgi complex and acid phosphatase is similarly localised. It appears that the Golgi complex contain both lipids and acid phosphatase.
The kinetoplast is made up of proteins and contains DNA and possibly also RNA but this latter possibility could not be established with certainty.

The cytoplasm of the parasite does not appear to contain any polysaccharide, in the leptomonad form, but the leishmania form probably contains glycogen.
PLATE I

Leishmania donovani (Photomicrographs)

Fig. 1  L. donovani, leishmania form, stained with Leishman's stain.
Fig. 2  Leishmania in section of bone marrow (Feulgen's reaction).
Fig. 3  Leishmania in section of liver (Reaction for alkaline phosphatase).
Fig. 4  Leptomonads (living) of L. donovani, under phase contrast microscope.
Fig. 5  Leptomonads stained by Leishman's stain.
Fig. 6  Leptomonads stained with iron haematoxylin.
Fig. 7  The Feulgen reaction in the leptomonads.
Figs. 8 & 9  Reaction for alkaline phosphatase in the leptomonads.
Figs. 10-12  Leptomonads acted on by ribonuclease in the fresh state, stained with Leishman's stain.

Magnification: Figs. 1, 3, 6-9, ca X 600; Figs. 4 & 12, ca X1000; Figs. 5, ca X1250; Figs. 7 & 8, ca X1000; Figs. 10, 11, & 12(b), ca X2500;

PLATE II

Leishmania donovani

Figs. 1-3  Mitochondria of leptomonads of L. donovani stained with Janus green-B.
Figs. 4 & 5  The Golgi complex visualised by supravital staining with neutral red as seen under phase contrast microscope.
Figs. 6 & 7  Leptomonads stained with brilliant cresyl blue.
Figs. 8-12 Leptomonads stained by the Feulgen method.

Figs. 13-19 Fresh culture of L. donovani subjected to the action of ribonuclease and finally stained with Leishman's stain.

Figs. 20-23 Control specimens of above.

N.B. — Figures not drawn to scale.

PLATE III

Leishmania donovani: structure of the nucleus

The figures are not drawn to scale. The figs. 5, 7 and 16 are drawn semidiagrammatically to emphasize the pattern of distribution of DNA and alkaline phosphatase.

The figs. 1—10 and 19—20 are from Feulgen preparation of leptomonal and leishmanial forms of L. donovani, respectively. The figs. 11—18 are from preparations demonstrating alkaline phosphatase.

Fig. 1 DNA present along the nuclear membrane and in the globular karyosome.

Fig. 2 Same, karyosome rod-shaped.

Fig. 3 Grains of DNA on the nuclear membrane; rounded karyosome at one pole.

Fig. 4 DNA arranged at intervals all round the nuclear membrane.

Fig. 5 Chromosomes arranged in anaphase at either pole.

Fig. 6 Chromosomes arranged in metaphase pattern at the central zone of nucleus.

Figs. 7 & 8 Double chromosomes arranged separately all along the nuclear membrane.

Fig. 9 No DNA in the nuclear membrane; globular karyosome containing DNA.

Fig. 10 Chromosomes irregularly arranged in a group of small rods (? skein formation) in the nucleus.
Fig. 11 Alkaline phosphatase present in the nuclear membrane and rounded karyosome.

Fig. 12 No alkaline phosphatase in the nuclear membrane; karyosome elongated and granular showing positive reaction.

Fig. 13 Leptomonad with divided parabasal body; alkaline phosphatase present in the nuclear membrane and elongated karyosome.

Fig. 14 Alkaline phosphatase in globular karyosome only.

Fig. 15 Alkaline phosphatase showing distribution similar to figs. 7 and 8.

Fig. 16 Alkaline phosphatase in chromosomes at either pole of the nucleus, corresponding to fig. 5.

Figs. 17 & 18 Leptomonads showing division of the nucleus and the parabasal body; chromosomes show marked reaction for the enzyme.

Figs. 19 & 20 Leishmania form showing DNA along the nuclear membrane and in rounded (19) or rod-shaped (20) karyosome.

N.B.—The kinetoplast shows positive reaction for DNA and alkaline phosphatase in both the leptomonad and the leishmania forms of L. donovani. Cytoplasmic granules showing strong reaction for alkaline phosphatase are seen, and the flagellum and the cytoplasm show weak reaction for the enzyme in the leptomonads.