P A R T II

CYTOCHEMICAL STUDIES OF THE GREGARINES

STUDIED IN PART I

INTRODUCTION

Gregarines have been the objects of great interest and much work has been done on their systematics. However, little information has been done on their cytochemical nature.

The polysaccharide content of the gregarines have been of interest to many workers in the field. It has been found to be present in a smaller or larger quantity in all the gregarines studied so far. The knowledge on the chemical nature of the paraglycogen has been extended by Mercier, Schrevel and Stark (1973), who have classified the reserve polysaccharide in Gregarina blattarum as an amylopectin. In the present study the presence of such reserve bodies reacting positively for polysaccharides has been traced in the different stages of the life cycles of various gregarines of earthworms. Such study on an earthworm gregarine was undertaken earlier by Sathanantnan (1977) who worked on Zeylanocystis burti.

The presence of DNA in the nucleus and RNA in the nucleolus and cytoplasm is well known. The presence of

extranuclear DNA in sporozoa has evoked the interest of many investigators in the field. (Preer 1950, Lwoff 1952, Ray and Gill 1955, Dasgupta 1959). They assumed these structures to be of bacterial or viral nature. In the present study toluidine blue and Fluorescence microscopy have been used for studying the Feulgen-positive and methyl green positive bodies in the cytoplasm of gregarines.

In this part of the investigation (Part II), a complete cytochemical study on the polysaccharide and nucleic acid contents in the different stages of the life cycles of the gregarines including the abnormal trophozoites that have been studied in Part I of the thesis is reported.

MATERIAL AND METHODS:-

Earthworms from various localities in Darjeeling district were brought to the laboratory, dissected, and smears of the coelomic fluid as well as of the seminal vesicles and cysts were prepared on clean dry glass slides.

Where sections were required seminal vesicles were taken out and fixed in freshly prepared Carnoy's fixative for about ten minutes, dehydrated and infiltrated with cedarwood oil overnight, cleared in Xylol and embedded in paraffin for one hour at 60°C. Sections were cut at 6 μ and 3 μ thickness, the latter being used for fluorescence microscopy.

The following histochemical staining methods were used.

A. Feulgen Reaction (Feulgen and Rossenbeck 1924, modified after Pearse 1968) to detect the presence of DNA.

B. Detection of DNA and RNA by

(i) Methyl green-pyronin stain after Kurnick 1955

(ii) Acridine Orange method for nucleic acids (DNA and RNA) by Fluorescence method after Armstrong, 1956.

C. Periodic Acid- Schiff method after Glick 1949, was used for the detection of Polysaccharides.

D. Carmine stain for glycogen (see Glick 1949) and Bauer Feulgen (see Glick 1949) for glycogen was used.

E. Toluidine Blue stain (see Pearse 1968) was used for studying basophilia.

PERIODIC ACID/SCHIFF REACTION IN SOME OF THE GREGARINES STUDIED IN PART I

(Plates XXV Figs 1 - 3)

Apolocystis n.sp(a)

Trophozoites:-

The reacting substance occurred as red spherical bodies. Such bodies filled up the cytoplasm. The nuclear area appeared colourless.

Gametocytes:-

Cytoplasm of the gametocytes was filled up with spherical bodies similar to those seen in the trophozoites. The wall of the gametocyst was negative to PAS technique.

Gametes:-

The cytoplasm of the gametes took up deep red colour.

Spores:-

Spore was faintly positive to PAS reaction.

Apolocystis n.sp.(b)

The colour reaction in the trophozoites, gametocytes, gametes and sporoblasts was similar to that seen in the corresponding stages of the parasite, Apolocystis n.sp.(a).

The colour reaction in the spores differed from that seen in the spore of the parasite described earlier, in that the polar ends of the spores took up deep red colour while the main part of the walls of the spores was negative to the reaction.

In both the above cases the reacting materials could be eliminated by treatment with saliva at 37°C, for an hour.

Apolocystis n.sp. (c)

Due to the paucity of materials, cytochemical work on Apolocystis n.sp.(c)could not be carried out.

Apolocystis n.sp.(d)

Normal trophzoites:

The cytoplasm was filled with minute spherical bodies positive to PAS reaction. A negative reaction was noted in the nucleus.

Abnormal trophozoites:

Black patches described in page127, seen in the abnormal forms of the parasite were negative to the reaction, but took up Delafield haematoxylin which was used as a counterstain. Certain granules in between the reacting bodies were also stained with haematoxylin. When treated with saliva at 37°C for about an hour the reacting bodies disappeared.

A(a) n.gen.,n.sp.

Trophozoites:

The two forms of the parasite (larger and the smaller form) varied in their reaction.

Larger forms:

The cytoplasm of these forms were packed with [illegible] bodies which took up deep red colour while the nucleus did not react to the stain.

The nature of the reacting substance could be confirmed with Bauer Feulgen method.

Smaller forms:

With the PAS technique the reacting substance was found to occur as red spherical granules but these granules

were fewer in number and widely scattered.

The rest of the body looked dull blue - when Delafield haematoxylin was used as a counterstain. In both the above cases the reacting substance could be removed by treatment with saliva.

Stomatophora n.sp.(a)

Trophozoites:

Cytoplasm was packed with deeply staining granules. In some cases these granules lay scattered in the cytoplasm.

Gametocyst:

The wall of the gametocyst did not take up stain. The residual cytoplasm in the gametocyst occurred as deep red mass.

Gametes and Zygotes:

Cytoplasm of the gametes and zygotes looked red.

Spores:

Spores were faintly positive to the reaction. In all the stages mentioned above, the reacting material disappeared when treated with saliva for about an hour at 37°C.

Stomatophora n.sp.(b)

The staining reaction of this parasite was the same as in the corresponding stages of Stomatophora n.sp.(a).

Zygocystis n.sp.(a)

Normal trophzoites:

The trophozoites both solitary and in a state of syzygy showed strong affinity to periodic acid/Schiff method. The intensity of staining reaction varied from one individual to another. Under high magnification, the reacting materials were found to be deeply stained on the periphery and lightly in the middle. The trophzoites stained red with Best Carmine method.

Abnormal trophozoites:

The abnormal trophozoites were distinctly negative to periodic acid/Schiff method. In cases where the infection occurred in patches, the normal cytoplasm stained red with the stain, but the infected areas remained colourless.

Gametocyst:

The wall of the gametocyst was negative to the reaction; the residual cytoplasm was however strongly positive to the reaction.

Sporozoites:-

The cytoplasm was positive to the periodic acid/Schiff reaction.

Spores:-

A weak reaction was detected in the wall of the spore. Two plug-like structures present at the two ends of the spore were negative to the reaction.

The positive reaction in all the stages mentioned above could be removed by the treatment with saliva at 37°C.

B (a) n.gen. n.sp.

Cytoplasm of the gregarine was filled with large elongated bodies that took up red colour very deeply. In between the reacting materials, haematoxylin-stained round bodies were visible. Saliva controls could not be tested due to the lack of sufficient material.

Nematocystis n.sp(a)

This parasite presents a very remarkable study as the cytoplasm hardly contained any reacting substances. Some cases were encountered where there were no signs of the reacting material, and yet others

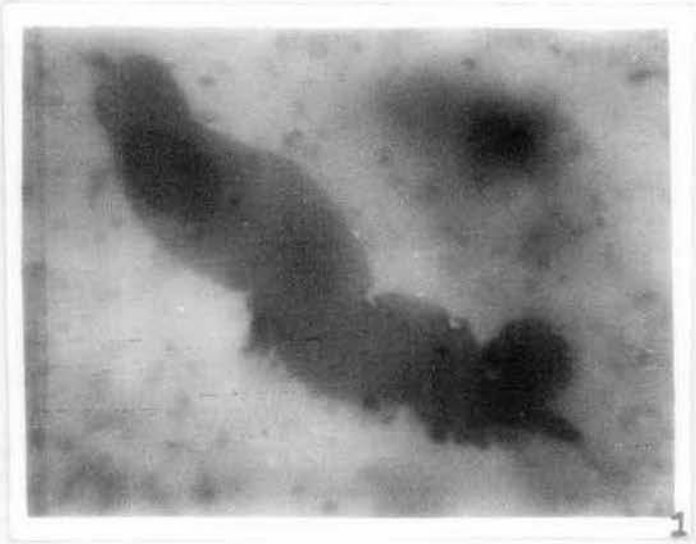
P L A T E XXV

Fig. 1. Photomicrograph of Nematocystis n.sp(a) showing the rarity of paraglycogen grains. About 6 can be seen in the cytoplasm. 6 X 40X

Fig. 2. Photomicrograph of B(a) showing the paraglycogen granules. Note the black bodies in the cytoplasm. Nucleus is masked by the paraglycogen granules 10 X 10X

Fig. 3. Photomicrograph of A (a) showing the paraglycogen granules. 10 X 10X.

PLATE XXV



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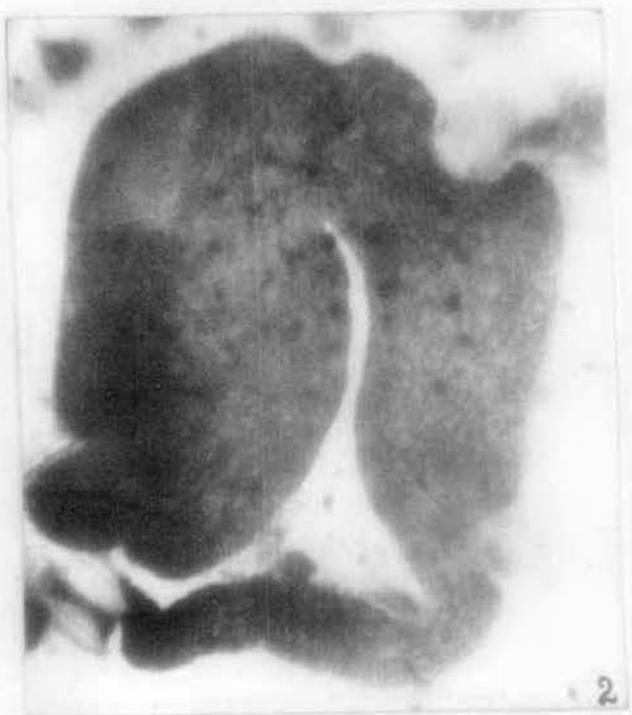

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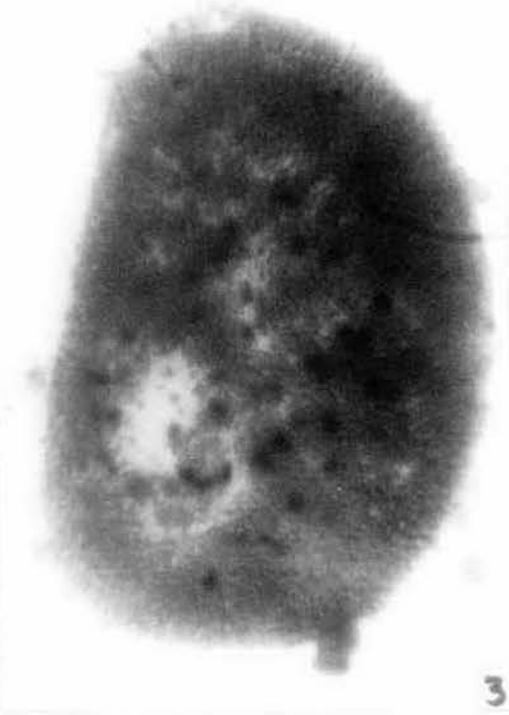

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were present which possessed about six or seven such granules. Saliva controls could not be tested for paucity of material.

Nematocystis n.sp.(c)

Trophozoites:-

Cytoplasm was strongly positive to periodic acid/ Schiff reaction. A negative reaction was noticed in the nucleus.

B (b)n.gen.,n.sp.

Trophozoites:-

Cytoplasm was strongly positive and appeared purplish red to periodic acid/ Schiff reaction. The nucleus was negative to the reaction.

DISCUSSION

Paraglycogen is known to constitute the largest granular inclusions in the cytoplasm of gregarines in general, and the most widely distributed polysaccharides of parasites is undoubtedly glycogen (von Brandt 1966). Such material has also been considered to represent amylopectin in some of the gregarines (Mercier, Schrevel and Stark 1973). However , in most of the

cases studied here, the polysaccharide present could be identified as glycogen by using saliva digestion method. In A(a)n.gen., n.sp. and Zygocystis (a) the presence of glycogen could also be confirmed by using Bauer-Feulgen and Best Carmine methods respectively. The results recorded here are also in contrast to that reported by Dutta (1962), who stated that paraglycogen present in Stomatophora diadema consisted of neutral polysaccharides other than glycogen. In the course of the present study the reacting materials in the two Stomatophorids Stomatophora n.sp.(a) and Stomatophora n.sp.(b), could be identified as glycogen by using saliva digestion method.

In the course of the present investigation it has been noted that the granules reacting positively to the periodic acid/Schiff technique varied in shape and size from one parasite to another. For example in Apolocystis, n.sp. Stomatophora, Zygocystis and in A(a) these were spherical and granular in shape, but varied from spherical to elongated in the different species of Nematocystis, and elongated in A(a). In most instances the paraglycogen granules present in this parasite were slightly curved. All these granules lay scattered and were not localised in any

specific area in the cytoplasm of any of these parasites. The number and the concentration of the granules varied from one individual to another, of the same species.

The heavy deposition of the glycogen in the cytoplasm of the parasites studied here could mean either that these were used up as energy by the parasites or that these represented reserve food materials to be used in future. According to von Brand (1952) it is an universally accepted fact that the endogeneous glycogen masses have an exogeneous source i.e., these are derived from carbohydrates imbibed as food from the host. The same author also notes that the production of energy is not always the sole purpose of glycogen, and that glycogen can be used also for the synthesis of chitin of exoskeleton and the chitinous egg membrane. Gill and Ray (1954) assumed that in all probability the glycogen in E. tenella contributed to the formation of various structures developing within the oocysts. The same authors believed that the presence of some positively reacting material in the sporozoites could mean that these were used up as energy to enable them to penetrate the tissues of the host.

Sathananthan (1977) stated that the materials reacting positively to PAS technique, on the walls of the spores could be a precautionary measure on the part

of the parasite to tide over the unfavorable conditions outside the host, implying thereby that these stages would remain dormant and survive long periods before infecting another host.

In Zygocystis n.sp.(a), the entire area of the cytoplasm in some cases assumed a brilliant colour, while yet others presented the reacting materials as numerous granules scattered all over the cytoplasm.

In the abnormal individuals of the same parasite, the infected patches failed to respond to the periodic acid/Schiff method; the only plausible explanation that can be given is that the waste products and secretions given out by the hyperparasites represented areas which yielded negative reaction to the PAS technique.

In Apolocystis n.sp.(d), the presence of the patches of cytoplasmic inclusions, appeared to have no effect on the number and concentration of the paraglycogen granules lying elsewhere in the cytoplasm of the host.

In the studies made regarding the distribution of paraglycogen granules in the different stages of the life cycle, it has been noted that the amount is heavy

in the trophozoite and the gametocyte stages. During gametogenesis, the residual cytoplasm shows strong positive reaction to PAS technique. Later on the positively reacting material is entirely used up when the spores start developing.

The trophozoite imbibes as much food as possible and builds up a good glycogen reserve which is utilized subsequently by the parasite. As the gametes and the spores start to develop these reserve food materials are gradually used up. Finally when the spores are fully formed all the glycogen reserve materials are used up.

No differences in the amount of polysaccharides was noted when studies were made in the different seasons of the year.

Nematocystis n.sp.(a) presented a very interesting case in that the glycogen granules were few and could be counted easily. Instances where just one or two granules were present were many and in a few individuals a complete absence of the reacting material was noted. A survey of literature reveals that all gregarines should have a smaller or larger store of polysaccharides.

The rapidity with which Nematocystis n.sp.(a)moves and the manner in which it carries out its metabolic activities with hardly any reserve food materials as a source of energy is a matter which needs further investigation.

In the smaller forms of A(a) the amount of glycogen reserves were far less in amount than in the bigger forms. This could mean that these were the younger stages of the parasite, and as they grew up they accumulated more and more of the glycogen which is further stored up in the cytoplasm.

SUMMARY

In the above investigation which has been conducted to study the polysaccharide content in the different stages of the life cycles of gregarines of earthworms, the following conclusions have been reached:-

1. The polysaccharide present in the different stages of the life cycle is undoubtedly glycogen.

2. The polysaccharide content is maximum in the trophozoite and gametocyte stages and decreases gradually during development.

3. Infected areas of Zygocystis n.sp(a) failed to show any presence of polysaccharides.

4. Nematocystis n.sp(a) is unique in that it hardly posseses such reserve bodies in its cytoplasm.

THE STUDY OF NUCLEIC ACIDS IN SOME OF THE GREGARINES STUDIED IN PART I

(Plate 26, Figs.1-2)

(Plate 27, Figs.1-2)

Apolocystis n.sp.(a)

The nucleus of the trophozoite was distinctly negative to Feulgen reaction. Certain individual trophozoites were found to harbour Feulgen-positive bodies in the cytoplasm. Usually these bodies were in the form of minute granules. In two instances, however, young trophozoites showed patches of Feulgen-positive areas in the cytoplasm. The use of pyronin-methyl green stain produced comparable results in that the nucleus showed no trace of methyly green. The nuclear membrane and the area adjoining the membrane took up the pyronin stain deeply, becoming lighter towards the central region.

Violet metachromasy was noticed in the nuclear membrane and the area immediately beneath it. The central area of the nucleus was lightly stained. Several metachromatic granules were present in the ground matrix of cytoplasm as minute dots.

Gametocytes:-

The gametocytes were distinctly Feulgen-negative and metachromasy existed in the cytoplasm and periphery of the nucleus.

Gametes:-

Nuclei in a state of division in the gametocytes were very faintly Feulgen-positive and also took up faint methyl green stain. The nucleus of the fully formed gamete was strongly Feulgen-positive, and also took up strong methyl green stain. Gametes exhibited slight metachromasy within the gametocyst.

Zygotes:-

The nucleus of the zygote lying within the gametocyst was strongly Feulgen-positive, and stained strongly with methyl green.

Sporozoites:-

The nucleus of the sporozoite was Feulgen-positive.

In all the stages mentioned above, the use of

pyronin methyl green showed that the cytoplasm always stained deep red with pyronin. A faint and uneven stain with pyronin was also noticed in the nucleus of the trophozoite and the gametocytes.

Yellow fluorescence was noticed in the nucleus of all the different stages of the life cycle excluding the trophozoites and gametocytes in which a positive result with Feulgen method was not obtained.

Apolocystis n.sp.(b)

The colour reactions in the trophozoite, gametocytes, gametes and zygotes were the same as seen in the corresponding stages of the parasite described above, though with a difference i.e. the parasite Apolocystis n.sp.(b) has a very prominent nucleolus which took up a very strong pyronin stain and showed metachromasy with toluidine blue. With acridine orange, the nucleolus showed orange fluorescence and the nucleus appeared with a somewhat clear area around it.

Apolocystis n.sp.(d)

Trophozoites:

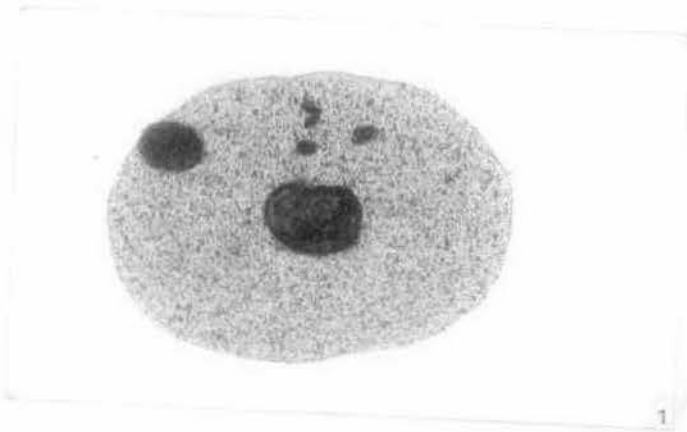
Nucleus was Feulgen- negative. Some trophozoites

P L A T E XXVI

Figs. 1 & 2.

The inclusion bodies in the cytoplasm of Apolocystis n.sp(d) show red metachromasy when stained with toluidine blue. 10 X 45

PLATE XXVI



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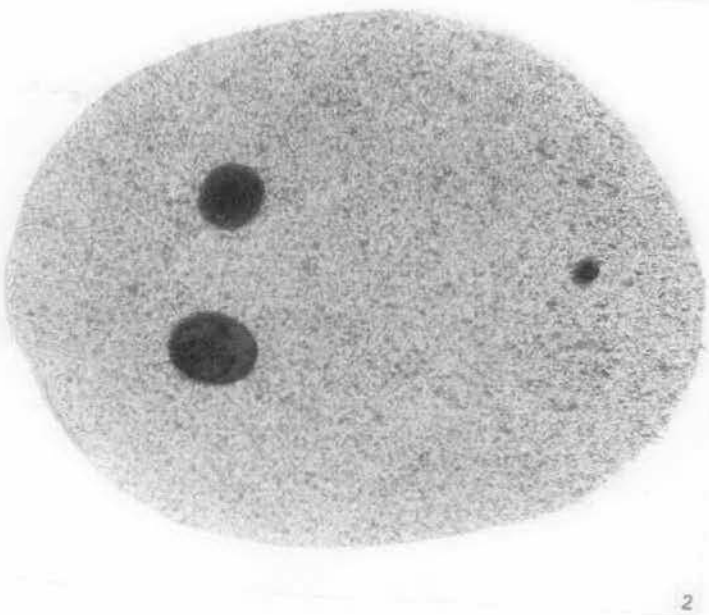

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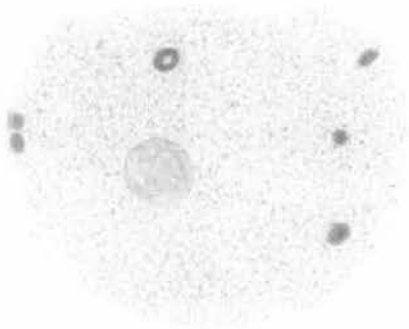
P L A T E XXVII

Figs. 1 & 2.

Feulgen positive inclusion bodies in the cytoplasm of Apolocystis n.sp(d)

10 X 45 X

PLATE XXVII



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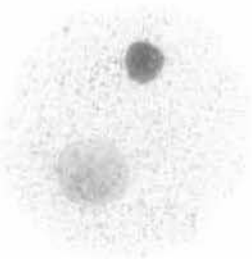

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were encountered, where Feulgen-positive bodies appeared in the cytoplasm. In one instance these Feulgen-positive bodies were so numerous that the gregarine took up a pinkish hue. The nucleus took up a faint pyronin stain and the cytoplasm took up the stain deeply.

The inclusions in the cytoplasm which presented various shapes (generally spherical) and which stained intensely with iron alum haematoxylin, corresponded to the Feulgen-positive areas. These areas occurring in patches did not appear homogeneous, as seen in iron alum haematoxylin stain. The patches appeared to harbour granular inclusions which were very strongly Feulgen-positive, and which occurred in a faintly Feulgen-positive matrix. Prominent metachromasy was observed in areas corresponding to the Feulgen-positive patches. The granular inclusions are believed to be inclusion bodies of bacterial or viral origin. These bodies did not stain with the alcian blue. In a few cases one or two ortho-chromatic dots were noticed when stained with toluidine blue. With pyronin methyl green the entire cytoplasm took up the pyronin stain while methyl green stained areas corresponded to Feulgen-positive areas noted above. The nucleolus took up pyronin stain very deeply.

Strong yellow fluorescence with acridine orange was noticed in areas in the cytoplasm which exhibited Feulgen - positive reaction.

Zygocystis n.sp.(a)

Trophozoites:

The cytoplasm as well as the nucleus of the trophozoites were Feulgen- negative. With pyronin methyl green stain, the cytoplasm took up faint red colour and the nucleus also took up a faint red colour. No sign of metachromasy in the cytoplasm of the trophozoite was noticed, but the nucleus exhibited faint violet metachromasy. With acridine orange the entire cytoplasm was orange including the nucleus which took up a slightly lighter colour compared to the surrounding cytoplasm.

Gametocytes:

The nucleus was Feulgen-negative and stained red with pyronin. Some of these showed black pigments. The residual cytoplasm showed red metachromasy (probably representing mucopolysaccharides).

Gametes:-

Nucleus of the gametes was faintly Feulgen- positive, and stained faintly with methyl green. The gametes showed faint metachromasy.

Zygotes:-

Nucleus of the zygote was strongly Feulgen - positive.

Sporozoites:-

Nucleus of the sporozoite showed positive reaction to Feulgen method and took up methyl green stain.

A (a) n.gen.,n.sp.

Trophozoites:-

In most of the cases, innumerable Feulgen - positive bodies occurring in dot like forms were found lying scattered in the cytoplasm. In one instance it was noted that these bodies were elongated in shape. Both the bigger as well as the smaller forms of the parasite appeared to have these Feulgen - positive bodies in the cytoplasm. The nucleus was Feulgen negative. These bodies also took up methyl green stain.

ytoplasm stained red with pyronin and the
i took up deeper pyronin stain. In some cases very

very faint Feulgen-positive patches were also observed in the cytoplasm.

With acridine orange the cytoplasm fluoresced bright orange. In this bright orange matrix there occurred minute granules that fluoresced bright yellow. These appeared to correspond to the Feulgen-positive bodies. The nucleoli looked pale orange with a slightly darker area in the centre. In some cases however, the nucleus fluoresced faintly.

The area of the sucker fluoresced orange only lightly and appeared transparent.

An interesting observation was made in the cytoplasmic region. Cytoplasm instead of being of uniform texture, seemed to show a blotchy picture. As such the orange colour also appeared in blotches.

Stomatophora n.sp(a)

Trophozoites:-

Nucleus was Feulgen-negative. In one or two instances there were a number of Feulgen-positive granules lying scattered in the cytoplasm. The regions of the sucker and the mucron were also Feulgen-negative. Cytoplasm and the nucleus took up pyronin stain.

With toluidine blue stain a large number of metachromatic granules were seen in the cytoplasm, and the nucleus appeared metachromatic.

Gametes:-

The nucleus of the gamete was faintly Feulgen - positive and stained faintly also with methyl- green.

Sporozoites:-

Nucleus of the sporozoite was Feulgen positive and also took up the methyl green stain.

Stomatophora n.sp.(b)

Nucleus was Feulgen - negative in the trophozoite and in the gametocytes, while the gametes, zygotes, sporoblasts and sporozoites had Feulgen- positive nucleus.

When subjected to fluorescence microscopy both Stomatophora n.sp.(a) and Stomatophora n.sp.(b) exhibited orange fluorescence in the cytoplasm and in the nucleoli. The nucleus however, showed faint fluorescence. Yellow fluorescence was also noticed in the cytoplasm in the form of granules.

In *Nematocystis* n.sp.(d) similar Feulgen-positive granules were observed, lying scattered in the cytoplasm. These granules stained with methyl green.

DISCUSSION

The nucleic acids, (DNA and RNA) have been of great interest to investigators in the field of Protozoology. Feulgen and Rossenbeck (1924) designed and utilised their well known reaction for the detection of DNA. The use of basic dye pyronin along with methyl green to distinguish between the two nucleic acids is well known (see Pearse 1968). Basing observations gained by means of Feulgen reaction it has been found that the nuclei of most of the parasitic species contain DNA. On the other hand, the ground cytoplasm of all the stages of the life cycle, especially of the gregarines, contain RNA. RNA also occurs in the nucleoli (von Brand 1966).

In the course of the cytochemical work carried out on various protozoa, metachromasy has often been noticed in the nucleoli and also in the cytoplasm. In most cases there was violet metachromasy in the nucleolus and a fainter violet stain in the rest of the

nucleus. Red metachromasy was observed in the residual cytoplasm of the gametocyst and gametes of Zygocystis n.sp.(a). While violet metachromasy could represent nucleic acids, the red metachromasy could possibly be due to the presence of mucopolysaccharides. Dasgupta (1961) conducted a series of studies on certain sporozoans wherein he encountered mucopolysaccharides.

Sirlin (1960), and von Brand (1966) have proved that the nucleolus is the chief site of RNA. The use of toluidine blue and pyronin- methyl green have confirmed this finding. With toluidine blue, metachromasy was observed in the nucleolus and also faint orthochromatic stain was seen in the nucleus. Pyronin, stained the nucleolus very deeply and the nucleus faintly. Since pyronin stains RNA and the lower polymers of DNA as well, it can be said that RNA is present in the nucleolus in heavy concentration. Metachromasy in the cytoplasm as well as pyronin staining in the same implies that the nucleic acid (RNA) is present in a lesser or greater quantity in the cytoplasm also.

In Apolocystis n.sp.(a), metachromasy existed in

the nuclear membrane and the area immediately adjoining it. Comparable results were obtained in the same species stained with methyl green pyronin. The central area of the nucleus took up the stain lightly. The nucleolus is considered to be the site for RNA, this structure being apparently absent in this species of Apolocystis, it is possible that RNA (essential for protein synthesis) is localised in the area specified for this parasite in the above description.

In the course of the present investigation on earthworm gregarines I have encountered more or less a uniform pattern of distribution of nuclear DNA in the various stages of the life cycle. For example, all the trophozoites, and the gametocytes had their nuclei negative to the Feulgen-reaction. During gametogenesis, however, the daughter nuclei showed slight positive reaction to the Feulgen's method. These gametes are also stained faintly by methyl green. The nucleus of the zygote in all the parasites was strongly Feulgen-positive. The nucleus of the sporozoites too showed similar reaction with Feulgen method. In all the above cases (gametes, zygotes, sporoblasts and sporozoites) the nuclei were stained with methyl green.

Daniels (1938) reported Feulgen-negative reaction for DNA in gregarines. Dutta (1962) also reported Feulgen-negative reaction in the nucleus of *Stomatophora diadema*. Histological literature reveals that the nucleus generally always shows a positive reaction for Feulgen's technique. But histochemical work on most of the gregarines under report, show that this is not so. Sathananthan (1977) has however, detected DNA in the nucleus of the gregarine *Zeylanocystis burti*.

A case of Feulgen-negative reaction in the nucleus was reported by Garnham (1954) in *Hepatozoon argantis* where the zygote nucleus was Feulgen-negative, though the nuclei during the sporogony were Feulgen-positive. Dasgupta and Meedeniya (1958), and Dasgupta (1959) made comparable observations on *H.sciuri*.

Survey of literature reveals that external factors may somehow influence the result of Feulgen reation in protozoa *e.g.*, starvation, oxygen deficiency and low temperature may lead to a diminution of DNA content in the macronucleus of *Paramaecium aurelia* (Gromova 1941); or death and degeneration could mean the loss of Feulgen-positive appearance in the nucleus of the crithidiae of

Trypanosoma melophagium (van Thiel 1925). But in the course of the present study all the gregarines when subjected to the Feulgen technique have failed to show any trace of Feulgen-positive material in the nuclei. This finding was confirmed by staining with methyl green.

DNA is well known to be mainly responsible for nuclear division and to be closely associated with the chromosomes that are present in the nucleus. The use of Fluorescence microscopy revealed the presence of DNA in the nuclei of the trophozoites and the gametocytes, where a positive reaction of the Feulgen technique was not obtained.

Judging from the above, the apparent absence of DNA in the nucleus of all the gregarines studied here, does not necessarily mean the complete absence of DNA. Daniels (1938), suggested that the negative Feulgen reaction could be due to the fact that the chromatin in the gregarines probably existed in a very dispersed condition which could not even be seen with the oil immersion lens. Ray and Gill's work(1955) brought forward a similar arguement regarding the gametocytes of Eimeria. Dasgupta(1959)

made similar observations on *Plasmodium* and *Eimeria*, and von Brand (1966) referred to the same.

A plausible explanation for the negative Feulgen reaction in the trophozoites and the gametocytes, and the positive reaction in the gametes, zygotes and the sporozoites can be formulated thus: The amount of DNA in the trophozoites and the gametocyte stages being in a highly dispersed condition could not even be detected by the Feulgen method. During gametogenesis, there is a considerable increase in the nucleoprotein, which probably results in the deep Feulgen staining of the material.

Of greater interest is the extranuclear occurrence of DNA especially so in the cytoplasm. Extranuclear DNA has been found to coincide with the specific cytoplasmic organelles such as the blepheroplast-kinetoplast complex of the trypanosomes. Freer(1950) demonstrated microscopic Feulgen-positive bodies in paramaecia. Lwoff(1952) also described certain Feulgen-positive bodies, the pro-virus particles, in certain lysogenic bacteria. Ray and Gill (1955) showed the presence of DNA particles in the cytoplasm of 10% of the oocysts of *E. tenella*. Dasgupta(1959) showed

a similar occurrence of DNA in the cytoplasm of the stages of E.stiedae. The present study is an added example to the text.

Many Feulgen-positive materials have been noticed in the cytoplasm of Apolocystis n.sp.(a), Apolocystis n.sp.(b) Stomatophora n.sp.(a), Stomatophora n.sp.(b), Nematocystis n.sp.(d) and A(a). These were generally in the form of minute granules and in a few cases like in Apolocystis n.sp(a) and A(a), they were present in the form of lightly stained patches. In Apolocystis n.sp.(d) however, Feulgen-positive granules were present but in an aggregated mass in the cytoplasm. It may be presumed that these Feulgen-positive materials are of viral or bacterial nature. Occurrence of such microbes were earlier reported in the oocysts of E.labbeana by Yakimoff and Timofeef (1940). Dasgupta (1959) also assumed such a case in the cytoplasm of the oocyst of Plasmodium cynomolgi. Lately, Mackenzie & Walker (1979) reported the presence of such microbes in the cytoplasm of G.garnhami. The nature of these Feulgen-positive bodies are even more convincing in the case of A(a) as some of the bodies show rod-like or coccoid shapes.

SUMMARY

In the course of the present investigation it has been found that the nuclei of all the gregarines studied showed a negative response to methyl green and Feulgen's technique. This could be dus to the fact that the chromatin in the gregarines existed in such a dispersed condition that it could not be seen even under oil immersion lens. However, the use of Fluorescence microscopy showed the presence of DNA in the nucleus of most of the gregarines.

A large number of extra- nuclear Feulgen-positive bodies were noticed in some of the gregarines studied in Part I. These were either scattered as minute spherical bodies or present as inclusion bodies in the cytoplasm of Apolocystis n.sp.(d). These bodies were Feulgen-positive, methyl green positive, showed red metachromasy and fluoresced yellow when subjected to Fluorescence microscopy. These could be of bacterial or viral nature.