GLYCOGEN IN THE CILIATE PARASITES OF FROG

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Abstract

The carbohydrate bodies present in the endoplasm of ciliate parasites of frog, viz. Nyctotherus macropharyngeus and Balantidium helenae, are composed of glycogen. The appearance of fixation artifacts during the preservation of glycogen in these ciliates is discussed.

Introduction

Well-defined carbohydrate bodies which serve as reserve food material of the parasitic ciliates Nyctotherus macropharyngeus and Balantidium helenae have been reported by the author earlier (Dutta, 1958 and 1959). Horning (1927), Richardson and Horning (1931), and Khajuria (1930) named these bodies as ‘vegetative granules’ in Nyctotherus. Barfurth (1885) was probably the first to observe the glycogen nature of these bodies in Nyctotherus and he described them as ‘glycogen particles’. Fauré-Fremiet and Thaureaux (1944) reported the presence of glycogenous bodies in free living (Paramecium, Glaucoma, Vorticella, Stauro, etc.) as well as parasitic (Ophyryoscolesiidae, Nyctotherus and Balantidium) ciliates. In Balantidium coli, Sen Gupta and Ray (1955) recognized the presence of a few paraglycogen or mucoprotein granules, in addition to the numerous glycogen granules. Apart from these bodies, they reported the presence of RNA, simple protein containing tyrosine, polysaccharides (mainly glycogen) and lipids in the endoplasm.

These bodies were identified as paraglycogen grains in Balantidium rectorum, B. cecumum, B. anomali, B. coli, Nyctotherus crocodilum, N. hylae and N. oralis by Lom (1955), and in Ichthyophthirius by MacLennan (1936).

Auerbach (1953) reported the presence of mitochondria and Golgi apparatus in Balantidium coli.

Material and Technique

The ciliates Nyctotherus macropharyngeus and Balantidium helenae obtained from the rectum of frog were fixed in Carnoy, Bouin, Helly and Lewitsky fluids. The paraffin sections of these ciliates were stained in periodic acid-Schiff (Hochstein, 1948 and Pearse, 1960), and Best’s carmine (Best, 1906) for the study of glycogen. The control sections were treated with salivary amylase or malt diastase solution (Pearse, 1960 and Lillie, 1954), and subsequently stained in periodic acid-Schiff or Best’s carmine. The glycogen in the sections was completely digested with this treatment. Glycogen also stains brown in Lugol’s iodine when used on fresh material. The living specimens of

Figs. 1-4 are photomicrographs of N. macropharyngens.

1. Living specimen, crushed; photomicrograph under the phase contrast.
2. Helly, Best's carmine (section).
3. Lewitsky, Best's carmine (section).
4. Fresh specimen, crushed. Lugol's iodine staining.

Abbreviations.—C = carbohydrate body; L = lipid body; M = mitochondria; N = macro-nucleus.
Results

The cytoplasmic inclusions of *Nyctotherus macropharyngeus* and *Balantidium helenae* have been investigated earlier by the author (Dutta, 1958 and 1959).

The cytoplasmic inclusions of *N. macropharyngeus* seen under the phase contrast (Fig. 1) are the mitochondria, lipid bodies and carbohydrate bodies.

The carbohydrate bodies appear of light contrast. They stain with periodic acid-Schiff and Best's carmine (Figs. 2 and 3). They are coloured brown with Lugol's iodine (Fig. 4). Since after treatment with salivary amylase or malt diastase solution, these bodies are completely digested (negative staining in periodic acid-Schiff and Best's carmine), it is concluded that the carbohydrate material present in them is glycogen. These bodies do not show positive reaction for lipids, proteins or nucleic acids.

In the stained preparations, these carbohydrate bodies show diverse structures depending upon the fixative employed. In the material fixed in Bouin, Carnoy or Helly, the carbohydrate bodies of small size show the peripheral part as stained, while the larger bodies reveal a honeycomb-like appearance. The individual honeycomb-like bodies also coalesce to form the large masses (Fig. 2). After fixation with Lewitsky fluid, however, these bodies stain homogeneously (Fig. 3). They show the same appearance as seen in the living material or after treatment with Lugol's iodine. From these preparations it can be concluded that Lewitsky fixes glycogen in its natural form, whereas other fixatives precipitate the proteins in which the glycogen is held up.

Figs. 5 and 6 are photomicrographs of *B. helenae*.

5. Bouin, Best's carmine (section).
6. Helly, Best's carmine (section).

Figs. 5 and 6 of *Balantidium helenae* also show that the carbohydrate bodies composed of glycogen are not fixed properly in Helly and Bouin fluids.
Baker (1956) has reported that acetic acid, formaldehyde, ethanol, picric acid, osmium tetroxide and potassium dichromate are not fixatives for carbohydrates, but most of them only precipitate the protein which prevents the glycogen from being dissolved in water. It has been experienced that the fixatives like Bouin, Carnoy or Hell, the constituents of which are acetic acid, formaldehyde, ethanol, picric acid, and potassium dichromate, actually produce extreme artifacts as far as the fixation of glycogen is concerned.

Bauer (1933) has concluded that chromic acid is the only substance used in cytology that actually fixes glycogen, that is, renders it insoluble in water. During the present investigation also, it has been observed that glycogen is well preserved in Levitsky which contains chromic acid as one of its constituents.

The presence of glycogen in the carbohydrate bodies has been recognized earlier also in some species of *Nyctotherus* and *Balantidium* by Barfurth (1885), and Fauré-Fremiet and Thaureaux (1944), while in other species of these ciliates these bodies are composed of paraglycogen as reported by Lorn (1955). Sen Gupta and Ray (1955) have, however, observed the presence of both the glycogen granules and the paraglycogen or mucoprotein granules in *B. coli*. They have recognized the presence of glycogen in the cytoplasm as well. Other authors such as Horning (1927), Richardson and Horning (1931), and Khajuria (1950) have named these bodies as ‘vegetative granules’.

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Abstract

The life-cycle of Eimeria tenella in the experimentally infected chicks has been investigated. The endogenous phase of the life-cycle includes two successive schizogonous cycles followed by a gametogonous cycle, while the exogenous phase includes the sporulation of the oocyst after they have been passed out of the body of the chick. The second schizogonous cycle produces merozoites of two types, small and large; these merozoites probably develop into micro- and macrogametocytes respectively. The average duration of the life-cycle is 7 to 9 days, but often the cycle is completed in 6 days; or it is delayed even up to 11 days. These variations are either due to the lagging behind of sporozoites or merozoites during the course of development or to the slow development of trophozoites and gametocytes. The stages of development of the parasite sometimes show variations even in the different regions of the same caecum. Chicks up to the age of 50 days are equally susceptible to this infection although the mortality is higher in younger chicks.

Introduction

The observations recorded earlier by Tyzzer (1929), and Gill and Ray (1957), on the life-cycle of Eimeria tenella in the experimentally infected chicks, differ with regard to the number of schizogonous cycles, or to the types of schizonts, and merozoites in the second schizogonous cycle.

Again, some sort of regularity in the timings of the life-cycle has been reported by earlier authors; but during the course of present investigations, a wide range of variations has been observed in the timings of the life-cycle of this coccidian in the experimentally infected chicks. On close examination these variations have been found to be significant and of frequent occurrence.

Material and Technique

The chicks, infected experimentally with the sporulated oocysts of Eimeria tenella, were dissected at various intervals of time from 1–12 days; and the caeca were removed in physiological saline solution (Baker, 1944) to study the stages of life-cycle of the parasite.

Some stages of the life-cycle have been drawn from the living material scraped from the inner lining of the caeca and studied under the ordinary and phase-contrast microscopes. The rest of the stages have been selected from the preparations made for the routine cytological and cytochemical study. The fixatives employed are: Lewitsky, Zenker, Carnoy, 1% cadmium chloride solution, etc. For staining, in addition to simple Heidenhain's iron haematoxylin, other techniques used are acid fuchsin (Cain,
The life-cycle of *Eimeria tenella* consists of endogenous and exogenous phases. The endogenous phase includes the stages from the entry of sporozoites into the epithelial cells of the caeca of the chick up to the formation of oocysts, while the exogenous phase includes the sporulation of the oocysts outside the body of the chick. These phases have been investigated in detail by Tyzzer (1929), and Gill and Ray (1957); therefore, they will be described here briefly.

**I. Endogenous phase**

This phase of life-cycle includes two successive schizogonous cycles followed by a gametogonous cycle:—

(A) **First schizogonous cycle.**—This cycle includes the first generation schizonts and merozoites I (Figs. 1 to 6).

(B) **Second schizogonous (merogonous) cycle.**—This cycle includes the second generation schizonts and merozoites II (Figs. 7 to 12). The schizonts of the second generation are of two types, producing small and large merozoites respectively. The schizonts mostly develop in the submucosa, but sometimes the schizonts (generally of small size), which produce small merozoites, develop in the epithelial cells along with the growing gametoeytes.
Nath, Datta, and Sagar: Life-cycle of Eimeria tenella
(C) Ga... cycle.—The gametogonous cycle includes:
(a) Microgametocyte and microgamete (Figs. 14 and 15).
(b) Macrogametocyte and macrogamete (Figs. 16 to 20).
(c) Formation of oocyst (Figs. 21 to 23).

II. Exogenous phase
This phase of the life-cycle includes the sporulation of oocysts after they have been passed out from the body of the chick (Figs. 24 to 26).

I. Endogenous phase
(A) First schizogonous cycle.—The sporozoites are liberated from the oocysts due to the action of digestive juices during their passage down the alimentary canal of chick. Mayhew (1937) recorded their liberation from the oocysts in the duodenum. The first schizogonous cycle starts with the entry of these sporozoites in the epithelial cells of the caeca of chick (Fig. 1). Within the epithelial cell, each sporozoite grows into a trophozoite (Figs. 2 and 3), and occupies a position below the nucleus of the host cell. With further growth, the nucleus of the trophozoite undergoes a series of divisions to produce a multinucleate schizont I. Each nucleus in the schizont is surrounded by a fragment of cytoplasm (Fig. 4), thus forming merozoites I, which later become spindle-shaped (Figs. 5 and 6). The host cell containing the mature schizont is squeezed out from the epithelium into the lumen. A siderophilous body, which is present in the sporozoite, persists in the growing schizont up to the formation of merozoites, where it is seen in the residual cytoplasm (Fig. 5). This siderophilous body is characteristic of the first schizogonous cycle. The merozoites are liberated in the lumen of the caeca.

(B) Second schizogonous cycle.—This cycle starts when a first generation merozoite (Fig. 6) attacks a new epithelial cell. The parasite, which at this stage occupies the same position as the sporozoite, i.e. below the nucleus of the host cell, rounds up and, as it grows into the schizont, it produces an injurious effect on the host cell; it becomes loose and begins to move down from the epithelial layer to the submucosa or even deeper. The nucleus of the trophozoite undergoes division during schizogony; the trophozoite now becomes a multinucleate schizont (Figs. 7 and 8). Due to the pressure exerted by the

Figs. 16 to 26. Camera lucida drawings of the stages of life-cycle of E. tenella.
16, early macrogametocyte, the nucleus shows a karyosome: Carnoy/mercuric-bromphenol blue.
17, growing macrogametocyte, showing increased number and size of lipid bodies: Lewitsky/acid fuchsine.
18, growing macrogametocyte: Zenker/pyronin G/methyl green.
19, late macrogametocyte: Lewitsky/iron haematoxylin.
20, macrogamete, showing the peripheral arrangement of lipid bodies: Lewitsky/acid fuchsine.
21, early oocyst, outer cyst wall is complete, inner cyst wall is developing: Zenker/pyronin G/methyl green.
22, oocyst, inner cyst wall is developing: Lewitsky/acid fuchsine.
23, oocyst: Lewitsky/iron haematoxylin.
24 to 26, oocyst undergoing sporulation: 1% cadmium chloride, coloured with Sudan black in 70% ethanol.
Xath, Ihittn, and Sagar: Life-cycle of Eimeria tenella
... growing schizonts, and also because of the loosening of the epithelial cells, the blood capillaries are ruptured and haemorrhage ensues. The chicks at this stage show typical symptoms of coccidiosis.

The schizonts of the second generation, when mature, produce merozoites II of two types, the large (Fig. 9) and the small (Fig. 10) (cf. Gill and Ray, 1957).

In a few cases, mature small merogonous schizonts have been noticed in the epithelial cells of the caeca, which develop side by side with the growing gametocytes. The small merozoites produced from these schizonts resemble the merozoites of III generation described by Tyzzer (1929). More than 25 chicks were sacrificed to study the stages of gametocytes, and since such merozoites (merozoites III of Tyzzer) have been noticed in a few cases, it may be concluded that the production of these merozoites is not a regular feature of the life-cycle, but it is probably due to the lagging behind of merozoites I and their delayed entry into the epithelium. The schizonts developed from them remain small and do not descend down into the mucosa but produce these small merozoites in situ.

Both the small and large merozoites of the second generation (Figs. 11 and 12) are liberated in the lumen of the caeca. Each of them later enters a healthy epithelial cell, rounds off; it now represents an undifferentiated gametocyte (Fig. 13). These gametocytes, which occupy a position below the nuclei of the host cells, differentiate into micro- and macrogametocytes. Gill and Ray (1957) have suggested that the gametocytes developed from small merozoites II produce microgametocytes, while those produced from large merozoites II differentiate into macrogametocytes.

1. Gametogonous cycle

(a) Microgametocyte and microgamete.—The microgametocyte, developed from the undifferentiated gametocyte, undergoes a series of nuclear divisions (Fig. 14). Each nucleus with a small amount of cytoplasm forms a microgamete (Fig. 15).

(b) Macrogametocyte and macrogamete.—The early macrogametocyte (Fig. 16), which develops from the undifferentiated gametocyte, grows (Figs. 17 to 19), and side by side its cytoplasmic inclusions also grow. The authors (Nath, Dutta, and Sagar, 1961) have reported elsewhere the structure of the macrogametocyte, macrogamete, and the formation of the oocyst in this species. The cytoplasmic inclusions described in these stages are mitochondria and lipid bodies. The lipid bodies of large size occupy a peripheral position in the macrogamete (Fig. 20).

(c) Formation of the oocyst.—Subsequent to fertilization, the lipid bodies coalesce along the cell membrane of the zygote, to form the double wall of the oocyst. During this process the lipid bodies form plaques which contribute to the formation of the outer wall of the oocyst (Fig. 21). Some small lipid bodies seen at this stage likewise form the inner cyst wall (Figs. 22 and 23).

II. Exogenous phase

The oocysts formed in the epithelial cells of the caeca are liberated in the lumen, from where they pass outside the body of the host.
Sporulation. —The oocysts collected from the caecal epithelium and preserved in 2.5% potassium dichromate solution undergo sporulation in nearly 45h at an optimum temperature of 28-29°C (Figs. 23 to 26). The mature oocyst reveals four spores, each containing two sporozoites. The sporoblasts as well as sporozoites possess a characteristic siderophilous body. A polar granule is developed within the oocyst during sporulation.

Discussion

The life-cycle of *E. tenella* has been worked out extensively by Tyzzer (1929), and Gill and Ray (1957). A difference of fundamental nature, however, exists as far as their findings of the schizogonous cycles of the asexual phase are concerned. Tyzzer (1929) reported the presence of three successive schizogonous cycles, Gill and Ray (1957), who re-investigated this cycle, concluded that there were only two schizogonous cycles, the third as described by Tyzzer being absent. They observed two types of schizonts of second generation, i.e. the large and the small, which produced large and small merozoites respectively. They believe that the small schizonts described by them correspond to the third generation schizonts of Tyzzer, and likewise the small merozoites described by them correspond to the small merozoites of third generation described by that author.

The present investigation also shows that there are only two schizogonous cycles as described by Gill and Ray (1957), and the merozoites produced in the second generation are of two types, the small and the large. In some cases these small merozoites were also seen in the epithelial cells developing along with the gametocytes as also mentioned by Gill and Ray (1957): they correspond to the third generation merozoites of Tyzzer. Such merozoites are probably produced from the first generation merozoites which lag behind in their development, and enter the epithelial cells at a later stage and develop *in situ*.

Variations in the life-cycle

The authors were interested in the cytochemical investigation of the gametogonous cycle, particularly of the late macrogamete of *E. tenella*, for which a large number of chicks had to be sacrificed. Contrary to the expectations, the stages of late macrogamete were seldom observed at the scheduled timings of the life-cycle described by Tyzzer (1929), and Gill and Ray (1957); more often stages other than these, i.e. either early gametocytes or mature oocysts, were observed in the caecal epithelium. Such irregular variations in the timings of the development of the mature macrogamete led us to investigate the variations in the whole of the life-cycle.

1. Life-cycle. —The time taken from the feeding of the oocysts to the chicks up to the formation of oocysts in the caecal epithelium varies from 6 to 11 days, the average time being from 7 to 9 days.

2. First schizogonous cycle. —Tyzzer (1929) has reported that the sporozoites develop into trophozoites by the end of 2nd day and undergo schizogony by the middle of 3rd day. Gill and Ray (1957) have observed growing trophozoites at 5th and the liberation of merozoites from 5th to 5th and rarely at 8th hour.

It has been observed by us that the sporozoites liberated from the sporulated oocysts enter the epithelial cells of the caeca in about 24h: grow into large trophozoites after 48h: and the first schizogonous cycle takes from 72h to 84h for completion when merozoites I are liberated.
But in some experiments, the development is considerably slowed down and fully grown trophozoites I along with merozoites I are seen at 100h. In still other cases only trophozoites I are developed at 100h, but they form merozoites I by about the end of 5th day.

In such experiments there is a lag of about 2 days in the completion of first schizogonous cycle. This lag in the development of the first cycle can be due to the delayed entry of sporozoites, or to the slow development of the parasite, or to both these factors. Gill and Ray (1957) have also reported a lag in the developmental cycle due to the delayed entry of sporozoites in the cecal epithelial cells.

3. Second schizogonous cycle.—Tyzzer (1929) has reported that the second schizogonous cycle starts by the end of 3rd day and is completed by the 5th day with the liberation of merozoites II. Gill and Ray (1957) have observed the completion of second cycle with the liberation of merozoites from 100h to 124h or even in some cases up to about 144h.

The present investigation also shows that the second schizogonous cycle is completed in 5 days (118h) with the liberation of merozoites II. But in some experiments, the developing schizonts II were seen even up to 6 or 7 days (i.e. 144h and 161h). Gill and Ray (1957) also noticed these schizonts by the end of 7th day. Consequently the production of merozoites II was noticed by them in large numbers up to seven and a half days (182h).

In such cases there is also a lag of about two and a half days in the completion of the second schizogonous cycle, with the result that the formation of gametocytes and oocysts is correspondingly delayed.

4. Gametogonous cycle.—Tyzzer (1929) has observed the maturation of gametocytes and production of oocysts in large numbers by 7 to 8 days. Gill and Ray (1957) have observed sexual forms developing by the end of the 5th day, and fertilization taking place late on 6th day or early on 7th day; but the discharge of oocysts which started late on 7th day continued on 8th and 9th days and decreased on 10th day.

It has been recorded by us that the gametocytes are very numerous after 6 to 7 days (144, 146, 168h); while the oocysts mostly appear from 7 to 9 days. In some experiments the maturing gametocytes are seen as late as the 9th day (214h) or early on the 10th day; and they produce the oocysts on the 10th day or even on the 11th day. In other cases, the development of gametocytes and the formation of oocysts were completed much earlier; the gametocytes appeared as early as 129h and the oocysts after 6 days (146h). The maximum number of oocysts were collected on 8th day.

5. The various regions of the caeca of an infected chick sometimes reveal different stages of the parasite. It has been observed that in a chick with 10 days old infection, the sections of the middle region of the infected caeca were infiltrated mostly with oocysts and a few very late stages of gametes; those of the distal region presented stages varying from early to late gametocytes along with the developmental stages of oocysts; and those of the extreme distal end revealed only the early developmental stages of the gametocytes. Such variations in the development of the parasite in the same caecum are probably caused by the lagging behind of some of the sporozoites or merozoites as described above.

6. Chicks up to the age of 50 days have been found to be equally susceptible to this infection; but the mortality is higher in the younger chicks.
Literature Cited


OBSERVATIONS ON THE MACROGAMETOCYTE LEADING TO THE FORMATION OF THE OOCYST IN EIMERIA TEN ELLA, RAILLIET AND LUCET, 1891, IN EXPERIMENTALLY INFECTED CHICKS

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Abstract

In the developing macrogametocyte, macrogamete and oocyst of Eimeria tenella, obtained from the caeca of the experimentally infected chick, two distinct types of cytoplasmic inclusions, viz. the mitochondria and lipid bodies, have been identified. Some of the lipid bodies assume large size in the late macrogametocyte, and later occupy a peripheral position in the macrogamete; they form the outer wall of the oocyst. The smaller lipid bodies seen in the oocyst at this stage form the inner wall. Many fixatives and unmasking agents have been employed for the demonstration of lipids. Lipid bodies also show positive reaction for proteins, RNA and acid mucopolysaccharides of the type of hyaluronic acid and chondroitin sulphuric acid.

Introduction

In view of the high pathogenicity of the various species of Eimeria, numerous parasitologists have been interested in their study in the past; and a good deal of cytological or cytochemical observations have been recorded. The oocyst being the infective stage of the parasite, the earlier workers have taken particular interest in the structure of the macrogametocyte, the macrogamete, the development of the oocyst wall and the subsequent development of the oocyst. But in spite of these investigations our knowledge of the cytoplasmic inclusions of macrogametocyte and macrogamete is still inadequate. The only type of cytoplasmic inclusions, which most of the earlier authors have described in the developing macrogametocyte, are the large cytoplasmic globules under different names such as ‘plastic granules’ (Pattillo and Becker, 1955), ‘haematoxylinophilic granules’ (Morgan and Hawkins, 1952), ‘large granules’ (Clarkson, 1958, 1959a and 1959b), and ‘peripheral globules’ (Gill and Ray, 1954a, 1954b, 1954c and 1954d, and Cheissin, 1958). These large globules, which form the wall of the oocyst, always occupy a peripheral position in the mature macrogamete.

Pattillo and Becker (1955) have observed at the periphery of the macrogamete of Eimeria acervulina and E. brunetti some smaller granules of uncertain role, which lie between the large plastic granules. They have also described an accumulation of small fat bodies around the nucleus as well as in the cytoplasm of old macrogametocyte. In addition to this they have summarized the previous work on the cytoplasmic inclusions of the macrogamete and the formation of the wall of the oocyst in Eimeria. The accumulation of small fat globules around the nucleus in the macrogamete of E. maxima has been reported by Cheissin (1940) also. Gill and Ray (1957) have reported that the large ‘peripheral globules’ are non-fatty in nature; and they have also described discrete fat globules in the cytoplasm of the macrogamete of E. brunetti.

Grasse (1953) describes in *Eimeria* two types of granules, viz. eosinophile granules ("grains plastinoides") and small granules staining with methylene blue and haematoxylin, which form the outer and inner walls of the oocyst respectively. Cheissin (1959) observed two types of granules composed of protein and mucoprotein in the macrogamete, which form the inner and outer walls of the oocyst respectively, in *Eimeria magna*, *E. intestinalis* and *E. media*. Lapage (1956) also describes an outer chitinous cyst wall and an inner lipoidal cyst wall, both of which are derived from the granules that are developed in the macrogamocyte.

**Material and Technique**

The caeca of the chicks, infected experimentally with *Eimeria tenella*, were fixed in the various fixatives such as Lewitsky, Lewitsky saline (Baker, 1956), 4% formaldehyde, formaldehyde-calcium with or without postchroming (Baker, 1946) and formaldehyde-saline (Baker, 1949). In addition to these fixatives the material was also fixed in the various unmasking agents such as Flemming's, with or without postchroming (Bradbury and Clayton, 1958), and saturated mercuric chloride solution and 1% cadmium chloride solution (Clayton, 1959).

The frozen gelatine sections of the material fixed according to the above techniques were coloured with Sudan black in 70% ethanol for the demonstration of lipids. The buffered osmium technique of Wigglesworth (1957) was found very useful for the study of the cytoplasmic inclusions.

Other techniques used for the study of mitochondria are: Lewitsky/iron haematoxylin staining, Lewitsky/Cain's acid fuchsine staining (Cain, 1948), and the Nadi reaction (Moog, 1943) for the localization of cytochrome oxidase.

For the identification of proteins the mercuoric bromphenol blue (Mazia and others, 1953) and coupled tetrazonium (Pearse, 1960) tests have been employed. For the study of nucleic acids, the pyronin/methyl green technique (Jordan and Baker, 1955) with salivary ribonuclease control for RNA (Bradbury, 1956) has been used. The techniques used for the study of carbohydrates are periodic acid-Schiff with acetylation and KOH reversal controls (Pearse, 1960; Hotchkiss, 1948; McManus and Cason, 1950), salivary amylase digestion for glycogen (Pearse, 1960), Hale's method (Hale, 1946), and metachromatic techniques like Schmorl's thionin and toluidine blue (Pearse, 1960).

Vital stains such as Janus green B and neutral red stain the mitochondria and lipid bodies respectively.

**Results**

During the present cytochemical investigations on *Eimeria tenella* in the experimentally infected chick, we have identified, in the macrogamocyte and the macrogamete, two distinct types of cytoplasmic inclusions. The first type of cytoplasmic inclusions are the mitochondria (Figs. 1-7 and 9-13); these stain with special mitochondrial techniques such as (1) supervital staining with Janus green, (2) Lewitsky/iron haematoxylin preparations, (3) Lewitsky/acid fuchsine (Cain, 1948), (4) buffered osmium technique (Wigglesworth, 1957), and (5) Nadi reaction (Moog, 1943) for cytochrome oxidase. They have been seen in the living cell under the phase contrast microscope as small rods of dark contrast. The oocyst of *E. tenella* also reveals typical mitochondria (Figs. 9-13). Recent investigators of *Eimeria tenella* (Edgar and others, 1944; Tsunoda and Ichikawa, 1955;
IN AND CYTOCHEMISTRY OF PARAMYLIUM BODIES IN KHAWKINEA SP.

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Abstract

The cytoplasmic inclusions identified in a colourless euglenoid flagellate, viz. Khawkinea sp., are the mitochondria, lipid bodies, paramylum bodies and a stigma. It has been demonstrated that the paramylum bodies arise from the lipid bodies. The cytochemical reactions of paramylum bodies have been discussed.

Introduction

The paramylum (or paramylon) bodies which are synthesized by both the colourless and green euglenoid flagellates represent the characteristic reserve carbohydrate material of the euglenoids. As far as it is known, in the green forms the paramylum bodies may arise on the surface of pyrenoids (Hall, 1953) which are usually associated with the chloroplasts (Kudo, 1954). With electron microscopy, Ueda (1958) has actually demonstrated that in Euglena gracilis a chloroplast has one or two pyrenoids on the surface of which the paramylum bodies are formed.

Contrary to these observations, Jahn (1946) has put forward the view that the formation of paramylum is not limited to pyrenoids; and in many forms the paramylum is not formed in contact with the chloroplasts (e.g. Phacus, Lepocinclis, some species of Euglena, and all colourless euglenoids). The same idea was probably first put forward by Czurda (1928) who clearly mentioned that the paramylum bodies in Euglena dicei and E. cicilis are formed in association with some definite cytoplasmic structures which are sometimes distinguishable and have wrongly been termed pyrenoids.

Lwoff (1951), while discussing the biochemistry of phytoflagellates, observes that the colourless flagellates synthesizing starch or paramylon must possess a leucoplast (colourless chromatophore) or its biochemical equivalent. He also mentions that nothing is known so far about the intermediaries concerned in the metabolism of paramylon. Pringsheim (1948) is, however, doubtful of the presence of any leucoplasts in the non-pigmented (colourless) flagellates.

Results

During the present investigation on a colourless euglenoid flagellate, Khawkinea sp., we have observed that this flagellate does not possess any chloroplast or pyrenoid or leucoplast; nevertheless the synthesis of paramylum takes place in this form. We have clearly observed the stages of the formation of paramylum from spherical bodies, which we have identified as lipid bodies, in the living as well as in the fixed material. In addition to the lipid bodies which synthesize paramylum, the
other cytoplasmic inclusions identified in this colourless euglenoid flagellate: chloroplasts and a stigma. The mitochondria are rod-like (Figs. B and C) and special mitochondrial techniques such as (1) supravital staining with Janus green, (2) Regaud/iron-haematoxylin, (3) Regaud/Cain's acid fuchsin (Cain, 1948), (4) Hoyer's post-osmication technique (Baker, 1957), (5) buffered osmium tetroxide technique (Worth, 1957), (6) Nadi reaction (Moog, 1943) for the localization of cytochrome oxidase. 

The lipid bodies which appear in the earliest stage as homogeneous granules grow into spheres (Figs. A–C, L₁) which stain with neutral red supravital. These spheres assume a duplex appearance (Figs. B–D, L₂), the externum being colourable with Sudan black B (for lipids), and coupled tetrazonium (for proteins): the internum remains unstained. The externum may be of even thickness or of uneven thickness with the result that the lipid bodies appear as rings or crescents in optical sections (Fig. B, L₂). The externum at a later stage appears as a thin crescent of large size (Figs. A and C, L₃), while the material synthesized in the internum represents the parylum body. During the formation

![Figs. A, B, and D. Photomicrographs of fresh specimens treated with 2% osmium tetroxide solution for a few minutes. Fig. C, camera lucida drawing of a specimen fixed in Lewitsky fluid and coloured with Sudan black B.]

A. A young flagellate. Nucleus appears as a clear area. B. A growing flagellate. C. A fully grown flagellate showing mitochondria, lipid bodies and parylum bodies. D. A fully grown flagellate. In the reservoir is seen the bifurcation of the flagellum and at the point of bifurcation is a swelling which appears as a dark body.

Abbreviations: F = flagellum; L₁–L₃ = the lipid bodies showing the formation of parylum bodies; M = mitochondria; N = nucleus; P = parylum body; R = reservoir.
of rod-like paramylum bodies, the cortex appears as two parallel rods enclosing a developing paramylum body (Fig. C, I). With further growth the paramylum body attains a large size and the sudanophil cortex or externum (Fig. D, L) disappears. The fully grown paramylum bodies (Figs. B and C, P), therefore, do not give positive tests for lipids or proteins.

The carbohydrate nature of paramylum has been established biochemically by Habermann (1874), Kutscher (1898), and Hutner and Hockett (1936) who found that on hydrolysis it yields mostly glucose. Gottlieb (1860) has determined that paramylum and starch have the same empirical formula. Lwoff (1951) has, however, observed that the paramylum bodies are different from starch. Paramylum has been described earlier as a polysaccharide (Hall, 1953; Kudo, 1954), a higher polysaccharide (Jahn, 1946), and as reserve carbohydrates (Lwoff, 1951; and Hutner and Provasoli, 1955). The available books on cytological and histochemical techniques do not give any information to establish the carbohydrate nature as such of the paramylum bodies. The paramylum bodies of *Khauskinea* do not stain with periodic acid-Schiff and its modifications. They remain unstained in Lugol’s iodine, Best’s carmine and lead tetra-acetate-Schiff (for polysaccharides). They are not digested with amylase. They remain unstained in Hale’s and metachromatic techniques (for acidic mucopolysaccharide) and pyronin/methyl green (for nucleic acids). The only technique which establishes the polysaccharide nature of the paramylum bodies of *Khauskinea* and with which they are stained is the induced y-metachromasia with the sulphation technique of Kramer and Windrum (1954), employing chlorosulphonic acid in pyridine and subsequent staining in Azure A (Spicer, 1960). For details of the histochemical techniques employed here reference may be made to Pearse (1960).

**Literature Cited**


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Gill and Ray, 1954a, 1954b, 1954c and 1957; and Ray and Gill, 1955), of *E. brunetti* and *E. necatrix* (Pattillo and Becker, 1955), of *E. stiedae* and *E. magna* (Cheissin, 1935), of *E. magna* (Cheissin, 1940), of *E. intestinalis* (Cheissin, 1958), and of *E. magna*, *E. intestinalis* and *E. media* (Cheissin, 1939); however, have not reported the presence of mitochondria in the macrogametocyte or the macrogamete or the oocyst.

The second type of inclusions are represented by the lipid granules, which grow in size with the growth of the cell; they are distributed in the cytoplasm of the young macrogametocyte at random, and stain with neutral red supervitally. During the final growth phase of the macrogamete, these lipid granules assume a very large size and arrange themselves at the periphery of the cell touching its limiting membrane. Nevertheless a large number of smaller lipid granules and spheroids continue to exist in the inner regions of the cytoplasm. Such lipid bodies are reported in other Protozoa (Dutta, 1959; Nath and Dutta, 1962) and Metazoa (Baker, 1959; Nath, 1960). It is interesting to note that the lipid spheroids of the growing macrogametocyte are homogeneous, not showing any duplex structure. The homogeneous appearance of these lipid spheroids seems to be related to the fact that they are actively synthesizing lipids, proteins, acid mucopolysaccharides and RNA in their interior, to be transformed into the ‘plastic granules’ or ‘peripheral globules’ of other authors.

For the demonstration of lipids, the osmium staining technique (Wigglesworth, 1957) and Flemming’s unmasking technique, followed by colouring with Sudan black (Bradbury and Clayton, 1958), have yielded useful results. With these techniques, a series of stages of the growth of lipid granules into large lipid bodies in the macrogametocyte and macrogamete (Figs. 1-7) have been traced. In the early macrogametocyte, the lipid bodies are seen as granules (Figs. 1 and 2), while in the growing macrogametocyte there are also present lipid bodies of large size distributed throughout the cytoplasm (Figs. 3-6). These large lipid bodies later arrange themselves at the periphery of the macrogamete, touching its limiting membrane (Fig. 7). Up to this stage the lipid bodies are homogeneously sudanophil as well as osmiophilic. They also show positive reaction for proteins, RNA and acid mucopolysaccharides of the type of hyaluronic acid and chondroitin sulphuric acid, and represent the ‘plastic granules’ or ‘peripheral globules’ described by the earlier workers.

Gill and Ray (1954b and 1954c), Ray and Gill (1955), and Tsunoda and Ichikawa (1955) have also described earlier the presence of RNA, chondroitin sulphuric acid and hyaluronic acid type of polysaccharide and alkaline phosphatase in the peripheral globules (identified as lipid bodies by us) of the macrogamete of *E. tenella*. Edgar and others (1944) thought that in *E. tenella* some small globules arranged along the border of the macrogamete contained fat. No other worker on *E. tenella* or other species of *Eimeria* has ever reported the presence of lipids in the large bodies of the macrogamete which represent the ‘plastic granules’ or ‘peripheral globules’, etc., of the earlier authors. The discrete fat globules or bodies described by Cheissin (1940), Pattillo and Becker (1955), and Gill and Ray (1957) in the macrogametocyte or macrogamete correspond to the small lipid bodies.

These enlarged peripheral bodies or ‘plastic granules’ are not periodic acid-Schiff positive (Fig. 8), containing as do acid mucopolysaccharides (hyaluronic acid and chondroitin sulphuric acid). These conclusions are supported by Pearse (1960) who observes that ‘it is clear from the work’ of Leblond and others (1957), and other authors that ‘the acid mucopolysaccharides, assumed by myself and others to be capable of giving a
Yath, Duffa and Sagar: Macrogametocyte and Oocyst of *Eimeria tenella*
positive though weak reaction with PAS, in fact do not react at all. The weak PAS reaction of intensely metachromatic tissue components containing acid mucopolysaccharides is thus due to the carbohydrate-protein complex of the type mentioned above. The \textit{in vitro} tests carried out by Braden (1955) on various acid mucopolysaccharides confirm this impression, as do those of Hoogwinkel and Smits (1957) who find that both CSA and hyaluronic acid are PAS negative.

With periodic acid-Schiff test, the cytoplasm of the macrogamete shows strong positive reaction, but the small peripheral granules, reported with this technique by Pattillo and Becker (1955) in the macrogamete of \textit{Eimeria brunetti} and \textit{E. acervulina}, have not been identified in \textit{E. tenella}. Even after the digestion of glycogen by pretreatment with salivary amylase, no such granules are revealed.

There are contradictory accounts in the literature with regard to the initial site in the cell where the lipid bodies or the ‘plastic granules’ originate. These bodies first originate at random in the form of small lipid granules in the cytoplasm of the macrogametocyte of \textit{E. tenella}. This observation is supported by the previous observations of Hadley (1911) in \textit{E. avium}, Roudabush (1937) in \textit{E. separata}, Lapage (1940) in \textit{E. caviae}, and Hossoda (1928) in \textit{Isospora lacazii} as quoted by Pattillo and Becker (1955). All these workers report that the plastic granules first arise at random in the cytoplasm of the macrogametocyte. Gill and Ray (1957) also support these observations in \textit{E. tenella}.

The macrogamete, subsequent to fertilization, develops a double cyst wall and forms the oocyst. During the formation of the outer cyst wall, the amount of lipids in the peripheral lipid bodies decreases considerably and they begin to spread as plaques along the cell membrane (Fig. 9). At a later stage (Fig. 10), the plaques reveal only weakly sudanophil rims, and in the final stages of transformation, the plaques become completely non-sudanophil, and their material forms the outer wall of the oocyst.

The smaller lipid bodies present in the developing oocyst at this stage, also later, occupy the peripheral position (Fig. 11), and spread to form the inner wall of the oocyst which retains its lipoidal nature, unlike the outer cyst wall (Fig. 12). Similar lipoidal nature of the inner cyst wall has been reported earlier by Lapage (1956) and others. Free lipid bodies continue to exist in the cytoplasm of the oocyst (Fig. 13).

It appears that the two types of granules described by Grasse (1953) and Cheissin (1959), which according to them form the outer and inner walls of the oocyst, are probably represented in \textit{E. tenella} by the lipid bodies as described above.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figs_1-13.png}
\caption{Camera lucida drawings of some stages of \textit{E. tenella} from the sections of the infected caeca of chicks.}
\end{figure}

\textbf{Abbreviations.} CW \textsuperscript{2} Double cyst wall; CW \textsuperscript{1} Outer cyst wall; CW \textsuperscript{0} Inner cyst wall; L Lipid body; M Mitochondria; N Nucleus.
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LITERATURE CITED


Printed at the Baptist Mission Press, 114 Acharyya Jagadish Bose Road, Calcutta 16, India.
The cytoplasmic inclusions of Paramecium caudatum, Colpoda cucullus and Vorticella are lipid bodies, mitochondria and a variable number of food vacuoles. The lipid bodies mostly appear as duplex spheres, though a few homogeneous spheres are also sometimes seen. The externum of the duplex lipid bodies is composed of phospholipids and lipoproteins in P. caudatum, of triglycerides, phospholipids and proteins in C. cucullus, and of phospholipids and proteins in Vorticella. The composition of the internum of the duplex bodies is not known. These bodies correspond to the ‘vacuome’ or the Golgi complex described in these ciliates by the previous workers. The mitochondria vary in shape; in P. caudatum they are oval or rod-like, in C. cucullus they are more or less oval, while in Vorticella they are rod-like. The macro- and micronuclei show the presence of DNA, RNA and proteins. The cytoplasm shows diffused reaction for RNA, proteins and glycogen.

Previous Literature

Paramecium.—Rammeloven (1925) demonstrated the presence of carbohydrate reserves in the form of soluble glycogen dissolved in the protoplasm of Paramecium. Park (1929) described in P. caudatum a blackening of the contractile vacuoles and of certain knob-like bodies on the surface of the meganucleus, in the Golgi techniques. Both these structures according to him represented the Golgi bodies. Hall and Nigrelli (1937) observed in P. bursaria a number of small neutral red-stainable globules which they described as ‘vacuome’. They also reported that the elements of ‘vacuome’ were consistently osmiophile and distinct from the mitochondria.

The mitochondria of Paramecium were described as spherical by Faure-Fremiet (1910) and as rod-shaped by Causey (1925-26) and Horning (1926). Recently the mitochondria of Paramecium have been extensively investigated with electron microscopy. Ornstein and Pollister (1952) described the presence of microvilli within the mitochondria of P. caudatum, Tsujita and others (1955) reported the occasional appearance of a ‘plicate structure’ within the mitochondria of Paramecium, Sedar and Rudzinska (1956) identified mitochondria in P. multimicronucleatum, Powers and others (1956) reported the presence of mass of tubules in the mitochondria of P. aurelia, some of which opened into the cytoplasm, and, finally, Dalton and Felix (1957) described the typical internal structure in the mitochondria of P. caudatum.

Using the electron microscope, Tsujita and others (1954) described the mitochondria of Paramecium caudatum as granules, spherical, ellipsoidal or filamentous, scattered in the cytoplasm. In the ectoplasmic region, immediately beneath the pellicle he observed...
almost parallel arrangement of mitochondria. The endoplasmic mitochondria, according to him, stain with Janus green, and also show a positive Nadi's reaction, thus indicating the presence of cytochrome oxidase and cytochrome complex. The mitochondria are also specially stained by Altman's or Altman-Kull's test.

Watanabe and others (1954) isolated the mitochondrial granules of *Paramecium caudatum*, and measured the activities of cytochrome oxidase and succinic dehydrogenase in the mitochondrial fraction. They reported that the mitochondrial granules are intimately related to these respiratory enzymes.

Tsujita and others (1955) consider that the ectoplasmic mitochondria of *Paramecium caudatum* seem to have some relation to the formation of trichocysts and they also believe that the mitochondria may be somehow related to the energy source for ciliary movement.

Sen Gupta and Ray (1958) reported sudanophil granules corresponding to mitochondria and the Golgi complex in *P. polycaryum*. According to them, the endoplasm contained RNA and glycogen, while the ectoplasm along with the cilia and basal granules gave a positive reaction for protein.

The vesicles of endoplasmic reticulum have been reported in the cytoplasm of *P. multimeronucleatum* by Sedar and Porter (1955), and Sedar and Rudzinska (1956).

Moses (1950) reported that the quantitative cytologic analysis of macro- and micronuclei of *P. caudatum* revealed that both the nuclei were alike in their nucleic acid-protein composition. According to him these nuclei contained similar concentrations of total protein, non-histone protein, DNA and RNA. He also calculated that the nuclei contained larger amounts of RNA than that of DNA. Wiehterman (1953) reported in the macronucleus of *Paramecium* the presence of fine threads and tightly packed discrete chromatic granules of variable size embedded in an achromatic matrix.

Watanabe (1957) studied the variation in the number of micronuclei in *Paramecium caudatum* and he described a possible process leading to the formation of amicronucleate or bimicronucleate individuals.

*Colpoda.*—Causey (1925-26) is probably the only worker who has investigated the cell inclusions in *Colpoda saprophylla* and he reported the presence of rod-shaped mitochondria in and about the food vacuoles, throughout the binary fission and conjugation.

The nuclear structure of *Colpoda* has been extensively investigated. A lobated karyosome or ‘cariosoma lobata’ in the macronucleus was considered a specific character of *C. cucullus* by Enriques (1908). Kidder and Oliver (1938) studied the nuclear changes in the life-cycle of *C. cucullus* and described the chromatin in macronucleus distributed as irregular plaques surrounding and extending into the colourless nucleoplasm. Burt (1940) classified the macronucleus in the various species of *Colpoda* into two groups: (1) the type found in *C. steinii* and *C. steinii var. piekarskii*, with the chromatin more or less evenly distributed about a homogeneous plasmosome, and (2) the type present in *C. cucullus* in which the chromatin is collected in plaques applied to the inner surface of the nuclear membrane.

The author (Dutta, 1959a) described briefly the distribution of nucleic acids in *C. cucullus*. In the macronucleus was reported a coarse network of DNA, in addition to a number of large Feulgen negative spaces which contained mainly RNA. The micronucleus contained both DNA and RNA. RNA was also demonstrated in the cytoplasm and mitochondria.
**Observations**

The cytoplasmic inclusions of *Paramecium caudatum, Colpoda cucullus* and *Vorticella sp.*, seen in the living condition under the phase contrast microscope, are the lipid bodies, mitochondria and a variable number of food vacuoles. There is nuclear dimorphism in these ciliates: the macronucleus is comparatively much larger than the micronucleus. The micronucleus often lies embedded on the surface of the macronucleus and in such cases it is difficult to locate the micronucleus. The various cytochemical tests used are given in Appendices I to III.

**Material and Technique**

The ciliates, *Paramecium caudatum, Colpoda cucullus* and *Vorticella sp.*, were obtained from the cultures of grass leaves or hay infusions. Living specimens were studied in the culture solutions, using vital stains, Janus green and neutral red in very dilute concentration (1:5000 to 1:100000) (Baker, 1938). The cytochemical tests (Appendices I to III) were employed on the wet whole mount smears, or the paraffin or frozen gelatine sections of the material. The thickness of the sections varied from 5-10 μ. For the identification of the mitochondria, Cain’s acid fuchsine (Cain, 1948) staining on Lewitsky or Helly fixed material was used. For the routine cytological study, the material fixed in Lewitsky or Helly was stained with Heidenhain’s iron haematoxylin.

The cytoplasmic inclusions of *Paramecium caudatum, Colpoda cucullus* and *Vorticella sp.*, seen in the living condition under the phase contrast microscope, are the lipid bodies, mitochondria and a variable number of food vacuoles. There is nuclear dimorphism in these ciliates: the macronucleus is comparatively much larger than the micronucleus. The micronucleus often lies embedded on the surface of the macronucleus and in such cases it is difficult to locate the micronucleus. The various cytochemical tests used are given in Appendices I to III.

**Lipid bodies.—**Under the phase contrast microscope, the lipid bodies appear in the living ciliates as duplex spheres distributed at random throughout the cytoplasm and the endoplasm. The number of the lipid bodies varies with the age of the ciliates. In the younger ciliates the lipid bodies are lesser in number, but their number increases with...
the growth of the ciliates. The lipid bodies first appear as small granules which grow and assume a duplex appearance, with a differentiation into an externum and an internum. When the material of externum is evenly distributed the bodies appear as rings in optical section but, when the material is unevenly distributed, they appear as crescents. The lipid bodies of *P. caudatum* and *C. cucullus* stain with neutral red supervitally, but in *Vorticella* sp. they are not stainable with neutral red. The food vacuoles also stain with neutral red. The lipid bodies of *P. caudatum* aggregate towards the anterior and posterior ends of the ciliate as a result of the fixation.

In the fresh material treated with 2% osmium tetroxide solution, the externum of the lipid bodies is blackened due to its lipoidal nature (Figs. 1, 6 and 12), while the internum is non-osmiophile.

The externum of the lipid bodies colours with Sudan black (Figs. 2, 7 and 13) and mercuric-bromphenol blue (Figs. 3 and 14), thus showing the presence of lipids and proteins in the externum. The duplex nature of the lipid bodies persists on colouring with Sudan black at 60° C, or even after the unmasking techniques of Ciaccio (1926) using 1% phenol at 37° C for 24 h (Dutta, 1958a), and of Bradbury and Clayton (1958) using fixation in Flemming's fluid followed by postchroming in dichromate-calcium. Crescentic lipid bodies are very clearly revealed in Sudan black after fixation in Lewitsky fluid.

The blackening of the lipid bodies in 2% osmium tetroxide solution, and a positive reaction with performic acid-Schiff, shows the presence of unsaturated lipids in them.

The externum of the lipid bodies of *P. caudatum* shows a positive acid haematein test, thus showing the presence of phospholipids. A part of the lipid material in the externum is not extracted with cold acetone or ethanol or ether, as after these extractions the externum shows a weak sudanophil reaction. The unextracted lipids are forming a complex with protein, i.e. lipoprotein, which resists these extractions. The cytochemical reactions show the presence of phospholipids and lipoprotein in the externum of the lipid bodies.

In *C. cucullus* the externum of the lipid bodies stains violet with Nile blue sulphate, thus showing the presence of some neutral lipids in addition to the acidic ones. The presence of neutral lipids is also confirmed because these bodies are colourable with Sudan III and IV, and Fettrot. After extraction with cold acetone, the lipid bodies appear as fine sudanophil crescents (Fig. 8), stainable blue with Nile blue sulphate, which shows the presence of some acidic lipids, the neutral lipids having been extracted. With acid haematein, the lipid bodies are darkly stained, but after extraction with pyridine they become weakly positive: this weak positive reaction is due to the presence of proteins, while the decrease in staining reaction after pyridine extraction is proportional to the amount of phospholipids which have been extracted. On extraction with cold ethanol,
Dutta: Cytochemistry of Paramecium, Colpoda and Vorticella
the lipid bodies become completely non-sudanophil, showing thereby that both the neutral
lipids and phospholipids are completely extracted.

In *Vorticella*, the lipid bodies are positive in acid haematein (Fig. 15) and brom-
phenol blue (Fig. 14), thus showing the presence of phospholipids and proteins respectively.
The phospholipids are not extracted with cold acetone, but they are easily extracted
with cold ethanol showing thereby that the phospholipids are not forming a complex
with proteins.

The internum of the lipid bodies in all these ciliates remains uncoloured in all the
techniques that were used for the study of lipids, proteins, carbohydrates and nucleic
acids, and probably it is watery in nature. The internum of lipid bodies in *P. caudatum*
and *C. cucullus*, however, stains with neutral red supervitally.

**Mitochondria.**—The mitochondria of the ectoplasmic region are distributed in a
peripheral layer below the pellicle, but in the endoplasm they are distributed at random.
The ectoplasmic mitochondria are arranged in a characteristic pattern which corresponds
with the ciliary lines. The endoplasmic mitochondria are specially seen undergoing
constant movement in the living state. They do not at any stage enter the food vacuole,
but they aggregate around the growing contractile vacuoles.

The mitochondria stain with Janus green B supervitally which shows the presence of
oxidative enzymes in them. They stain specifically with acid fuchsine (Figs. 4, 9 and 10)
according to the special technique of Cain (1948). They stain darkly in Regaud or
Helly or Lewitsky/iron haematoxylin preparations.

The shape of mitochondria varies in different ciliates; in *P. caudatum* they are oval or
rod-like (Figs. 3 and 4), in *C. cucullus* they are more or less oval (Fig. 9) and in *Vorticella*
they appear as rods (Figs. 14 to 16).

**Cytoplasm.**—Both the ectoplasmic and endoplasmic regions of the cytoplasm show a
positive reaction with PAS, Best’s carmine and lead tetraacetate-Schiff but, after diges-
tion with salivary amylase or malt diastase, these reactions show completely negative
results, thus demonstrating the presence of glycogen in these forms. The glycogen is
dispersed in a dilute colloidal form throughout the cytoplasm. Owing to the presence of
glycogen, the cytoplasm also stains brown in Lugol’s iodine. The periphery of the food
vacuoles usually shows stronger reaction for glycogen than the endoplasm.

The cytoplasm stains with pyronin component of pyronin/methyl green, but it shows a
completely negative reaction after salivary ribonuclease extraction, thus showing the
presence of RNA. The presence of protein in the cytoplasm is inferred as it stains with
mercuric-bromphenol blue.

The cytoplasm contains a variable number of food vacuoles in these ciliates, but in
*C. cucullus* the food vacuoles practically pack up the whole of the endoplasm (Fig. 11).

**Nuclei.**—The nuclei in these ciliates are dimorphic. The macronucleus is compara-
tively large as compared to the micronucleus. In *P. caudatum* (Fig. 5) and *Vorticella* sp.
(Fig. 17) Feulgen positive material is present in fine granular state, but in *C. cucullus*
(Fig. 10) it is in the form of plaques applied to the inner surface of the nuclear membrane.

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6. Fresh material treated with 2% osmium tetroxide solution; 7, Formaldehyde-calcium, Sudan
black; 8, Formaldehyde-calcium, acetone extraction, Sudan black; 9, Helly, Cain’s acid fuchsine;
10, Carnoy, Feulgen; 11, Living specimen.
The remaining spaces of the macronucleus contain RNA. Feulgen positive material also stains with methyl green thus confirming the presence of DNA in it. The staining of macronucleus with mercuric-bromphenol blue shows the presence of proteins.

The micronucleus of *P. caudatum* (Fig. 5) and *C. cucullus* (Fig. 10) shows reactions for DNA, RNA and proteins.

**DISCUSSION**

**Lipid bodies.**—The lipid bodies, which have been identified in *Paramecium caudatum*, *Colpoda cucullus* and *Vorticella*, are in the form of homogeneous or duplex spheres, the latter showing differentiation into an osmiophile externum and an osmiophobe internum, like the typical Golgi bodies of other protozoan and metazoan cells. The lipid bodies are distributed at random in both the ectoplasmic and the endoplasmic regions of the cytoplasm.
In *Paramecium caudatum*, these bodies which stain supervitally with neutral red have been described by Hall and Nigrelli (1937) as neutral red-stainable globules (vacuome) in *P. bursaria*. Koehring (1930) observed a clustering of these neutral red-stainable globules of *Paramecium* around the food vacuoles. Sudanophil granules corresponding to these lipid bodies have been described as Golgi complex by Sen Gupta and Ray (1938) in *P. polyzoa*.

Park (1929) observed a blackening of the contractile vacuoles and certain knob-like bodies on the surface of meganucleus of *P. caudatum*; he, therefore, regarded both these structures as the Golgi bodies. During the present investigation on the same species, no osmiophile nature of the contractile vacuole or any other structure on the surface of meganucleus has been observed. Hall and Nigrelli (1937) also reported a complete non-osmiophile and non-argentophil nature of the contractile vacuole in *P. bursaria*.

The neutral red-stainable globules (vacuome) of *Vorticella* have been described as osmiophile by Hall and Dunihue (1931), Finley (1934), and Hall and Nigrelli (1937). Present investigation shows that the osmiophile inclusions of *Vorticella*, comparable morphologically and in distribution to the vacuome of these authors, are represented by the lipid bodies. Contrary to observations of earlier authors, these lipid bodies, however, do not stain supervitally with neutral red, although food vacuoles are easily stained. This non-staining of the lipid bodies with neutral red is probably due to some difference in species. In similar preparations, the lipid bodies of amoebae could be easily stained with neutral red.

Some blackened crescent-shaped inclusions, described in addition to the vacuome in *V. concavata* and *V. campanula* by Finley (1934), also represent the lipid bodies. These lipid bodies correspond to the dictyosomes and reveal characteristic ultrastructure with electron microscopy, similar to the Golgi apparatus of metazoan cells. Such Golgi dictyosomes have been identified among the ciliates by Noirot-Timothé (1957), and Fauré-Fremiet and Rouiller (1958).

Mitochondria.—The mitochondria of the ciliates have been extensively investigated by both the light and electron microscopy. With the morphological technique the mitochondria have been identified in *Paramecium* by Fauré-Fremiet (1910), Causey (1925-26), Horning (1926), and Sen Gupta and Ray (1938), in *Colpoda saprophylla* by Causey (1925-26), and in *Vorticella* by Hall and Nigrelli (1930), Hall and Dunihue (1931), and Finley (1934).

Watanahe and others (1954) demonstrated the cytochrome oxidase and succinic dehydrogenase activity in the isolated mitochondria of *Paramecium caudatum*. Tsujita and others (1954), using vital stain, Janus green and Nadi reaction, suggested the presence of cytochrome oxidase and cytochrome complex. These authors also used Altman’s or Altman-Koff’s test for their identification. The dehydrogenase activity has been described in the mitochondria of *Opalina caudinca* (Hunter, 1955) and *Stylochiria postulata* (Hunter, 1959). The respiratory enzymes have been located in the mitochondria of *Stentor coeruleus* (Weisz, 1950) also. The author (Dutta, 1961) has also concluded that in *Amoeba* sp, the mitochondrial enzymes are located in the peripheral-differentiated layer of mitochondria which probably corresponds to the double limiting membranes of the mitochondria revealed with electron microscopy.

The present investigation shows that the mitochondria of *Paramecium caudatum* are oval and rod-like. Of *Colpoda concavata* are more or less oval, and those of *Vorticella* are
The demonstration of the endoplasmic reticulum in *P. multimicronucleatum* by Sedar and Porter (1955), and Sedar and Rudzinska (1956) also supports the basophil nature of the cytoplasm, as the granular component of the endoplasmic reticulum is considered rich in RNA.

**Nuclei.**—The micronucleus of *Paramecium caudatum* is very rarely seen and probably it lies embedded in the macronucleus, and there is every possibility that this may be even an amicronucleate condition arising out of irregular division of *Paramecia* as suggested by Watanabe (1937). In *Vorticella*, also, the micronucleus has never been observed.

The quantitative analysis of macro- and micronuclei of *P. caudatum* was made by Moses (1959), who estimated similar concentrations of total protein, non-histone protein, DNA and RNA in both the nuclei. During the present investigation, the distribution of the nucleic acids and proteins has been studied in *P. caudatum*, *C. cucullus*, and *Vorticella*. Discrete chromatic granules embedded in achromatic matrix of the macronucleus of *Paramecium*, as described by Wichterman (1933), have also been recognized. In *C. cucullus*, as described by Burt (1940), the chromatin of macronucleus is often seen red-like. Supravital staining with Janus green B is considered specific for demonstrating mitochondria (Dutta, 1958, 1959, 1959c, and 1961).

The mitochondria consisting of double limiting membrane and the *eistru mitochondriales* have been studied by electron microscopy in almost all types of cells. The ultrastructure of mitochondria of Protozoa has been reviewed by Powers and others (1956), Sedar and Rudzinska (1956), and Dalton and Felix (1957). *Paramecium* (Ornstein and Pollister, 1952; Metz and others, 1953; Tsujita and others, 1954; Tsujita and others, 1955; Sedar and Porter, 1955; Sedar and Rudzinska, 1956; Powers and others, 1956; and Dalton and Felix, 1957), *Vorticella* (Dalton and Felix, 1957), *Spirotrichomonas ambiguum* (Randall, 1956), *Caeclostium* (Randall, 1956), *Tetrahymena pyriformis* (Rudzinska and Porter, 1954; Sedar and Rudzinska, 1956), and *Euplotes patella* (Roth, 1956; Powers and others, 1956).

In all the three ciliates investigated, the mitochondria are arranged in a characteristic pattern in a subpellicular region of the ectoplasm. Such a peculiar arrangement of mitochondria corresponds to the insertion of cilia in ciliary lines. The author also supports the views of Tsujita and others (1955) that the mitochondria in the ectoplasmic region might be providing energy for the ciliary movement.

**Cytoplasm.**—The cytoplasm, which is differentiated into the ectoplasmic and the endoplasmic regions, is rich in RNA, protein and glycogen. The glycogen in these ciliates is not accumulated into well-defined bodies, but it is present in dilute soluble form. The investigations of Barfurth (1885) on *Paramecium* and *Vorticella*, and those of Rammler (1925) on *Paramecium*, revealed the presence of carbohydrate reserves in the form of soluble glycogen dissolved in the protoplasm. Sen Gupta and Ray (1958) also demonstrated the presence of RNA and glycogen in the endoplasm of *P. polycaryum*. The ectoplasm according to them shows reaction for proteins. Faure-Fremiet and Thaurouex (1944), however, described the glycogen in *Paramecium* and *Vorticella* in the form of well-defined bodies. The present investigation shows that the glycogen along with RNA and protein is dispersed throughout the cytoplasm in *P. caudatum*, *C. cucullus* and *Vorticella*.

The demonstration of the endoplasmic reticulum in *P. multimicronucleatum* by Sedar and Porter (1955), and Sedar and Rudzinska (1956) also supports the basophil nature of the cytoplasm, as the granular component of the endoplasmic reticulum is considered rich in RNA.

**Nuclei.**—The micronucleus of *Paramecium caudatum* is very rarely seen and probably it lies embedded in the macronucleus, and there is every possibility that this may be even an amicronucleate condition arising out of irregular division of *Paramecia* as suggested by Watanabe (1937). In *Vorticella*, also, the micronucleus has never been observed.

The quantitative analysis of macro- and micronuclei of *P. caudatum* was made by Moses (1959), who estimated similar concentrations of total protein, non-histone protein, DNA and RNA in both the nuclei. During the present investigation, the distribution of the nucleic acids and proteins has been studied in *P. caudatum*, *C. cucullus*, and *Vorticella*. Discrete chromatic granules embedded in achromatic matrix of the macronucleus of *Paramecium*, as described by Wichterman (1933), have also been recognized. In *C. cucullus*, as described by Burt (1940), the chromatin of macronucleus is often seen
collected in the form of plaques applied to the inner surface of the nuclear membrane, but in some stages it is seen as a coarse network.

**ACKNOWLEDGEMENTS**

The author is grateful to Dr. Vishwa Nath, Professor of Zoology, Emeritus, Punjab University, for the supervision of this work, and to Dr. G. P. Sharma, Professor and Head of the Department of Zoology, Punjab University, for providing the laboratory facilities.

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Dutta: Cytochemistry of Paramecium, Colpoda and Vorticella

### Showing the cytochemical reactions of Paramecium caudatum

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**Key:** b & r = blue and red; C = Carnoy; c = crescents; F = formaldehyde solution; FCa = formaldehyde-calcium; G = gelatine; H = Helly; P = paraffin; PC = with postchroming; r = rings; WB-PE = weak Bouin followed by pyridine extraction; Z = Zénker; ~ ~ ~ = slightly positive; --- = moderately positive; ---- = strongly positive; --- = negative; * = after treatment with; × = not observed.
### Appendix II

*Showing the cytochemical reactions of Colpoda cucullus*

| Technique | Fixation | Embedding medium | Thick. of section | Reference | Lipid bodies | Mitochondria | Chloroplast | Microglo- | Micro- |
|-----------|----------|------------------|-------------------|-----------|--------------|--------------|-------------|-----------|
| SB (Sudan black) in 70% ethanol at ordinary temp. and at 60°C | L, FCa, FCa–PC | G | 10 μ | Baker, 1944, 1946, 1956 | — | e | — | — | — |
| SB in propylene glycol | L, FCa, PC–PC | G | 10 μ | Chalfin and Pott, 1931 | — | e | — | — | — |
| SB* acetone | Fresh, FCa | G | 10 μ | Krishna, 1940; Pearse, 1950 | — | e | — | — | — |
| SB* ether or ethanol | Fresh, FCa | G | 10 μ | | — | — | — | — | — |
| SB | L | G | 10 μ | Cain, 1947, 1948a | — | — | — | — | — |
| Nile blue | FCa, FCa–PC | G | 10 μ | | — | — | — | — | — |
| Sudan III and IV in 70% ethanol/acetone | FCa, FCa–PC | G | 10 μ | kay and Whitehead, 1941 | — | e | — | — | — |
| Fettirn in 70% ethanol | FCa, FCa–PC | G | 10 μ | Pearse, 1960 | — | e | — | — | — |
| AH (Acid haematin) | FCa–PC | G | 10 μ | Baker, 1940 | — | — | — | — | — |
| AH* PE (Pyridine ext.) | WB–PE | G | 10 μ | Pearse, 1960 | — | e | — | — | — |
| Flechler’s reaction | F, FCa | G | 10 μ | Pearse, 1960 | — | e | — | — | — |
| Flechler’s reaction | F, FCa | G | 10 μ | | — | — | — | — | — |
| Feyrer’s mounting-staining method | F, FCa | G | 10 μ | | — | — | — | — | — |
| Cholesterol reactions | FCa | G | 10 μ | Schulz, 1924, 1925; Pearse, 1960; Gomori, 1952; Romieu, 1957 | — | — | — | — | — |
| Plasminogen | FCa | G | 10 μ | Pearse, 1960; Lillie, 1953 | — | — | — | — | — |
| Caesalpinia’s technique | FCa–phenol | G | 10 μ | Bradbury, 1956 | — | — | — | — | — |
| Fleming’s unmasking | Fleming–PC | G | 10 μ | Bradbury and Clayton, 1958 | — | — | — | — | — |
| PAS (periodic acid/Schiff) | C | P | 10 μ | Hotchkiss, 1948; Pearse, 1950 | — | — | — | — | — |
| PAS* acetylation | C | P | 10 μ | | — | — | — | — | — |
| PAS* restoration with 0.1N KOH | C | P | 10 μ | | — | — | — | — | — |
| Beat’s carmine | C | P | 10 μ | Pearse, 1950 | — | — | — | — | — |
| PAS or Beat’s carmine* salivary amylase | C | P | 10 μ | | — | — | — | — | — |
| Mercuric–bromphenol blue | PCa, C | P or G | 10 μ | Mania and others, 1953 | — | — | — | — | — |
| Feulgen | C | P | 10 μ | Pearse, 1960 | — | — | — | — | — |
| Feulgen* trihaloacetic acid | C | P | 10 μ | Schneider, 1945 | — | — | — | — | — |
| FMG (pyronin G/methyl green) | Z | P | 10 μ | Jordan and Baker, 1935 | red | red | b & r | dark red | — |
| FMG* salivary ribonucleic acid | Z | P | 10 μ | Bradbury, 1956a | — | — | — | — | — |

Key: b & r = blue and red; C = Carnoy; e = crescents; F = formaldehyde solution; FCa = formaldehyde-calcium; G = gelatine; L = Lewitsky; P = paraformaldehyde; PC = propylene glycol; PCa = propylene glycol–calcium; PE = pyridine–ethanol; Z = Zenker; W = weak; B = branched; PE = pyridine–ethanol; F = formaldehyde; A = alcohol; R = red; G = green; B = blue; C = cyan; S = strong; M = moderate; N = negative; * = after treatment with; X = not observed.

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**References:**
- Mazia and others. 1953
- Pearson, 1960
- Schneider, 1945
- Jordan and Baker, 1955
- Bradbury, 1950a
- Schultz, 1924, 1925
- Pearse, 1960
- Gomori, 1952
- Romieu, 1927
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## Table of Cytochemical Reactions of Vorticella sp.

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<td>Ciavino’s unmasking</td>
<td>FCa-phosphol</td>
<td>G</td>
<td>10 μ</td>
<td>Nath, 1957</td>
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<tr>
<td>Flemming’s unmasking</td>
<td>Flemming-PC</td>
<td>G</td>
<td>10 μ</td>
<td>Bradbury, 1956</td>
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<tr>
<td>PAS (periodic acid/Schiff)</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>Hotchkiss, 1948; Pearson, 1960</td>
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<tr>
<td>PAS* acetylation</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>McManus and Cason, 1959</td>
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<tr>
<td>PAS* restoration with 0-1N KOH</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>Pearson, 1960</td>
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<tr>
<td>Best’s carmine</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>Shimizu and Kumamoto, 1952</td>
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<tr>
<td>Lead tetraacetate/Schiff</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>Pearson, 1960</td>
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<tr>
<td>PAS or Best’s carmine or Lead tetraacetate/Schiff*</td>
<td>C and Z</td>
<td>P</td>
<td>10 μ</td>
<td>Pearson, 1960</td>
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### Appendix III—contd.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Fixation</th>
<th>Embedding medium</th>
<th>Thickness of section</th>
<th>Whole mount</th>
<th>Reference</th>
<th>Lipid bodies</th>
<th>Mitochondria</th>
<th>Cytoplasm</th>
<th>Macronucleus</th>
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<tr>
<td>MBB (mercuric-bromphenol blue)</td>
<td>FCa</td>
<td>G</td>
<td>10 µ</td>
<td>8</td>
<td>Mizia and others, 1953</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MBB* cold ethanol</td>
<td>Fresh</td>
<td>C</td>
<td>-</td>
<td>8</td>
<td>Pearson, 1960</td>
<td>-</td>
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<tr>
<td>Feulgen</td>
<td>Z</td>
<td>S</td>
<td>Schneider, 1945</td>
<td>-</td>
<td>-</td>
<td>red</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Feulgen* trichloroacetic acid</td>
<td>Z</td>
<td>S</td>
<td>Jordan and Baker, 1955</td>
<td>-</td>
<td>-</td>
<td>blue</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P/MG (pyronin G/methyl green)</td>
<td>Z</td>
<td>S</td>
<td>Bradford, 1936b</td>
<td>-</td>
<td>-</td>
<td>blue</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P/MG* salivary ribonuclease</td>
<td>Z</td>
<td>S</td>
<td>Schneider, 1945</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Key: C = Carnoy; e = crescents; F = 4% formaldehyde; FCa = formaldehyde-calcium; G = gelatine; L = Lewitsky; P = paraffin; P/MG = with postchroming; r = rings; S = whole mount smear; WB-PE = weak Bouin's followed by pyridine extraction; Z = Zenker; - = weak reaction; - - = moderate reaction; - - - = strong reaction; - - - - = negative reaction; * = after treatment with.

Date: Cytochemistry of Paramecium, Colpoda and Vorticella
THE CONTRACTILE VACUOLE OF PROTOZOA AND THE GOLGI APPARATUS

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(Received 28 July 1961)

Abstract

The homology of the contractile vacuolar complex of Protozoa to the Golgi apparatus has been discussed in the light of recent morphological, cytochemical and electron microscopical investigations, and strong evidence has been put forth against this homology. The contractile vacuoles or their feeding canals have no relation whatsoever to the Golgi apparatus.

The hypothesis that the contractile vacuolar complex of Protozoa represents the Golgi apparatus has been favoured from time to time by the light and electron microscopical investigations. The light microscopists who favour this view have mainly relied on the cytochemical techniques of silver and osmium impregnation which are now considered capricious and highly unreliable. Baker (1954) regards these impregnation techniques as 'very reliable to be misleading morphologically and quite useless histochemically'. Baker (1957) has reported that a wide variety of different objects like cell membrane and various cuticular structures, the nuclear membrane, and mitochondria and also various bodies like parabasal bodies, the contractile vacuoles and their reservoirs in Protozoa are often blackened by the Golgi techniques. Concerning the Golgi techniques, Hill (1933) reports that 'it is extremely difficult to give exact criteria for the Golgi apparatus; in fact, no basis for the identification of Golgi material in Protozoa has yet been agreed upon'. MacLennan (1939) is of the view that in Protozoa, 'the term Golgi body, being based upon non-specific criteria, ... includes a heterogeneous group of structures, including scattered endoplasmic granules and granules associated with the contractile vacuoles or fused to form heavy and permanent vacuolar membranes ...'. MacLennan (1943) has also observed that both lipoidal and non-lipoidal bodies in Protozoa react identically to the Golgi techniques. Kudo (1954) remarks: 'It seems impossible at present to indicate just exactly what the Golgi apparatus is, since the so-called Golgi techniques ... are not specific and the results obtained by using the same method often vary a great deal'.

The homology of the contractile vacuole of Protozoa to the Golgi apparatus was first suggested by Nassonov (1924 and 1925). Nassonov (1924) demonstrated the impregnation of the walls of contractile vacuoles or their feeding canals in Zoathanum vicinum, Vorticella, Limnaea stagnalis, Nassula lateritidae and Paramecium caudatum by the Golgi methods of preparation, and he concluded that the membrane so impregnated secreted or excreted the fluid found in the contractile vacuoles at diastole. Nassonov (1925) mentioned that the wall of the contractile vacuole of Chilodon sp. and Dogielia sphasii did not blacken with osmic acid impregnation, although he noticed the blackening
of a ring-shaped region of protoplasm surrounding the vacuole. He concluded that this osmiophile material together with the contractile vacuole in Protozoa was the homologue of the Golgi apparatus in metazoan cells and the whole formed a secretory mechanism. Gatenby (1929) accepted Nassonov's homology and suggested that 'particularly all modern cytologists are now disposed to believe Nassonov's views'. Gatenby (1938) suggested modification of Nassonov's hypothesis and he considered that osmiophile material alone represented the Golgi apparatus and that its association with the contractile vacuole was only a secondary association. Other examples, which show the osmiophile nature of the cortices of contractile vacuoles or the surrounding protoplasm and thus support the Nassonov's homology, are given in Table I.

**Table I**

<table>
<thead>
<tr>
<th>Name</th>
<th>Reaction of the cortex of contractile vacuole to the Golgi techniques</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Euplotes</em></td>
<td>Osmiophile contractile vacuole and feeding canals</td>
<td>Gelei (1928)</td>
</tr>
<tr>
<td><em>P. caudatum</em></td>
<td>Osmiophile</td>
<td>Park (1929)</td>
</tr>
<tr>
<td><em>Euglena</em></td>
<td>Osmiophile</td>
<td>Smyth (1941)</td>
</tr>
<tr>
<td><em>Vorticella</em></td>
<td>Osmiophile</td>
<td>Smyth (1934)</td>
</tr>
<tr>
<td><em>Vorticella</em></td>
<td>Osmiophile</td>
<td>Gatenby (1941)</td>
</tr>
<tr>
<td><em>Vorticella</em></td>
<td>Very thick osmiophile cortex</td>
<td>Hall and Burnie</td>
</tr>
<tr>
<td><em>Vorticella</em></td>
<td>Osmiophile</td>
<td>Gatenby (1941)</td>
</tr>
<tr>
<td><em>Vorticella</em></td>
<td>Osmiophile</td>
<td>Finley (1934)</td>
</tr>
</tbody>
</table>

King (1933) described the Golgi bodies in *Euplotes* sp. as represented by small accessory osmiophile vacuoles which are associated with the contractile vacuole. According to him, these accessory vacuoles arise from the osmiophile radiating canals of the contractile vacuole. The same author (1935) described in *Paramecium multimicronucleatum* a 'specialized' excretory protoplasm surrounding the vacuolar canals, but at the same time he denied the homology of Nassonov. Gelei (1933) reported in *Spathidium* the osmiophile nature of the wall of contractile vacuole and of smaller osmiophile vacuoles present in the vicinity of contractile vacuole as well as scattered in the cell. Bush (1934) identified the Golgi material in *Haplophrys* as a long osmiophile tube which forms part of the vacuolar system and extends the full length of the eiliate. Gatenby (1941) reported a very thick osmiophile (Golgi) cortex round the contractile vacuole of *Vorticella*. He also observed that during the division of the eiliate the contractile vacuole with its osmiophile membrane was passed on completely to one daughter organism, while the other daughter organism formed a new vacuole.

The application of osmium impregnation techniques to some flagellates also supports Nassonov's homology. Gatenby and Singh (1938) described the Golgi apparatus in *Corynomaus subtilis* and *Euglena* sp. as osmiophile granules associated with the osmoregulatory mechanism of the cell, while in *Euglena viridis* they described it as osmiophile bodies closely applied to the so-called contractile vacuole. In *Chilomonas paramecium*,...
Gatenby and Snyth (1940) observed a cortical substance (Golgi apparatus) surrounding the contractile vacuole, which had the power of reducing osmium tetroxide and thus impregnating black.

The electron microscopists have also provided evidence in favour of the Nassonov's homology, but the evidence provided by them is not convincing. Gatenby and others (1955), particularly, have interpreted the ultrastructure of the cortices of the contractile vacuole or their feeding canals in *Paramecium* and *Vorticella* as double membranes which they consider is homologous to the ultrastructure of the Golgi apparatus. They report that, 'in the case of the contractile vacuole of *Vorticella* sp., the vacuole in partial systole contains a small cavity surrounded by a spongy mass of double membranes very similar to that of the cortex of the vacuole canal of *Paramecium*'. Dalton and Felix (1957) have identified membranes and granules comparable to the Golgi complex of the vertebrate cell in the wall of the contractile vacuole of *Chlamydomonas*. Grimstone (1961), who has reviewed the fine structure of Protozoa, considers that the evidence produced from the electron microscopy by Dalton and Felix (1957), and Gatenby and others (1955) in support of the homology of the contractile vacuole to the Golgi apparatus is 'unconvincing'. Lutfy (1958) has expressed the opinion that the ultrastructure of the cortices of the contractile vacuoles of protozoans, the parabasals of flagellates and the dictyosomes of sponge (*Grantia*) choanocytes revealed in all of them double lamellae or flattened closed vesicles which resemble the metazoan Golgi apparatus. Although Gatenby and Tahmisian (1959) have interpreted the lamellated osmiophile membranes surrounding the apparently non-contractile vacuoles of the marine sponges as the Golgi apparatus, their study of the ultrastructure of the contractile vacuole of freshwater sponge (*Ephydatia fluviatilis*), however, does not support Nassonov's homology since they have suggested that in the freshwater sponge there is absolutely no relation between the contractile vacuole and the Golgi apparatus; the latter according to them is represented by the dictyosomes (Golgi bodies).

As against the views of Nassonov and his supporters, there is available a mass of evidence which does not lend any support in favour of the homology of the contractile vacuolar complex to the Golgi apparatus. The investigations of a large number of authors who employed the classical techniques of osmium or silver impregnation provide a strong evidence against the Nassonov's homology since they did not notice the impregnation of the walls of the contractile vacuoles or of the adjacent protoplasm in a large number of protozoans (see Table II).

The author considers that the impregnation techniques are most unreliable since indiscriminate deposition takes place in forms like *Paramecium*, *Vorticella* and *Agyro*thecus, etc. The author has employed Kolatchev technique on these forms and consistent results have never been obtained with this technique. The author has also observed that the treatment of protozoans with 2 per cent osmium tetroxide for a short time does not show the osmiophile nature of the cortices of the contractile vacuole or their feeding canals or of their surrounding protoplasm; although with this treatment the lipid bodies of forms like *Agyro*thecus macropharyngus (Dutta, 1958a), *Balantidium helena* (Dutta, 1958a), *Amoeba* sp. (Dutta, 1959a and 1961a), *Paramecium caudatum* (Dutta, 1961b), *Vorticella* (Dutta, 1961b), and *Calyptoplasma circinatum* (Dutta, 1961b), which appear as homogenous or duplex bodies, are always blackened due to their lipoidal nature. Moreover, even the cytochemical investigations of these forms have never revealed the lipoidal
nature of the cortices of the contractile vacuole or their feeding canals although the lipid bodies are demonstrable.

The electron microscopists have produced a strong evidence against the Nassonov's homology of the contractile vacuole of Protozoa to the Golgi apparatus. They have established beyond doubt that in Trachdomonas sp. (Ueda, 1960), Amoeba proteus, Pelomyxa carolinensis and Hartmannella rynodes (Pappas and Brandt, 1958), A. proteus (Mercer, 1959), A. proteus, Pelomyxa carolinensis and Hartmannella rynodes (Pappas, 1959) the ultrastructure of the cortices of the contractile vacuoles is altogether different from the typical Golgi dictyosomes of Protozoa described in Chlamydomonas (Sager and Palade, 1957), Amoeba proteus (Cohen, 1957; Mercer, 1959), A. spumosa (Cigada Leonardi, 1958), A. proteus, Pelomyxa carolinensis and Hartmannella rynodes (Pappas and Brandt, 1958) and ciliates (Noirot-Timothee, 1957; Faure-Fremiet and Rouiller, 1958). Pappas (1959) has particularly mentioned that the contractile vacuole and its components bear no morphologic resemblance to the metazoan Golgi complex. The author has also reported earlier that in Amoeba sp. (Dutta, 19596) there is apparently no direct relation between the formation of the contractile vacuole and the lipid bodies or the so-called Golgi bodies.

In the living amoeba, when examined under the phase-contrast microscope, the contractile vacuole is seen to arise by the coalescence of small accessory vacuoles which slowly aggregate into three or four vacuoles, the latter finally uniting to form a large contractile vacuole.

The investigation of Rudzinska (1958) on the ultrastructure of the contractile vacuole of Tetrahymena infusorum reveals that there is no homology between the contractile vacuole and the Golgi apparatus, since she has reported in this species independent Golgi apparatus
in the form of 'dietvosome-like bodies' composed of 'piles of packed vesicles' distributed at random throughout the cytoplasm. She has reported a continuity between some of these vesicles and the contractile vacuole and, therefore, suggests that these vesicles might be responsible for the drainage of water from the cytoplasm into the contractile vacuole. Studies on the ultrastructure of the amoebae (Pappas and Brandt, 1958; Pappas, 1959; Mercer, 1959) provide a strong evidence against the suggestion made by Rudzinska (1958) since these authors have demonstrated the origin of contractile vacuole by the simple coalescence of small vacuoles or vesicles which do not arise from the Golgi apparatus but ‘may be derived from pinocytosis vacuoles or components of the endoplasmic reticulum’, as reported by Pappas (1959), or produced by the activity of mitochondria which aggregate round the contractile vacuole as described by Mercer (1959). Similar arrangement of mitochondria in a layer surrounding the contractile vacuole has also been reported by Pappas and Brandt (1958) and Pappas (1959). According to Mercer (1959) the activity of these mitochondria causes local changes in the cytoplasmic space between them and the membrane of the contractile vacuole which lead to the separation of droplets of water enclosed in membranes. He describes that the membranes of these small vacuoles or vesicles and of the contractile vacuole itself are morphologically identical in their ultrastructure. Pappas and Brandt (1958) have demonstrated that these vesicles contribute both their contents and their membranes to the growing contractile vacuole during diastole.

Das and Tewari (1955) suggested the origin of the contractile vacuole from the Golgi bodies in *Amoeba verrucosa*. The Golgi bodies described in this form are (1) dark solid granules, (2) small vacuoles with circular or crescent-shaped dark rims, and (3) a few (three or four) large vacuoles. According to them the fully formed Golgi bodies which are in the form of large osmiophilic vacuoles coalesce to form the fully developed contractile vacuole, the wall of which does not show any osmic impregnation. The observations of the author on *Amoeba sp.* (Dutta, 1956) do not support the views of Das and Tewari (1955) since there is clear evidence from the living study that the lipid bodies of the amoeba, which are granular, are not in any way transformed into the contractile vacuole.

The study of the parasitic Protozoa further provides a strong objection to Nasonov's homology since Golgi bodies have been demonstrated in many forms in which the contractile vacuoles are altogether absent, e.g. *Sporozoa*. *Opalina ranarum* provides an interesting example in which, in spite of the absence of contractile vacuole, there are present Golgi bodies corresponding to the lipid bodies of Dutta (1956) and the Golgi dictyosome of Noirot-Timotheé (1958) (quoted by Grimstone, 1961). The evidence presented above from the morphological, cytological and electron microscopical investigations, therefore, strongly suggests that there is no homology between the contractile vacuolar complex and the Golgi apparatus.

Acknowledgements

The author is grateful to Dr. Vishwa Nath, Professor of Zoology, Emeritus, Panjab University, for the supervision of this work, to Dr. G. P. Sharma, Professor and Head of the Department of Zoology, Panjab University, for providing research facilities and to the National Institute of Sciences of India for the award of a Research Fellowship, during the tenure of which this paper was prepared.
Dutta: Contrasted Varieties of Protozoa and Golgi Apparatus

Literature Cited

The cell inclusions of the trophic and cyst forms of the colourless euglenoid flagellate, Khawkinea sp., are the mitochondria, lipid bodies, paramylum, stigma and the nucleus. The mitochondria reveal certain thickened end-granules which separate and develop into independent granules. These granules grow into homogeneous and duplex lipid spheres. In the absence of chloroplasts, pyrenoids or leucoplasts, the paramylum bodies are synthesized or segregated in the internum of the duplex lipid bodies. The lipid granules, spheres and the externum of the duplex bodies, contain phospholipids and lipoproteins. Paramylum is composed of some peculiar polysaccharide. Stigma shows positive reaction for carotenoids. The cytoplasm, which shows positive staining for RNA and proteins, is devoid of any polysaccharides. The pellicle and the bifurcated base of the flagellum, however, contain poly, polysaccharides in addition to proteins. The present investigation does not support the homology of the stigma to the Golgi apparatus.

Introduction
The majority of the euglenoid flagellates, including colourless as well as green forms, possess a typical metaplasmic reserve material, the paramylum (paramylon), which is not found outside the order Euglenoidina. As regards the origin and cytochemistry of the paramylum bodies, the authors (Nath and others, 1960) have already reported that in the colourless euglenoid flagellate, Khawkinea sp., the paramylum bodies, which are composed of a peculiar neutral polysaccharide, are synthesized or segregated in the internum of the duplex lipid bodies; it has been emphasized that the presence of chloroplasts, pyrenoids, leucoplasts or their biochemical equivalent is not absolutely essential for the synthesis of paramylum.

The present paper deals with the detailed cytochemical study of Khawkinea sp. For the earlier work on the cytoplasmic inclusions of the euglenoids, reference may be made to Patten and Beams (1936), Gatenby and Singh (1938), and the review on euglenoid flagellates by Jahn (1946).

Material and Technique
The colourless euglenoid flagellate, Khawkinea sp., appears in large numbers in the culture of dry leaves in tap water. The trophic form appears in the culture after 24 h while the cysts are present in the old cultures.

Living study.—The trophic form of the living flagellate was examined under the phase-contrast microscope in the culture medium. Janus green B was used as a vital stain for mitochondria, and neutral red for lipid spheres. The vital dyes were dissolved in the culture medium in a dilution of 1 : 50000 to 1 : 100000. The inclusions of the cyst form are not stained vitally as the cyst wall is impermeable to them.

Cytological techniques.—For the cytological study the following techniques were used.

Cytochemistry of *Khaivkinea* sp.

Fixed in Zenker, Helly, Regaud or Lewitsky, and stained in Heidenhain's iron haematoxylin for the routine study of cell structures.

Fixed in Helly, Regaud or Lewitsky, and stained in acid fuchsine (Cain, 19486) for the study of mitochondria.

Used Hermann's postosmication technique (Baker, 1957) for the study of mitochondria.

Used buffered osmium staining technique (Wigglesworth, 1957) for the study of mitochondria.

Used Kolatchev technique (Kolatchev, 1917) for the study of lipoidal inclusions.

**Cytochemical tests**—For the cytochemical study the specimens were studied as whole mounts, or sometimes paraffin or gelatine sections were employed. The various fixatives employed and the cytochemical tests used for the study of lipids, proteins, carbohydrates, nucleic acids, enzymes and carotenoids are given in Appendices I to IV.

In addition to the routine tests for the demonstration of lipids, the following unmasking techniques were tried for the demonstration of bound lipids:

- Phenol unmasking technique (Ciaccio, 1926).—The material was fixed for 6 h in formaldehyde-calcium and treated with 1% phenol at 37°C for 24 h as described by Dutta (1958).

- Clayton's unmasking techniques (Clayton, 1958):

<table>
<thead>
<tr>
<th>Fixation</th>
<th>Reagent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flemming</td>
<td>90% ethanol</td>
<td>6 h</td>
</tr>
<tr>
<td></td>
<td>100% dioxane</td>
<td>1 h</td>
</tr>
<tr>
<td></td>
<td>1% phenol</td>
<td>24 h</td>
</tr>
<tr>
<td></td>
<td>5% pyrogallol</td>
<td>1/2 h at 60°C</td>
</tr>
<tr>
<td></td>
<td>5% hydroquinone</td>
<td>1 h at 60°C</td>
</tr>
</tbody>
</table>

- Flemming’s unmasking technique (Bradbury and Clayton, 1958).—Fixed in Flemming’s for 24 h and postchromed in dichromate-calcium (Baker, 1946).

- Clayton's unmasking techniques (Clayton, 1959).—The material was fixed in 1% cadmium chloride or saturated solution of mercuric chloride for 18 h.

  After these unmasking techniques, the material was coloured with Sudan black. There was no improvement in colouring with Sudan black after these techniques.

  In addition to these, Berenbaum's method (Berenbaum, 1958), employing burnt Sudan black on Carnoy-fixed material, was also used for the study of bound lipids. This technique also did not reveal any bound lipid.

**Observations**

The cell inclusions, which have been identified during the living study of the trophic form of *Khaivkinea* sp. (Fig. 1) under the phase-contrast microscope, are the mitochondria, lipid bodies, paramylum bodies, stigma and nucleus. The chloroplasts are completely absent in this form. This flagellate is permanently colourless and remains so even if the culture is exposed to sunlight. The proximal end of the flagellum which passes through the reservoir shows a bifurcation; at the point of bifurcation there is present a paraflagellar body. The pellicle, which covers the body, shows characteristic striations.

In the older cultures the flagellates undergo encystment, and during this process the flagellar apparatus disappears. The cysts develop a double cyst wall and reveal the
same inclusions as the trophic form. The cytochemical reactions of the various inclusions are given in Appendices I to IV.

**Mitochondria.**—The mitochondria of the trophic form are distributed throughout the cytoplasm, in addition to peripheral layer below the pellicle, while in the cyst they are distributed at random. The mitochondria appear as short filaments with end-granules. These mitochondria are demonstrable with the special mitochondrial techniques such as Regaud-jon haematoxylin, Hermann’s postosmication technique (Baker, 1957) (Fig. 2), buffered osmium staining technique (Wigglesworth, 1957) (Fig. 3), Lewitsky or Regaud or Helly/acid fuchsin (Cain, 1948). They stain with Janus green B supervitally. The demonstration of cytochrome oxidase activity in them with the Nadi reaction (Moog, 1943) also points to their mitochondrial nature.

The mitochondria, which in both the trophic and cyst forms colour with Sudan black B (SBB) (Figs. 4 to 8), are acid haematein (AH) positive. Some of the mitochondria are Sudan III and IV positive; they also stain violet with Nile blue sulphate (NBS). These reactions show the presence of phospholipids in addition to some triglycerides in them.

After extraction with cold acetone or ethanol, the mitochondria become comparatively less sudanophil; this shows that the lipids which remain unextracted are forming some lipoprotein complex. The presence of proteins in the mitochondria is evident from their staining with mercuric bromphenol blue and coupled tetrazonium (CTZ) (Fig. 9). A negative CTZ reaction obtained after performic acid and benzoylation confirmed the presence of tryptophan. They further show negative reactions with Millon’s for tyrosine, and Sakaguchi for arginine. They also show positive reaction for RNA (positive staining with pyronin, negative after ribonuclease).

**Lipid bodies.**—The lipid bodies appear in the young trophic form as small granules, which appear to arise in association with the mitochondria in the form of surface granules or end-granules, separating subsequently. The lipid granules grow into spheres; when the synthesis of paramylum starts, these spheres assume a duplex appearance. The lipid bodies subsequently grow, and the material which is synthesized or segregated in the internum represents the paramylum. With further growth of paramylum in the internum, the material of the externum is consumed and it disappears altogether when the paramylum is fully formed. The sequence of development of the paramylum from the lipid granules shows that the primary function of the lipid bodies in this form is the synthesis or segregation of paramylum.

The cytochemical reactions of the lipid bodies of trophic form (Appendix I) include the reactions of lipid granules, spheres and externum of the duplex spheres, in the internum of which the paramylum bodies arise. The lipid bodies colour with SBB (Figs. 4 and 5). The internum of the duplex spheres remains uncoloured at ordinary temperature or even if the colouring is done at 60°C. This non-sudanophil nature of the internum of duplex bodies can be due either to the (1) presence of cholesterol and cholesterol esters which have a melting point higher than 60°C, or (2) the presence of masked or bound lipids, or (3) the non-lipid nature of the internum. Since the tests for cholestrols or cholesterol esters (Schultz, 1924, 1925; Romieu, 1927; and Okamoto’s method described by Pearse, 1960) are negative, a number of special unmasking techniques for the demonstration of bound or masked lipids (Ciaccio, 1926; Berenbaum, 1938; Bradbury and Clayton, 1938; and Clayton, 1938) were employed. Even after these
unmasking techniques, the presence of any lipid material in the internum could not be established. Moreover, the internum of the duplex bodies is non-osmiophile as revealed by Kolatchev and fresh osmium tetroxide preparations. Since the whole series of stages of development of the paramylum bodies in the internum of duplex bodies have been traced (Nath and others, 1960), it can be concluded that the duplex appearance of the lipid bodies in this form is due to the presence of non-lipid material (paramylum) in the internum.

The lipid bodies can remain unstained in NBS either due to the presence of cholesterol, cholesterol esters or some unsaturated lipids, but the negative Schultz test show the absence of cholesterol and their esters in them. The lipid bodies are AH positive, which shows the presence of phospholipids. To find out the exact nature of the lipids which remain unstained in NBS, a section of the flagellates, previously stained with NBS (where lipid bodies remain unstained), was coloured with SBB, when the lipids were clearly demonstrable in the lipid bodies. Subsequently, the colour of SBB was removed from this section by keeping it in 70% ethanol, and it was restained with AH, which showed a positive reaction, thus demonstrating the phospholipid nature of the lipids.

After extraction with cold acetone or ethanol, the lipid bodies remain weakly sudanophil, and this faint coloration is due to the presence of some unextracted lipids which are forming a complex with proteins (lipoproteins). These proteins are demonstrable on staining with mercuric bromphenol blue and coupled tetrazonium (Figs. 9 to 11).

In Kolatchev preparations, the phospholipids are not preserved but the lipoprotein component of these bodies is clearly demonstrable. It may be concluded that the lipid granules, spheres and the externum of duplex spheres show the presence of phospholipids and lipoproteins.

The lipid bodies of the cyst form are sudanophil (Figs. 6 to 8); they are also stained with mercuric bromphenol blue (Fig. 10).

The lipid bodies of the cyst, like the trophic form, show positive reaction for phospholipids and lipoproteins.

Paramylum bodies.—The paramylum bodies appear as transparent rounded, elliptical, oval or rod-like structures. They swell on treatment with dilute potassium hydroxide solution, but with concentrated potassium hydroxide or sulphuric acid they immediately dissolve.

The chloroplasts, pyrenoids or leucoplasts, generally considered responsible for the formation of paramylum in other euglenoids, are absent in Khawkinea. In this form the synthesis of paramylum takes place in association with lipid bodies. In the trophic form, the details of this synthesis have been described earlier (Nath and others, 1960).

Paramylum bodies do not stain with PAS or its various modifications (Appendices II and IV). They remain unaffected by salivary amylase or deamination. They also do not show any positive test for acid mucopolysaccharides. Moreover they do not show reactions for lipids, proteins or nucleic acids. These bodies, however, show induced metachromasia according to the technique of Kramer and Windrum (1954), used for the demonstration of neutral polysaccharides. According to this technique the neutral polysaccharides of paramylum are converted to acidic ones by sulphating them with chlorosulphonic acid in dry pyridine for 10 mins., they are stained subsequently in Azure A at pH 4.5 (Spicer, 1960). With this treatment the paramylum bodies show an induced
γ-metachromasia (Fig. 12). No such metachromasia could be induced with the technique of Lewis and Adesanya (1959) in this material.

The material of the paramylum appears to be a peculiar neutral polysaccharide which remains unstained in the usual techniques applied for the demonstration of polysaccharides.

**Stigma.**—The stigma of *Kawkinea* appears in the living condition as an orange-red, concavo-convex plate; its concave side facing the gullet (Fig. 1). In exceptional cases there are present two separate stigmas in the same individual. The stigma in the living individual often reveals certain granules embedded in the matrix.

The cytochemical tests (Appendix III) show the presence of carotenoids in the stigma. With Carr-Price test the stigma instantaneously turns sharp blue with a bright lustre. It gives the same reaction with Lugol’s iodine, perchloric acid and trichloroacetic acid, but it shrinks greatly.

The whole flagellate disintegrates on treatment with concentrated sulphuric acid at room temperature but, when the acid cooled below 4°C (as recommended by Pearse, 1950) was used, the stigma instantaneously turned sharp blue, disintegrated into granules and the colour faded away.

With concentrated hydrochloric acid or formic acid the stigma turned blue and then disintegrated; sometimes it disintegrated even before turning blue.

With aniline in excess of acid, the stigma turned into a dark red duplex sphere or it split into granules.

The lipid nature of the stigma is revealed by (1) its colouring with SBB, and (2) its osmiophile nature as shown in Baker’s HPO (Fig. 2), Kolatchev technique, and the treatment of fresh specimen with 2% osmium tetroxide solution. Staining with mercuric bromphenol blue indicates the presence of proteins.

The stigma reacts negatively to the routine tests for nucleic acids and carbohydrates.

**Nucleus.**—The nucleus in trophic as well as in cyst forms appears vesicular with a central endosome. In Zenker or Helly/iron haematoxylin preparations, the endosome is clearly visible in the centre of the nucleus, and the space between the endosome and the nuclear membrane is packed with chromatin granules. In acetocarmine preparations, the chromatin granules are stained, while the endosome remains unstained. These granules are Feulgen positive (Figs. 13 and 14), thus showing the presence of DNA in them. In pyronin/methyl green preparations, the matrix of the nucleus along with the endosome stains with pyronin due to the presence of RNA, while the peripheral Feulgen positive granules stain blue or bluish green due to the presence of DNA. Salivary ribonuclease extraction removes RNA from the endosome and the matrix of the nucleus, while trichloroacetic acid removes both the nucleic acids.

**Cytoplasm.**—The cytoplasm shows positive reaction for RNA (positive staining with pyronin, negative after salivary ribonuclease), and proteins (positive staining with mercuric bromphenol blue and CTZ) (Appendices I and IV). Occasionally the cytoplasm of the cyst and trophic form shows the presence of some diffuse lipids as revealed by colouring with SBB.

**Pellicle.**—The pellicle is a thin, delicate, flexible membrane, which allows the characteristic euglenoid movements of the organism in life. It reacts positively in periodic acid-Schiff and lead tetraacetate-Schiff tests (Fig. 15); these reactions are not affected
by pre-treatment with 1% malt diastase in acetate buffer. It does not show any meta-chromatic staining with the techniques used for the demonstration of acid mucopolysaccharides. It shows positive staining with mercuric bromphenol blue (Fig. 9) and CTZ tests for proteins. Further analysis shows that the proteins present are of tryptophan type. The various cytochemical reactions (Appendices I and II) show the presence of neutral mucopolysaccharide in the pellicle.

Reservoir.—The gullet opens anteriorly to the outside by a subterminal opening which is limited by a membrane; posteriorly it expands into a reservoir (Fig. 1). The wall of the reservoir is non-sudanophil and generally non-osmiophile, but in a few cases fine osmiophile granules are seen along the wall of the reservoir in Kolatcheev preparations.

Flagellum and Paraflagellar body.—The flagellum is nearly twice as long as the body of the flagellate, or even a little longer. The flagellum passes down the gullet and bifurcates. The paraflagellar body is a swelling on the flagellum at its point of bifurcation (Fig. 1) or slightly below it. This body appears dark under the phase-contrast microscope. Its osmiophile and sudanophil nature demonstrates the presence of lipids in it (Fig. 3), while its staining with mercuric bromphenol blue shows its protein nature (Fig. 9).

The bifurcation of the flagellum is clearly seen in Zenker/iron haematoxylin preparations. The base of the flagellum, i.e., its bifurcation, stains with lead tetraacetate-Schiff and mercuric bromphenol blue (Fig. 10), while the portion of the flagellum outside the body stains with mercuric bromphenol blue only.

Cyst wall.—The cyst is covered by a double cyst wall; the inner is thin and delicate, while the outer is thick and chitinous. The outer cyst wall is negative in mercuric bromphenol blue and CTZ, while the inner shows the presence of tryptophan type of proteins. In periodic acid-Schiff and lead tetraacetate-Schiff tests, the inner wall shows positive reaction for polysaccharides, and this positive reaction persists even after digestion with 1% malt diastase. Sometimes the inner wall colours with SBB.

The outer wall appears to be chitinous though it remains negative in the iodine and zinc chloride test (Lillie, 1954) for chitin.

DISCUSSION

Mitochondria.—The mitochondria have been identified in a number of euglenoids, such as Euglena (Causey, 1926; Brown, 1930a; Hall, 1931; Baker, 1933; Patten and Beams, 1936; Gatenby and Singh, 1938), Coleodium (Johnson, 1934), Astasia (Hall, 1934), Rhabdomonas (Hall, 1931), Peranema (Hall, 1929), Copromonas subtilis (Gatenby and Singh, 1938), Euglena gracilis (Ueda, 1938; Wolken and Palade, 1953), Euglena viridis (Hovasse, 1948), and Euglena gracilis and Poteriochromonas stipitata (Chrysomonadina) (Sedar and Rudzinska, 1956). Wolken and Palade (1953), Sedar and Rudzinska (1956) and Ueda (1958) identified them with electron microscopy.

In Khawkinia the mitochondria, in the form of short filaments with surface granules, have been identified by the authors with special mitochondrial techniques. Their cytochemical reactions show that the mitochondria are composed of RNA, phospholipids and lipoproteins with small amounts of triglycerides in some cases. The proteins identified are tryptophan. The presence of cytochrome oxidase in them points to their respiratory function in the cell. Respiratory enzymes have also been located in the mitochondria of other protozoans like Stentor coeruleus (Weisz, 1950) and Leishmania donovani (Guha and others, 1956).
It has also been claimed that the mitochondria of Protozoa give rise to a variety of structures, such as (1) the pyrenoids (Causey, 1926), (2) Golgi material, parabasal bodies, blepharoplasts and stigma (Alexieff, 1928), and (3) lipoids in gregarine (Joyet-Lavergne, 1927).

The mitochondria of *Kha wkinea* appear to play an important part in the origin of lipid bodies in that cell. The sudanophilic surface granules of the mitochondria grow, subsequently detach themselves, and become independent lipid granules, which in turn perform the function of the synthesis of paramylum. Similar mitochondrial origin of lipid bodies has been described earlier also in a variety of cells by Hirsch (1939 and 1948), Lacy (1954, 1956a and 1956b), Junqueira and Hirsch (1956), Kanwar (1958), Nath (1955), Nath and Chopra (1955), and Nath and Gupta (1956).

A more convincing evidence regarding the mitochondrial origin of lipid bodies was provided by the electron microscopical study of adrenal cortex by Lever (1955), who observed that 'it is thus highly probable that the mitochondria are the sites of elaboration or accumulation of lipids.' He has traced a series of stages from recognizable mitochondria, through mitochondria containing strongly osmiophile matrix, to frank lipid droplets. His later investigation with electron microscopy (Lever, 1956), on the rat luteum, confirmed his previous findings that the mitochondria are almost certainly the sites of lipid production or elaboration.

**Lipid bodies.**—The lipid bodies of the earliest stages separate as granules from the surface of mitochondria, and become independent inclusions in the cytoplasm. The granules grow into spheres. The lipid bodies at these stages stain with neutral red. The lipid granules stainable vitally with neutral red may be said to represent the presubstance of the Golgi material as described by Hirsch (1939). These lipid bodies are destined to perform the function of synthesis of paramylum as reported earlier by the authors (Nath and others, 1960). As the synthesis of paramylum starts, the lipid bodies assume a duplex appearance; in the internum of these bodies the paramylum material is synthesized and segregated. The material of the externum is consumed during this process of synthesis.

The lipid bodies have been described as Golgi bodies in other euglenoids. Brown (1930b) described the Golgi bodies of *Euglena* as osmiophile spheres with black borders and clear centres. Hall (1931) identified similar Golgi bodies which stained with neutral red also, and described them as 'vacuome.' Baker (1933) also described Golgi bodies similar to those observed by Brown (1930b), but he identified neutral red stainable bodies (vacuome) as distinct inclusions. The elements of vacuome (neutral red stainable granules) were also reported in *Peranema* (Hall, 1929; Chadefaud, 1938), *Colacium* (Johnson, 1934), and *Phacus* (Dangeard, 1928). The blackening of these vitally stained elements of vacuome by osmium tetroxide, together with their blackening by the usual Golgi methods of preparation, and their general occurrence in Protozoa, led Hall (1930, 1931 and 1936) to identify them as Golgi material.

Patten and Beams (1936) homologized the neutral red stainable bodies of *Euglena* to the volutin granules of Baker (1933) due to their metachromatic behaviour. In spite of the osmiophile nature of the neutral red stainable bodies which appeared as osmiophile rings, spheres and crescents, they mentioned that they could not record any evidence which could support the homology of these bodies to the Golgi bodies.
Gatenby and Singh (1938) also support the views of Patten and Beams (1936) and they believe that the neutral red stainable bodies are comparable to volutin. They consider that, in *Copromonas subtilis* and *Euglena* sp., the Golgi material has no relation to the neutral red stainable bodies; the Golgi material was associated as osmiophile material with the wall of the so-called contractile vacuole.

The present investigation on *Khawkinea* sp. shows that the lipid bodies, stainable vitally with neutral red, are consistently osmiophile in the Golgi techniques, while the wall of the reservoir (flagellar vacuole) rarely shows osmiophilic material in the form of fine granules associated with it.

**Paramylum bodies.**—The authors (Nath and others, 1960) have reported earlier that the participation of chloroplasts, pyrenoids, or leucoplasts is not absolutely essential for the synthesis of the paramylum in the euglenoids, since convincing evidence has been put forward, showing that in this permanently colourless euglenoid, *Khawkinea* sp., the lipid bodies are centres of synthesis of paramylum. As the active lipid synthesis is in progress, the paramylum bodies start developing in the internum of the lipid bodies. Patten and Beams (1936) in *Euglena* reported that with sufficiently long impregnation by Mann-Kopsch method, they were able to blacken some lipid bodies, though this blackening could be easily removed by bleaching. In *Khawkinea* sp., it has been noticed that the fully formed paramylum bodies do not impregnate with osmium tetroxide, but the exterum of the developing lipid bodies is osmiophile.

As regards their cytochemical composition the paramylum bodies are composed of some peculiar neutral polysaccharide (Nath and others, 1960), which is different from the known polysaccharides like glycogen, starch and cellulose described by Pearse (1960), as judged by its negative reaction to periodic acid-Schiff and its various modifications. The polysaccharides like galactogen and dextrans, described by Casselman (1959) in addition to the above-mentioned ones, are also positive to periodic acid-Schiff test. It seems that the polysaccharides present in paramylum are very resistant to oxidation with periodic acid. The polysaccharides such as cellobiose and certain methyl glycosides also have been reported by Janczyk (1959) to be very resistant to periodic acid, even though they satisfy the structural requirement for oxidation.

**Stigma and Paraflagellar body.**—The stigma of *Khawkinea* sp., which is reddish orange in colour due to the presence of carotenoids, reveals granules embedded in a concavo-convex matrix. Jahn (1946) also described a similar structure of the stigma. Wolken (1956a) observed that the orange red stigma of a large number of phytoflagellates contained a pigment of carotene type. The ultrastructure of the stigma of *Euglena gracilis* (Wolken and Palade, 1952 and 1953; Wolken, 1956a and 1956b), *Chlamydomonas reinhardi* (Sager and Palade, 1954 and 1957), and *Chromulina psammobia* (Fauré-Fremiet and Rouiller, 1957) reveals that it is formed by a regular juxtaposition, underneath the membrane of the chromoplast, of a series of elements or chambers, limited by double membranes. These chambers contain a pigment of the carotene type.

As regards the origin of stigma, Alexeiev (1928) considers it to be of mitochondrial origin, while Mangenot (1926) and Rothert (1914) regard it as a modified chromoplast. Chadefaud (1931 and 1935) and Hollande (1938) are of the view that the eye-spot (stigma) is either a differentiated part of a plast as in Chlorophyceae or Xanthophyceae, or a small chloroplast entirely transformed into it, as in Euglenidae. Fauré-Fremiet (1938) reported that it is not essential that the carotene-bearing organelle, i.e., the stigma, should
always be a modified plast (chromoplast): it may represent some other cytoplasmic inclusions of the Golgi type, for example.

As regards the photosensitivity of the euglenoids, Wager (1899) considers that either stigma or paraflagellar body is a photoreceptor.

Mast (1911), on the other hand, regards the flagellar swelling (paraflagellar body) as sensitive to light; he considers that the stigma, by its orientation in a beam of light, acts as a shield, preventing strong light from reaching the swelling. Faure-Fremiet (1938) also regards the paraflagellar body of the Euglenidae as a photoreceptor. He has also observed a connection of the stigma with the flagellum.

On the other hand, some workers like Franzé (1893 and 1908), Rothert (1914), Mast (1927 and 1938) and Wolken (1956a and 1956b) regard the stigma as photoreceptor. Pringsheim (1937) observed that colourless euglenoid forms with stigma are phototactic, while the non-stigma bearing Euglenidae and non-stigma bearing members of Astasiaidae and Peranemidae are not phototactic.

Wolken (1956a) observed that in *Euglena*, we can look upon the eye-spot + flagellum as really a "primitive eye".

The stigma of euglenoids has also been considered as Golgi apparatus on the basis of its supposed homology with the parabasal body of certain flagellates by Grassé (1925 and 1926), and Duboscq and Grassé (1933). According to Grassé (1925 and 1926), the stigma consists of a proteid (chromophobe) basis in which are scattered lipoid (chromophile) spherules. Brown (1936), and Patten and Beams (1936) are against the homology of the stigma with the Golgi apparatus. Mangenot (1926) has remarked that the impregnation of stigma with the Golgi techniques has no significance beyond the fact that carotenoid pigments will reduce osmium tetroxide. The ultra-structure of the stigma described by Wolken and Palade (1952 and 1953), Wolken (1956a and 1956b), Sager and Palade (1954 and 1957), Faure-Fremiet and Rouiller (1957) also does not prove any homology of the stigma with the metazoan Golgi apparatus.

The present investigation on *Khatkinea* sp. shows that due to the presence of carotenoids the stigma is of lipoidal nature and, therefore, it is impregnated in the Golgi techniques. The paraflagellar body like the stigma also shows sudanophil and osmiophile nature because of the presence of lipids in it.

**LITERATURE CITED**

Nath, Dutta, and Dhillon: Cytochemistry of Khawkinea sp.


Explanation of Figures

All the figures are the camera lucida drawings of the specimens of Khawkinea sp.

Trophic form (Figs. 1–5, 9, 11–13 and 15).

Cystic form (Figs. 6-8, 10 and 14).

Abbreviations: CW1 — outer cyst wall; CW2 — inner cyst wall; PL — flagellum; PV — flagellar vacuole; L — lipid body; M — mitochondria; N — nucleus; NL — eudosome; P — paramylum; PB — paratagacchar body; PL — pellicle; S — stigma.
## Appendix 1

**Showing the cytochemical reactions of trophic form of Khawkinea sp.**

<table>
<thead>
<tr>
<th>Technique</th>
<th>F ximation</th>
<th>Fixation media</th>
<th>Reference</th>
<th>Liquid bodies</th>
<th>Mitochondria</th>
<th>Peroxisome bodies</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
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</thead>
<tbody>
<tr>
<td>LIPIDS</td>
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<tr>
<td>SBB in 70% ethanol at room temperature and at 60°C</td>
<td>F Cs, F Cs+PC</td>
<td>Smears or Baker, 1941, 1946, 1949</td>
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<tr>
<td>SBB in propylene glycol</td>
<td>F Cs</td>
<td>Smears</td>
<td>Chiffelle and Putt, 1954</td>
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<td>SBB + cold acetone</td>
<td>F Cs or fresh</td>
<td>Smears or Pearson, 1960</td>
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<tr>
<td>Sudan III &amp; IV in 70% ethanol/acetone</td>
<td>F Cs, F Cs+PC</td>
<td>Smears or Key and Whitehead, 1941</td>
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<tr>
<td>XBS</td>
<td>F Cs, F Cs+PC</td>
<td>Smears or Cain, 1947, 1948</td>
<td>unstained (violet)</td>
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<td>(E —)</td>
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<tr>
<td>AH</td>
<td>F Cs+PC</td>
<td>Smears</td>
<td>Baker, 1946</td>
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<tr>
<td>Fiebiger’s reaction</td>
<td>F Cs, F Cs+PC</td>
<td>Smears or Pearson, 1966</td>
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<tr>
<td>Feyer’s mounting-staining method</td>
<td>F Cs, F Cs+PC</td>
<td>Smears or Schulz, 1924, 1925; Romieu, 1927; Okamoto (after Pearse, 1960)</td>
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<tr>
<td>Plasmal reaction</td>
<td>F Cs+PC</td>
<td>Smears or Nath, 1957</td>
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<td>PROTEINS</td>
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<tr>
<td>Hg-BPB</td>
<td>F Cs, Z, C</td>
<td>Smears or Mazia and others, 1933; Bandag, 1933</td>
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<td></td>
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<td>(E +)</td>
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<tr>
<td>Baker’s modification of Milton’s for tyrosines</td>
<td>F Cs, C</td>
<td>Smears or Baker, 1956</td>
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<tr>
<td>Sakaeguchi</td>
<td>C</td>
<td>P</td>
<td>(Modified from McLeish and others, 1957)</td>
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<tr>
<td>CTZ</td>
<td>C</td>
<td>P</td>
<td>Pearson, 1960</td>
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<tr>
<td>CTZ + DNFB</td>
<td>C</td>
<td>P</td>
<td>Pearson, 1960</td>
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<tr>
<td>CTZ + PFA</td>
<td>C</td>
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<td>CTZ + Benzoylation</td>
<td>C</td>
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<td>NUCLEIC ACIDS</td>
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<tr>
<td>PG/MG</td>
<td>C, Z</td>
<td>Smears or Jordan and Baker, 1955</td>
<td>pink</td>
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<tr>
<td>PG/MG + salivary ribonucleases</td>
<td>C, Z</td>
<td></td>
<td>Brodbury, 1966</td>
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<td>PG/MG + trichloroacetic acid</td>
<td>C, Z</td>
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<td>blue (E —)</td>
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<tr>
<td>Feulgen</td>
<td>C, Z</td>
<td></td>
<td>Feulgen and Rosenbeck, 1945</td>
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<tr>
<td>Feulgen + trichloroacetic acid</td>
<td>C, Z</td>
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</tbody>
</table>

**Key to Appendices I-IV:**

- AH = acid haematein; C = Carnoy; CTZ = coupled tetrazonium; DNFB = dinitrofluorobenzene; E = endosome; F Cs = formaldehyde calcium; Fm = formaldehyde saline; G = gelatine; Hg-BPB = mercuric bromphenol blue; HPO = Hermann’s postosmication; L = Lewitsky; LC = Lewitsky saline; XBS = Nile blue sulphate; P = paraffin; FAM = periodic acid-Schiff; PC = post-chroming; PL = pyridine extraction; PFA = performic acid; PG/MG = pyronin G/methyl green; SBB = Sudan black B; WB = weak Bouin; Z = Zenker; * = after treatment with; ** = weak positive reaction; *** = moderate.
### Appendix II

**Showing the cytochemical reactions of trophic form of Khawkinea sp.**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Fixation</th>
<th>Embedding medium</th>
<th>Reference</th>
<th>Paramylum bodies</th>
<th>Base of the flagellum</th>
<th>Pellicle</th>
<th>Lipid bodies</th>
<th>Mitochondria</th>
<th>Nucleus</th>
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</thead>
<tbody>
<tr>
<td><strong>CARBOHYDRATES</strong></td>
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<tr>
<td>PAS</td>
<td>C</td>
<td>P</td>
<td>Hotchkiss, 1948; Pearse, 1960</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>PAS * acetylation</td>
<td>C</td>
<td>P</td>
<td>McManna and Cason, 1950; Lillie, 1954</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>PAS * 0.1N KOH or 20% ammonia in 70% alc, at 37°C, reversal</td>
<td>C</td>
<td>P</td>
<td>McManna, 1948</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PAS without reducing bath</td>
<td>C</td>
<td>P</td>
<td>Glick, 1949</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>PAS with aqueous periodic acid</td>
<td>C</td>
<td>P</td>
<td>Lillie, 1947</td>
<td>-</td>
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<tr>
<td>PAS with fresh periodic acid</td>
<td>C</td>
<td>P</td>
<td>Pearse, 1960</td>
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<tr>
<td>PAS * 1% malt diastase</td>
<td>C</td>
<td>P</td>
<td>Shimizu and Kumamoto, 1952; Pearse, 1960</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Lead tetraacetate--Schiff</td>
<td>C</td>
<td>P</td>
<td>Lillie, 1954</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Lead tetraacetate--Schiff * malt diastase</td>
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<td>Red carmine</td>
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<td>Smears or P</td>
<td>Pearse, 1960</td>
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<td>Alcian blue</td>
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<td>Steedman, 1956</td>
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<td>Hale's Sulphation techniques</td>
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<td>Hale, 1946</td>
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<td>Azure A * chlorosulphonic acid in dry pyridine</td>
<td>C</td>
<td>P</td>
<td>Spierer, 1960; Kramer and Wadström, 1954</td>
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<td>Methylene blue * conc. H₂SO₄ glacial acetic acid or sulphury chloride</td>
<td>C</td>
<td>P</td>
<td>Lewis and Ade-anya, 1959</td>
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<td>‘Nad’ reaction</td>
<td>Fresh</td>
<td></td>
<td>Moog, 1943</td>
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For abbreviations please refer to key in Appendix I.
### Appendix III

**Showing the cytochemical reactions of stigma of *Khawkinea* sp.**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Fixation</th>
<th>Reference</th>
<th>Results</th>
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<tbody>
<tr>
<td>Carr-Price Test. with antimony chloride in chloroform Lager’s isobole</td>
<td>Fresh</td>
<td>Bourne, 1935</td>
<td>Stigma immediately turns very bright and sharp blue.</td>
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<tr>
<td>Cresyl blue</td>
<td>Fresh</td>
<td>Pease, 1960</td>
<td>Root of the animal is all clear and distorted.</td>
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<tr>
<td>Pearltinic acid</td>
<td>Fresh</td>
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<td>Stigma turns blue with short treatment; shrinks greatly.</td>
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<tr>
<td>Formic acid</td>
<td>Fresh</td>
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<td>Turns blue, dis-integrates into small granules before or after turning blue.</td>
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<tr>
<td>Trichloroacetic acid</td>
<td>Fresh</td>
<td></td>
<td>Turns blue, blue or black, disintegrating into granules which fade and disappear.</td>
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<tr>
<td>Cold conc. H₂SO₄</td>
<td>Smears</td>
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<td>Shrinks and turns blue.</td>
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<td>H₂O₂</td>
<td>Smears</td>
<td></td>
<td>Blue.</td>
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<tr>
<td>SBB in 70% ethanol</td>
<td>Smears</td>
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<td>Black.</td>
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<td>Hg-BPB</td>
<td>Smears</td>
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<td>PEG/Me₆</td>
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<td>PAS</td>
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<td>2° osmium tetroxide</td>
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For abbreviations please refer to key in Appendix I.
## Appendix IV

### Staining cytochemical reactions of cytoplasm of Khawkinsia sp.

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<th>Fixation</th>
<th>Embedding medium</th>
<th>Reference</th>
<th>Lipid bodies</th>
<th>Mitochondria</th>
<th>Nucleus</th>
<th>Plasma</th>
<th>Cytoplasm</th>
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<td>SHB in 70% ethanol, Sudan III and IV in 70% ethanol</td>
<td>FCa + PC</td>
<td>G, P</td>
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For abbreviations, please refer to key in Appendix I.
HOMOLOGUE OF THE GOLGI APPARATUS IN HERPETOMONAS MUSCARUM, GIARDIA INTESTINALIS, LOPHOMONAS BLATTARUM AND L. STRIATA

By

VISWA NATH, F.N.I., G. P. DUTTA and BALDEV DHILLON