Studies on *Nyctotherus ovalis* Leidy, with special reference to its nuclear structure[1].

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(With plates 2—4.)

Contents.

Introduction.

Through the painstaking studies by workers such as Fischer (1899), Mann (1902), Tellyesniczky (1898, 1927), Strangeways and Canti (1927), Bělař (1928) and others, information is now available with regard to the effects of various fixatives and of the processes involved in making permanent preparations upon certain living

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animal and plant cells, their nuclei and mitotic figures. The successful application of Schiff's reaction in detecting thymonucleic acid by Feulgen and Rossenbeck (1924) and the confirmatory findings of recent workers seem to leave little doubt concerning the identity of the intranuclear chromatin substance of certain animal cells and the thymonucleic acid. However, as far as the writer is aware, no studies have been made regarding how the constituent of the nucleus which manifests a positive nucleal reaction, appears in the resting nucleus in life. This is undoubtedly due to the minuteness and small amount of chromatin material as it usually occurs in a resting nucleus.

For some time, the writer has employed Feulgen's nucleal staining in studying the nuclei of various protozoans, and obtained results which confirm on the whole those of previous investigators. During the course of study, it was noted that the massive macronucleus of the ciliate, *Nyctotherus ovalis*, contains, as Stein observed so many years ago, occasionally large spherules which measure not rarely as large as 20 μ in diameter. These spherules give positive nucleal reaction and have, therefore, been considered as composed of the thymonucleic acid or the chromatin. With this material which can be obtained abundantly here in Urbana, an attempt was made to study as precisely as possible the individual chromatin spherules as they occur in the nucleus in life, the effects of certain reagents and fixatives upon them, and how the so-called nuclear stains differ from Feulgen's nucleal staining in practice. The results so far obtained are reported in the following pages.

General morphology of *Nyctotherus ovalis*.

Nyctotherus ovalis is a common inhabitant in the colon of *Blatta orientalis*. It occurs almost always in the anterior half of the host organ in association with several other species of Protozoa. These cockroaches are easily collected from the campus grounds from March up to the end of November, being most abundantly found in summer (Kudo, 1926). The ciliate has been observed in about 50 per cent of the host insects examined up to April, 1935. As a rule, the heaviest incidence of infection in each of the past ten years has been noticed in the summer months, which is obviously related to the activity and feeding habit of the insects. Compared with *Endamoeba blattae* (Kudo, 1926), *Lophomonas blattarum* and *L. striata* (Kudo, 1926 a), *Nyctotherus ovalis* may be said to be most common intestinal protozoan of the cockroach that has come under the writer's attention.

When the host insects are confined in a jar and given food, they become easily infected with the four species of protozoans mentioned above. This is due to the contamination of food material by the encysted forms of these organisms which are voided in fecal matter. Of several food matters tried, yeast cakes have been found to be the most excellent one and have been used almost exclusively for the past several years. Yeast cakes seem to possess a great nutritive value for both the host insects and the protozoans which live within them, as judged by the behavior and activity of the former and the abundance of the latter.

Cultivation of this and other protozoans of the cockroach in vitro has been carried on from time to time for several years in this laboratory. Of various culture media tried, a suspension of three fresh egg-whites in 200 cc. of sterile RINGER'S solution was found to be most satisfactory. Ten cc. of this medium is kept in a sterile PETRI dish, 4 cm in diameter and 1 cm high. The colon of a yeast-fed host insect is extracted and examined in a small drop of RINGER'S solution on a slide. If it contains Nyctotherus, the whole is transferred into the PETRI dish and the host organ is thoroughly broken up in the medium. The entire culture is conveniently examined under a dissecting microscope or a low power compound microscope without taking out any samples. The best culture is obtained when the H-ion concentration of the culture medium remains between 7.0 and 7.8. This is closely correlated with the condition of the natural habitat of the ciliate, for the colon of the host insect shows on an average pH 7.0 to 7.4, while the parts anterior to it possess pH 6.2 to 6.6. When the room temperature is maintained between 18° and 22° C, the ciliate retains its normal body from, structure and behavior for several weeks, during which time division forms appear from time to time. When bacterial growths become too abundant, about one-half of the culture medium is pipetted off from the surface and fresh medium is added. It is interesting to note that not all bacteria occurring in the culture are injurious to either this protozoan or *Endamoeba blattae*, because these protozoans are often found in large numbers actively moving about among the thickest bacterial growth in the culture. It should further be noted here that inclusion of the host's organ seems to be essential for a successful cultivation. For the past one and one-half year, the fresh egg-white has been replaced by coagulated egg-albumin (DIFCO), which has been found equally satisfactory. Ten grams of coagulated egg-albumin is emulsified in 200 cc. of

Ringer's solution and the whole is autoclaved for 30 minutes under 15 pounds pressure.

Aragão (1912) used a 5 per cent egg-white in 0.85 per cent salt solution in keeping alive *Nyctotherus cordiformis* of Brazilian frogs in vitro. Kept in Petri dishes at room temperature, the ciliates multiplied in three to four days, followed by encystment on the next day. However, some often lived two weeks before degeneration. Balch (1932) reported successful cultivation of *N. ovalis* of *Blattella germanica.* The medium he used was composed of 19 parts of 0.5 per cent salt solution and one part of rabbit serum. He succeeded in maintaining three cultures for 40 days. Subculturing was done at weekly intervals and the cultures were kept at room temperature.

In a side view in which the organism is almost always observed under a compound microscope, *N. ovalis* is ovoid in form (Pl. 2 Fig. 4). In the oral or aboral view it is pyriform with its sides slightly concave near the middle of the body, which condition is distinctly noticeable under a dissecting microscope in actively swimming individuals. The posterior region is broadly rounded, except the extremity which is slightly bluntly pointed due to the presence of a somewhat elevated lip which surrounds the cytopyge. Stein (1867) maintained erroneously that the body of Nyctotherus is dorso-ventrally flattened. Kent (1881—1882) defined the ventral surface as „one upon which the oral aperture debauches", which however is not correct, because in the majority of the species of Nyctotherus there is no definite surface upon which the organism rests under natural condition. In an ordinary preparation, the ciliate invariably lies on its side unless enough support of the coverglass is included in the preparation. Although Bütschli (1887—1889) distinguished the right and left sides, it was Entz (1913) who correctly observed and described the body shape and external characteristic of *N. piscicola* which resembles the present species closely.

The body is covered with a thin pellicle which is striated longitudinally (Pl. 2 Fig. 1). These striae are due to a regular and linear arrangement of insertion points of the cilia which cover the entire body surface. The comparatively narrow, but quite deep peristome begins near the anterior end (Pl. 2 Figs. 8, 9), runs posteriorly and gives rise to the cytostome situated near the middle of the body. From the cytostome extends a long cytopharynx which runs almost at right angles to the peristome for some distance, and then takes its course toward the posterior end of the body (Pl. 2

Figs. 1, 2). The adoral zone along the peristome continues down through the cytopharynx to its posterior end.

The ectoplasm is a uniformly thin layer located below the pellicle, but is considerably thickened along the peristome and cytopharynx, and gives rigidity to these cell-organs (Pl. 2 Fig. 6). The fibrillar connections between the opposite sides of peripheral ectoplasm which were described by ENTZ (1913) in *N. piscicola* and by TEN KATE (1927) in the present species as well as *N. cordiformis*, have not been seen by the writer, except the fibrillae which connect the macronucleus with the peristome, cytopharynx and ectoplasm as will presently be stated.

The endoplasm presents a unique appearance. Between the anterior end of the body and the macronucleus which is situated in front of the cytopharynx, the endoplasm is filled with numerous bodies which vary considerably in form and size. The most conspicuous bodies which occur commonly in large numbers are of more or less rounded disc-shape with irregular contours, measuring about 6 μ in diameter and about 1 to 2 μ in thickness (Pl. 2 Fig. 2). LUGOL'S solution stains these bodies mahogany-red, which suggests that they are made up of glycogenous substance. Under a low power objective, this region of the ciliate appears darkly granulated (Pl. 2 Figs. 1—4) and this apparently led STEIN (1867) to call it "Körnerfeld". In almost all species of Nyctotherus a similar condition has been noticed by various observers.

The endoplasm located between the macronucleus and the posterior end of the body is much lighter in color, is more or less alveolated and contains starch grains, yeast cells, glycogenous bodies, bacteria and the spores of *Coelosporidium periplanetae*, a haplosporidian, which is nearly always found in the Malpighian tubules of the host insect. The endoplasmic movement is apparently confined to this region. Near the cytopyge are seen one or more contractile vacuoles (Pl. 2 Figs. 3, 4).

In the endoplasm of some individuals are found small flagellates, many of which are seen turning arround very actively within the body of Nyctotherus and which when liberated by the rupture of the ciliate pellicle swim about actively. The body is nearly spherical to ovoid and measures 3 to 5 μ in diameter. The three flagella are almost equal in length (being 5 to 12 μ long) and thickness, and arise from the anterior end of the body, near which is located a comparatively large vesicular nucleus. Occasionally the body of the ciliate is found to be densely filled with encysted individuals

which are invariably ovoid and a little smaller than the actively motile forms. In all cases the infected ciliate shows a typical macronucleus and does not appear to be affected by the presence of these organisms. Walker (1909) described "spore-formation" in *N. parvus* Walker (of *Rana clamitans* and *R. palustris*), *N. multisporiferus* Walker (of *Cavia cobaya*) and *Balantidium falciformis* Walker (of *R. palustris*). His illustrations and descrition of the „spores" show a very close resemblance between his „spores" and the flagellates under notice.

Since the normal *N. ovalis* in vegetative stage is highly active and motile, it is impossible to measure it accurately in life, without bringing about abnormality of form and dimensions. Numerous active individuals from ten host insects were placed in Ringer's solution on slides to each of which was added 2 per cent solution of osmium tetroxide. As far as can be judged with inspection, there was no perceptible decrease in dimensions of the specimens through killing. Two hundred undeformed individuals taken at random measured 90 to 185 μ long and 62 to 95 μ wide near one-third from the posterior end. Giant forms which measure more than 300 μ long by 200 μ wide were occasionally recognized among the forms mentioned above in a small number of newly caught cockroaches. They show morphological characteristics similar to the individuals of average dimensions. In a certain number of host insects which had been fed on egg-meat medium (Difco), yeast cakes or yeast extract (Difco) for two months, these giant forms were more frequently encountered than in those newly caught or starved. They measured 320 to 360 μ long and 200 to 240 μ wide. It may be supposed that a change in diet resulted in producing large forms, but if so, why there are a number of smaller forms co-existing in the host's organ, cannot be explained at present. Many individuals which are in precystic stage appear usually much smaller than the active vegetative forms.

Leidy (1853) gave 136 μ (1—187 th inch) and 102 μ (1—250 th inch) as the length and width of *N. ovalis* he observed. On the other hand, Stein (1867) stated that the ciliate shows extremely different dimensions. Small individuals measured only about 68 to 87 μ ($^1/_{32}$ to $^1/_{27}$‴) long by 57 to 68 μ ($^1/_{38}$ to $^1/_{32}$‴) wide, while lager individuals were more than 363 μ ($^1/_6$‴) by 273 μ ($^1/_8$‴). However, the commonly observed individuals were 136 to 218 μ ($^1/_{16}$ to $^1/_{10}$‴) long by 121 to 186 μ ($^1/_{18}$ to $^1/_{12}$‴) wide. Thus Stein's forms are much larger than those observed by the present writer.

STEIN's description and illustrations of *N. ovalis* and the writer's observation upon the forms under consideration are so morphologically alike that there seems to be no question as to their specific identity. Why then is there auch a discrepancy in the dimensions? Do they represent different races? The present writer believes that the discrepancy here noted is due to a difference in circumstances under which the measurements were made rather than to differences in nature and quantity of food taken in by host insects or to racial difference. Measurement of each of fifty freshly killed specimens in normal body form and in highly flattened state shows that the flattened individuals give 15 to 30 per cent longer and 25 to 50 per cent wider dimensions than those in normal body forms. Judging from STEIN's figures (1867, Pl. 15, Figs. 11, 12), it appears that the specimens were somewhat flattened and consequently showed greater dimensions than naturally preserved specimens.

The encysted forms occur commonly in the host insect in which active vegetative forms are also present. The cyst is oval to ellipsoid (Pl. 2 Fig. 10). Its anterior end is broadly rounded, while its posterior end is slightly elevated. It is light yellow to light brown in color and the cyst membrane is uniformly thick throughout. Anterior to the middle, there is the macronucleus. The cytoplasm between the nucleus and the front end is in most cases loaded with glycogenous bodies. One or two contractile vacuoles remain visible in many cysts. The karyophore which is a characteristic structure in the trophozoite does apparently not occur in the encysted organism. Fully formed cysts measure 72 to 106 μ long by 58 to 80 μ broad. STEIN (1867) observed several cysts and mentioned that the largest cysts measured 121 μ by 73 μ ($^1/_{18}$‴ by $^1/_{30}$‴) and the smallest 62 μ by 40 μ ($^1/_{35}$‴ by $^1/_{54}$‴), while average cysts measured 73 to 91 μ long by 55 to 73 μ wide ($^1/_{30}$ to $^1/_{24}$‴ by $^1/_{40}$ to $^1/_{30}$‴). LUCAS (1927) gives 55 to 94 μ long by 25 to 70 μ wide as dimensions of the cysts she observed.

The micronucleus.

There is a single micronucleus in each individual. It is without exception attached very closely to the anterior surface of the macronucleus and is a spherical, ovoid or ellipsoidal body, measuring less than 10 μ in diameter (Pl. 2 Fig 5). As viewed in life in a slightly compressed ciliate, it is enveloped by a thin, but distinct membrane and contains a homogeneous mass or a group of several

small rounded bodies, suspended in the karyochylema, the whole being far more hyaline than the massive macronucleus.

In an actively motile individual, it is impossible to observe the micronucleus because of the glycogenous inclusions usually present in abundance anterior to the macronucleus. The micronucleus is most clearly observable in sectioned individuals. Its contents give a rose color with FEULGEN's nucleal staining, while the chromatin spherules in the macronucleus show a typical deep coloration by the same staining. HEIDENHAIN's iron haematoxylin or DELAFIELD's haematoxylin stains the micronucleus much less deeply than the macronuclear chromatin spherules or sometimes not at all so that it is conspicuously colored with the counter-stains.

Of the previous investigators of this ciliate, ZULUETA (1916) was the first to see the micronuclcus. He wrote: "El micronúcleo presenta un endosoma de aspecto homogéneo rodeado por una ancha zona clara limitada por la membrana." TEN KATE (1927) figured the micronucleus of this ciliate as seen in sections without however giving any description of it

The macronucleus as it appears in life.

The macronucleus of *Nyctotherus ovalis* lies anterior to the cytopharynx and the middle of the body. It is a very conspicuous structure and is therefore easily seen in an actively motile living individual under a low power dissecting microscope. Viewed from the oral or aboral surface, the macronucleus is a short or elongated rod-like body (Pl. 2 Figs. 3, 6). It may be of uniform width or its extremities may be much enlarged, with its axis, as a rule, at right angles to the body axis. It measures 30 to 70 μ long by 15 to 25 μ wide. Viewed from the habitual lateral surface the macronucleus is biconvex. Its anterior surface may vary considerably in convexity from almost plain to slightly arched condition, while the posterior surface is invariably highly convex (Pl. 2 Figs. 5, 7; Pl. 3 Fig. 15). The thickness of the macronucleus as measured at right angles to the body axis in side view varies from 30 to 50 μ. The macronucleus of *N. gyoeryanus* appears, according to GRASSÉ (1928), to possess a similar form and location within the body of the ciliate.

The macronucleus is delimited by a very thin membrane. In life the membrane is very difficult to see and one doubts ist presence. ZULUETA (1916) saw this condition and wrote: "La membrana nuclear es delgada y no siempre visible". However, it is distinctly seen in

many living specimens. Its presence can be demonstrated by subjecting the nucleus to the influence of 10 per cent sodium chloride solution, which results in swelling and transparency of the chromatin spherules. In such a preparation the nuclear membrane is clearly noticed (Pl. 3 Fig. 16) and when the nuclens is rolled under the coverglass, the membrane shows numerous folds on it (Pl. 3 Fig. 17).

Surrounding the macronucleus are two (anterior and posterior) membranes. In lateral view, these membranes are seen to extend toward both the oral and aboral surfaces and each ends independently in the ectoplasm. The anterior membrane often continues its course toward the anterior region at both the oral and aboral surfaces and at midway between the macronucleus and the anterior end of the body, it forms a transverse membrane (Pl. 2 Figs. 5, 7). That these are membraneous structures is clearly seen by their continuity in a living organism. It is further noticeable in the serial longitudinal (Pl. 2 Figs. 5, 7) as well as transverse sections (Pl. 2 Figs. 8, 9) of individual organisms.

In the oral or aboral view, the perinuclear membranes do not extend to the sides. The right and left extremities of the macronucleus and the peripheral ectoplasm directly opposite them are connected by several fibrillae. This condition has also been recognized in living as well as in serial longitudinal (Pl. 2 Fig. 6) and transverse sections (Pl. 2 Fig. 8). Besides these membranes and fibrillae, there are distinct fibrillae connecting the perinuclear membranes with the thickened ectoplasmic wall of the peristome and the cytopharynx (Pl. 2 Figs. 6, 8). The membranes described here are made up of numerous fibrillae and homogeneous cytoplasm, which could be seen in vivo and in section preparations.

A fibrillar system connecting the macronucleus and the ectoplasm had first been noticed by SCHUBERG (1888) in *Isotricha prostoma* and *I. intestinalis*. SCHUBERG named these fibrillae "Kernstiele" (1888, p. 382) or "Karyophoren" (1888, p. 394), and wrote: "Diese Kernstiele sind Fortsetzungen der inneren Körpermembran, die sich als kurze Stränge zum Nucleus begeben, um denselben deutlich membranartig zu umschließen". This author further compared his observations with those of STEIN (1867) on three species of Nyctotherus and suggested that the members of the latter genus possess a similar structure. Through the efforts of recent investigators, it is now well established that a fibrillar system occurs in several species of Nyctotherus. The karyophore has been observed in the following species: *N. piscicola* (ENTZ, 1913), *N. ovalis* (ZULUETA, 1916; TEN KATE, 1927), *N. buissoni*

(Pinto, 1926), *N. cordiformis* (ten Kate, 1927), *N. haranti*, *N. duboisii*, *N. velox*, *N. gyoeryanus* (Grassé, 1928) and *N. silvestrianus* (Kirby, 1932).

Of these authors who have recognized the karyophore, all agree that the structure completely envelops the macronucleus. However, as to the connection between the latter and the ectoplasm, observations and opinions vary. Entz and ten Kate consider that the suspension of the macronucleus is carried on by fibrillae, arranged in certain manners. On the other hand, Zulueta (1916) considered that the karyophore of the present species is "una lámina hialina que constituye un tabique o diafragma", which he compared with the ectoplasmic septum of the polycystid gregarine. The evidence in support of this view is inadequate; in fact Zulueta does not give the "diaphragm" as it is seen in the oral or aboral view or in transverse section. Grassé (1928) undertook a careful comparative study of five species of Nyctotherus and in four of them observed a karyophore similar to the one described above. In *N. gyoeryanus*, Grassé (1928, Pl. 2 Fig. 3) gives one of the membranes of the karyophore as composed of fibrillae and about one-fourth to one-third in width in relation to that of the body. Kirby (1932) observed similarly in *N. silvestrianus* that "the macronucleus is surrounded by membranes which are extended toward the periphery of the body as a karyophore". As was stated above, in *N. ovalis*, the two membranes are quite as wide as the length of the macronucleus, but do not form a complete septum, the macronucleus being suspended from the ectoplasm on its right and left sides by several fibrillae (Pl. 2 Fig. 8).

The membrane occurring between the macronucleus and the anterior end of the body has also been seen by previous observers of this and other species of Nyctotherus. ten Kate (1927) held that it was made up of a group of fibrillae which he named "links-rechts-Fibrille" in *N. ovalis*. On the other hand a structure similar to the one described above, present in *N. haranti*, was found to be a membrane by Grassé (1928) who named it "lame frontale".

Both Entz (1913) in *N. piscicola* and ten Kate (1927) in *N. ovalis* and *N. cordiformis*, observed that each fibrilla is in direct contact with a cilium of the body surface. This the present writer failed to confirm with the material on hand. The membranes and the fibrillae are, as seen in living individuals of *N. ovalis*, similar to the ectoplasm in their optical characteristics and measure at places 1 to 2 μ in thickness.

As to the function of the karyophore, Schuberg (1888) considered it as "eine Fixierung der Kerne", which view has been adopted by

recent workers. But if it serves for maintaining the constant position of the macronucleus in the anterior region of the body, how the macronucleus of *N. tipulae* in which according to GRASSÉ (1928) no visible karyophore occurs, is able to hold its position in the anterior region of the body is difficult to understand.

When a living individual is slightly flattened between a cover glass and a slide so that it moves about slowly, its macronucleus is seen to contain numerous spherical bodies suspended in a homogeneous karyochylema. These spherules are quite refractile under a low magnification, but less so when more highly magnified. With smooth contours they may be more or less uniformly small as is usually the case (Pl. 2 Figs. 2, 4; Pl. 4 Fig. 22), of extremely different dimensions (Pl. 2 Fig. 11; Pl. 3 Figs. 13, 19, 20) or of intermediate sizes (Pl. 3 Fig. 12). These spherules vary from a small fraction of micron up to as large as more than 20 μ in diameter. In some cases the ciliates obtained from one and the same host individual show more or less similar dimensional regularity of the spherules, while in others these spherules differ greatly among the ciliates taken from one host insect.

These spherules are, as a rule, perfectly spherical, although larger spherules appear occasionally to be ovoid (Pl. 3 Fig. 13). The smallest spherules appear to be homogenous and structureless. The spherules which are more than 1 μ in diameter are frequently alveolated, which condition is noticeable in the specimens which were obtained from the colon of an actively motile host and which appear normal in their structure and behavior. Larger spherules are often greatly alveolated (Pl. 2 Fig. 11; Pl. 3 Figs. 13, 20). Of these alveoli, one to several may be more distinctly contoured, larger and more centrally located than others and one or more small spherical bodies may occur in them (Pl. 2 Fig. 11; Pl. 3 Fig. 13). When these spherules are highly compressed, neither the alveoli nor the intraalveolar bodies are noticed, but when the pressure is removed the alveoli and the minute bodies which occur in them become visible again.

The karyochylema of the macronucleus is homogenous and structureless when examined in individuals which are held motionless by the mechanical pressure of a cover glass, in both bright and dark field microscopes (Pl. 3 Figs. 12, 19, 20). When an organism is subjected to mechanical pressure under a cover glass, the body breaks up and its nucleus becomes highly flattened. If the pressure is still more increased, the nuclear membrane ruptures and the karyochylema streams out slowly, carrying in it the spherules which may move

in different ways. When a drop or two of Ringer's solution are added to the preparation, the karyochylema contracts and brings back at least part of the numerous spherules into more or less solid masses. These changes were examined under both bright and dark field microscopes, but no formed structures were visible. Furthermore, in certain individuals, the spherules are seen undergoing active Brownian movements which also indicate that the nuclear sap is without any structure. The karyochylema therefore appears to be an optically homogeneous dispersion medium.

Almost all of the previous observations on the macronuclei of various species of Nyctotherus seem to be based upon stained specimens. With excellent illustrations, Stein (1867) described the appearance in vitro of the macronucleus of the present species as follows: "In der lichten Nucleussubstanz liegen gewöhnlich sehr zahlreiche kernartige Gebilde, zum Teil von ansehnlicher Größe". Stein noticed further a similar appearance in the macronucleus of *N. cordiformis* and *N. gyoeryanus*. Schneider (1886) in his work on *N. cordiformis*, described its nucleus as follows: "Coloré convenablement et éclairé, ce corps (nucleus) parait entièrement gorgé de chromatosphérites. A un examen avec un excellent objectif homogène de Prazmoswski, ces chromatosphérites sont bien isolés, et il est impossible de les interpréter comme des coupes optiques d'un filament nucléaire. Je me prononce très nettement sur ce point. Dans nombre d'exemplaires, le nucléus présente des places sans chromatosphérites qui apparaissent comme des vacuoles".

Bezzenberger (1903) found the chromatin of the macronucleus of *N. macropharyngeus* and of *N. magnus* to be finely granulated, those in the first species being somewhat larger than those in the second. Entz (1913) gives the following description of the macronucleus of *N. piscicola*: "Das Chromatin des Macronucleus besteht aus größeren und kleineren Kügelchen, welche miteinander mit Fäden (Linin) verbunden sind (?) und ohne jegliches System angeordnet zu sein scheinen. . . . In manchen Kügelchen lassen sich Granulationen beobachten, welche in einem Falle in Vierergruppen verteilt waren. Die Chromatinkügelchen des Macronucleus scheinen aus äußerst kleinen, runden Körperchen zusammengesetzt zu sein, welche miteinander mit feinen Fäden verbunden sind". Unfortunately living specimens were unavailable to Entz. Pinto (1926 a) gives illustrations of *N. cordiformis*, *N. cunhai* and *N. tejerai*, the macronuclei of which are filled with what appear to be uniformly small chromatin granules. ten Kate (1927) studied the fibrillar system of *N. ovalis* and *N. cordi-*

formis, but did not give any description of their nuclear structures. However, his Figs. 20 to 23 show the finely granulated macronucleus containing several large spherules, some of which are nearly one-third the diameter of the micronucleus shown in the same figures.

JÍROVEC (1927) included *N. cordiformis* in his study of various nuclei by means of the nucleal staining and stated that the macronucleus of this ciliate is made up of very small and somewhat numerous nucleoli lying in a very thick chromatin network which is stained purple-red — the nuclear structure which he saw in all macronuclei of the ciliates he studied. This is contrary to the observations made by STEIN, ARAGÃO, TEN KATE and others. GRASSÉ (1928) gives the following observation on the macronucleus of *N. haranti*: "il est bourré de granules chromatiques de taille uniforme, très ovides de couleurs basiques. On découvre, presque toujour à l'intérieur du noyau, un space (4 or 6 μ) circulaire incolore qui j'interprète comme un plasmosome." On the other hand, GRASSÉ saw only chromatin granules in the macronucleus of *N. tiputae* or *N. velox*. KIRBY (1932) writes that the macronucleus of *N. silvestrianus* contains spherules and granules of various sizes, ranging in diameter up to 1.5 μ, and stains red with neutral red and blue with cresyl blue, while the micronucleus remains unstained.

Thus it is apparent that the macronucleus of Nyctotherus is characterized by the possession of numerous more or less conspicuous spherical bodies. SCHNEIDER, ENTZ, GRASSÉ and others held them as chromatin bodies based upon their reactions to nuclear dyes. The spherules which occur in the macronucleus of *N. ovalis* and which were described above as seen in living organisms, have been found to show positive reactions to all known chromatin "tests" and are therefore regarded as chromatin spherules.

The macronucleus and FEULGEN'S nucleal staining.

As the macronuclear chromatin spherules vary in size among different individuals, a single ciliate was studied at a time in order to find out exactly how the spherules appear in life, upon fixation and after staining. For the nucleal staining, FEULGEN'S (1926) procedure was followed. Because of the large dimensions and quantity of the spherules occurring in the macronucleus, the progress of the staining in the fuchsin sulphurous acid could distinctly be followed under a dissecting microscope ($\times 17$). One minute after coming in contact with this mixture, the nucleus assumes a slight rose color, in two minutes, the color deepens, in three and four minutes, it

becomes crimson red and in five minutes it appears violet-red. Longer treatment increases the intensity of the color only slightly.

Fixation with a mixture of mercuric chloride and acetic acid (49:1) brings about certain changes upon the chromatin spherules. The spherules become quite distinctly outlined and in many cases appear angular rather than spherical, which changes are noticed in both large and small spherules. There is usually a small amount of shrinkage. The karyochylema does not seem to be affected at all and remains colorless and structureless after the whole process. When these spherules are subjected to the nucleal staining and mounted in toto, it is noted that they nearly recover their original form and size. The staining further shows that the alveoli seen in the chromatin spherules in life are devoid of chromatin substance except the varying number of minute chromatin spherules which are suspended in them. These intraalveolar chromatin bodies are arranged without order and no connection is observable among them.

In the case of sectioned material, there is almost always a greater decrease in size of chromatin spherules after completion of preparations. Measuraments of large spherules in life and in sections of twenty specimens show that the entire process causes about 10 to 15 per cent decrease in diameters of the chromatin spherules. When large chromatin spherules are sectioned, the alveoli are always without either the color of fuchsin or that of light green, which indicates that the alveoli do not contain either chromatin or plastin. These large spherules in section preparations are sometimes seen to be surrounded by a narrow light violet-red zone, while smaller ones in the same preparations do apparently not show this condition. Whether this is a natural condition not observed in life or due to a partial dissolution of the superficial portion of the spherules by the fixative and aftertreatment, cannot be determined at present. In sectioned nuclei, from which large chromatin spherules have been washed away during manipulation, fibrillar structures are often seen stretched among the remaining spherules, which take light green color. They appear to be precipitation product of the karyochylema.

The chromatin spherules and chromatin 'tests'.

As is stated above, it was found that the macronuclear spherules of *Nyctotherus ovalis* are made up of thymonucleic acid as judged by their reaction to Feulgen's nucleal staining. Three so-called tests for chromatin were tried in order to find out whether they were reliable or not.

1. Acidified methyl green.

Methyl green has long been recognized as one of the few specific chromatin stains and is often used in an acidified solution for staining the nucleus of a living protozoan. In the present study, 1 per cent methyl green in 1 per cent aqueous solution of acetic acid was used. The methyl green was in usual impure condition, as it is used in all cases in practice; it gives metachromatic reaction by virtue of methyl violet which always occurs in it in a small amount.

The acidified methyl green was added to the preparation under the cover glass, after the chromatin spherules were observed in life. When the dye reaches the organism, the latter is killed instantaneously, and the peripheral zone of the cytoplasm and the chromatin spherules become stained simultaneously, the former blueish-violet and the latter bright green. As a rule there is a perceptible increase in diameters of the spherules (Pl. 3 Figs. 20, 21). The largest spherule shown in Pl. 3 Fig. 20 measured 20.5 μ in diameter in life. When stained with the dye its diameter increased to 21.5 μ without apparent change in the thickness of the nucleus (Pl. 3 Fig. 21). In the same nucleus two smaller chromatin spherules measured in fresh condition 11.3 μ and 5 μ respectively, which however increased to 13.3 μ and 6 μ in diameters after being treated with the dye.

The alveoli within the chromatin spherules are either colorless or very light blue and become distinctly distended after the treatment (Pl. 3 Figs. 20, 21). The intraalveolar chromatin bodies are stained in the same color and intensity as is the main part of the spherules. When the nucleus which was stained with acidified methyl green is subjected to FEULGEN'S nucleal staining, the chromatin spherules show typical coloration. Thus the acidified methyl green may be considered a specific chromatin dye.

2. Ten per cent solution of sodium chloride.

Working on the constituents of animal as well as plant cell-nuclei, ZACHARIAS (1881) found that 10 per cent sodium chloride solution caused swelling of the (chromatin) granules, while the nucleolus remained unaffected. By longer action the granules appeared entirely dissolved by the solution. Although there are no distinct nucleoli in the macronucleus of *Nyctotherus ovalis*, this solution was used to determine its effect on the chromatin spherules.

The macronucleus of a living specimen was studied in side view (Pl. 3 Fig. 15) and the salt solution was added to the preparation. The diffusing solution killed the organism instantaneously and at the same time the nucleus became highly transparent, due to the sudden swelling and transparency of all chromatin spherules, without revealing any structures which might be interpreted as nucleoli. This brought about an enlargement of the entire nucleus (Pl. 3 Fig. 16), leaving the distended nuclear membrane very conspicuous. As the nucleus was rolled under the cover glass, the membrane formed numerous folds on it (Pl. 3 Fig. 17). Ten minutes after the application of the salt solution, the cover glass was lifted and the nucleus together with now deformed and disintegrating cytoplasmic mass was fixed in mercuric chloride and acetic acid and subjected to the nucleal staining. The enlarged nucleus was stained violet-red, while the nuclear membrane and the cytoplasm proper took light green. In the nucleus, the individual chromatin spherules were found to be much enlarged, but still retained individuality with fairly distinct outlines (Pl. 3 Fig. 18). Whether prolonged treatment with the salt solution would dissolve the chromatin spherules completely or not could not be determined, because this resulted in bursting of the nuclear membrane and complete disintegration of the body.

Several observations gave a similar result which indicates that 10 per cent sodium chloride solution brings about instantaneous and vigorous change in the physical make-up, and increase in size, of the chromatin spherules.

3. Pepsin and hydrochloric acid.

Artificial digestion has long been used for detection of chromatin. As early as 1881, Zacharias tried this method on Vorticella, Paramecium and Opalina. Concentration and proportion of the acid and pepsin used for this purpose appear to vary greatly according to investigators (Spalteholz, 1927). In the present study, 2 per cent pepsin (Difco) in 0.5 per cent hydrochloric acid was used. After observing the macronucleus in life, the actively motile ciliate was transferred to Nemeczek depression slide to which was added the pepsin-hydrochloric acid mixture. The preparations were kept at 35—37° C and observations were made at various intervals. Of numerous observations which gave similar results, one will be described here.

The macronucleus of a living specimen was observed and photographed (Pl. 4 Fig. 22). The chromatin spherules in this nucleus were fairly uniformly small and the largest ones measured about 3 μ in diameter. Both the nuclear membrane and the karyophore were distinctly visible. Five minutes after the addition of pepsin solution, the nucleus was found to have decreased in size, the cytoplasm, nuclear membrane and karyophore were already partially digested, while the chromatin spherules were distinctly contoured and more transparent than in life (Pl. 4 Fig. 23), which latter condition appeared at least in part due to the change which took place in the karyochylema. In one hour and a half, the nuclear membrane and the karyophore were greatly digested and the nucleus itself shrunk a great deal, its largest diameter in this view becoming about one-half that of the living state. Individual chromatin spherules were partially hydrolyzed and appeared smaller in size and indistinct in outline (Pl. 4 Fig. 24).

After 13 hours and a half, the cytoplasm, the karyophore and the nuclear membrane were completely digested and the chromatin spherules were seen fusing with one another, resulting in indistinct outlines of individual spherules (Pl. 4 Fig. 25). In 38 hours, the cytoplasm, except the inclusions, had entirely disappeared and the chromatin mass of the nucleus became less compact so that it was much enlarged and presented an irregular shape. Numerous chromatin spherules were further so affected by the enzyme that there were several enlarged spherical bodies apparently composed of hydrolyzed chromatin substance (Pl. 4 Fig. 26). Almost the same conditions were found 61 hours after the beginning of the experiment (Pl. 4 Fig. 27). Ten days later the conditions were nearly the same. For control 0.5 per cent hydrochloric acid alone was used. From this and similar experiments it appears certain that the macronuclear chromatin spherules of *N. ovalis* are digested to a certain extent by pepsin-hydrochloric acid and that they however resist artificial digestion much more strongly than any other part of the ciliate protoplasm.

The effects of fixatives upon the chromatin spherules.

Almost all of the previous attempts to observe the effects of fixatives upon the nucleus were confined to the nucleus as a whole or structures other than the chromatin bodies. For instance, STRANGEWAYS and CANTI (1927) studied the effects of various fixatives upon the outline and form of the nucleus, nucleoli and various cytoplasmic

structures of various tissue cells of the embryonic fowl in tissue cultures. In the present study, observation was directed especially to the chromatin spherules themselves as they occur in the resting macronucleus of *Nyctotherus ovalis*.

For each fixative several observations were made on specimens taken from different host insects or cultures on different dates. After the nucleus of a living individual was observed, a freshly prepared fixative was added directly to the preparation. The amount of the fixative used was approximately twice the amount of Ringer's solution or culture medium which had been used in making the preparation. The concentration, therefore, of the fixative when the latter reached and acted upon the organism, must have been somewhat lower than that indicated for each fixative. Since the living nucleus is more or less flattened under the cover glass to allow accurate observation, it is probable, of course, that some of the larger spherules may have been also compressed. When the fixative is added to the preparation, its diffusion under the cover glass tends to lift the latter before it reaches the organism which recovers its freedom and begins to move about for a second or two until the fixative reaches it. Several fixatives appeared to affect the water contents of the karyochylema greatly and this resulted in crowding together of the chromatin spherules. Consequently the same chromatin spherules were not observed, except in a few favorable cases, both in life and in fixed condition, although of course one and the same nucleus was under observation. It is therefore impossible to make a precise comparison between the individual chromatin spherules in life and after fixation.

Absolute alcohol. After fixation with absolute alcohol, the chromatin spherules become less distinct and much more irregular, especially angular, in outline. The karyochylema shrinks greatly. although the shrinkage of the chromatin spherules is not so great, Feulgen's nucleal staining shows that the chromatin spherules are less compact and less deeply stained as compared with those which were fixed with a fixative containing mercuric chloride. The karyochylema appears to be rose-colored.

Fischer (1899) using 96 per cent and absolute alcohol as fixatives, found that the precipitates of peptone, albumoses and nucleic acid were easily soluble in water and even if kept in alcohol for a week they did not coagulate. Tellyesniczky (1898) found in the immature male germ cells of salamander fixed with absolute alcohol and stained with Weigert's stain or safranin that "im Inneren des

mitgerissenen Kernes verdichtet sich das Chromatin ganz eigenthümlich und bedeckt in Form eines Kegels den Kern". MANN (1902) observed that the precipitate of nucleic acid formed by absolute alcohol is soluble in water, while that of nucleoalbumin or nuclein is insoluble. From the light, yet fairly distinct rose coloration of the karyochylema by the nucleal staining noted above and from the fact that the chromatin spherules are less compact and less deeply stained, it would appear that the nucleic acid of *Nyctotherus ovalis* is not well fixed with absolute alcohol so that it becomes partially dissolved during the after-treatment.

0.5 per cent acetic acid solution. With this fixative, the chromatin spherules become highly alveolated with indistinct outline. The nuclear membrane is not fixed so that the nucleus breaks easily into small masses of chromatin spherules during the manipulation. Nucleal staining shows deeply stained highly alveolated chromatin spherules deformed and fusing with one another.

FISCHER (1899) tried acetic acid in various concentrations in his experiments and found that the nucleic acid (of yeast) behaved in different ways, subject to the concentration of the acid and the reaction of the solution. MANN (1902) showed that the precipitates of nuclein, nucleoalbumin and nucleic acid produced by 10 per cent acetic acid solution was insoluble in water. BERG (1926) found in 1904 that nucleic acids from animal material were not precipitated by dilute acetic acid, while those from plant material were precipitated by it, although the latter varied in solubility. Using 5 per cent solution on the liver of guinea-pig to determine its rate of penetration, UNDERHILL (1932) remarked that the fixed nuclei in the periphery were well preserved and that in the center of the section which was so completely macerated as to be almost structureless, nuclei were present in some cells as distorted masses of chromatin. The present study tends to show that 0.5 per cent acetic acid used alone does not precipitate the chromatin spherules of this ciliate.

4 per cent formaldehyde solution. After addition of 4 per cent neutral formaldehyde solution, the chromatin spherules become less distinctly outlined. The alveoli in the chromatin spherules are partially lost. In some instances, the peripheral portion of the spherules become mottled. As a rule, the fixation brings about more numerous smaller chromatin spherules than in life. This may be caused by disintegration, and transformation into the smaller spherules, of the superficial portion of large spherules. FEULGEN'S staining produces deep typical coloration by which the individual spherules

are distinctly contoured. The intrachromatin alveoli are however less distinctly visible. The shrinkage of chromatin spherules due to the after-treatment with alcohols is very slight, but there is a very weak rose coloration of the karyochylema.

It is generally recognized that dilute formaldehyde does not precipitate the nucleic acid. Tellyesniczky (1898) working with salamander testes observed that treatment with 0.5 or 10 per cent formaldehyde resulted in a very poor fixation of both the cytoplasm and nucleus and stated that "am destructivsten (of simple fixatives he used) wirkt das Formalin, welches das Plasma wie auch den Kern gleich heftig alterirt". Mann's table (1902) shows that nucleic acid is not precipitated by 10 per cent solution. Baker (1933) showed that 4 per cent formaldehyde does not harden albumin or nucleoprotein, nor render it insoluble in water, and that the fixative made it no longer capable of being hardened by alcohol. The present study seems to show in agreement with Baker that 4 per cent formaldehyde does not precipitate the nucleic acid nor make it insoluble, as judged by the breaking up of some of the chromatin spherules by the treatment and the rose coloration of the karyochylema and that the reagent renders the nucleic acid no longer capable of being hardened by alcohol, which is noticeable in very slight shrinkage of the chromatin spherules as seen in the completed permanent preparations.

6 per cent solution of mercuric chloride. After fixation with this solution, the chromatin spherules are distinctly outlined, but slightly angular and smaller. Feulgen's nucleal staining brings out deeply and distinctly stained chromatin spherules. The nuclear membrane is well preserved, although the entire nucleus shows a high degree of shrinkage. Staining and further manipulation result in a still greater shrinkage of the nucleus.

Fischer (1899) showed that the precipitate of the nucleic acid in solution produced by 7 per cent mercuric chloride is insoluble in water. Using 2.5 per cent solution, Mann (1902) obtained a similar result. Mercuric chloride is widely known as a fixative which produces a great shrinkage of the protoplasm, which is also shown by the present study. The conspicuously noticeable shrinkage in stained permanent preparations is, however, not entirely due to the precipitating action of mercuric chloride, but also to the after-treatment with alcohols and xylol during the course of preparation. Of nuclear elements, the chromatin spherules themselves do not suffer as great a shrinkage as does the karyochylema.

2 per cent osmium tetroxide. Exposure to the vapor, or fixation by the solution, of 2 per cent osmium tetroxide, preserves the chromatin spherules excellently. They are more or less spherical and distinctly outlined, with no perceptible shrinkage though less refractive. The nucleal staining shows a typical deep staining of the spherules. The karyochylema remains colorless. The progress of staining of the nucleus in fuchsin sulphurous acid after fixation with osmium tetroxide as seen under a dissecting microscope, is as follows: In one minute there is a slight orange coloration, in two minutes the color becomes rose red, in three minutes the nucleus turns crimson red, in four minutes it assumes a deep red and after five minutes the nucleus appears to be violet-red. Longer treatment does not intensify the chromatin coloration much, the cytoplasm begins, however, to take rose color after twenty to thirty minutes.

SCHAUDINN'S fluid (Pl. 4 Figs. 28—30). This fixative is probably one of the most commonly employed in protozoology. The solution used has been made up of 66 cc. of 6 per cent solution of mercuric chloride, 33 cc. of absolute alcohol and 1 cc. of glacial acetic acid. When fixed with this fluid, the chromatin spherules become slightly angular, but distinctly outlined. They do not show any perceptible shrinkage, although the whole nucleus shrinks a great deal. The karyochylema shrinks which results in the crowding together of the chromatin spherules. FEULGEN'S nucleal staining reveals a typical deep coloration of the chromatin spherules. The staining and mounting appear to restore the slight shrinkage and deformity of the chromatin spherules which were apparent after the fixation. An intense coloration of the chromatin spherules is reached in about five minutes in fuchsin sulphurous acid solution. The karyochylema remains unstained, which shows that the fixation is complete and the chromatin is not dissolved during the after-treatment and mounting.

Mercuric chloride and absolute alcohol. The mixture used consists of two parts of 6 per cent solution of mercuric chloride and one part of absolute alcohol. When treated with this fixative, the chromatin spherules often become angular in form, but with distinct outlines and at the same time undergo a more or less recognizable amount of shrinkage. Intrachromatin alveoli are not well preserved; the karyochylema shrinks in many cases. FEULGEN'S nucleal staining is completed in about five minutes in fuchsin solution. The chromatin spherules are deeply and distinctly stained, while the karyochylema remains unstained.

Carnoy's mixture (three parts of absolute alcohol and one part of glacial acetic acid). After fixation with this fluid, the chromatin spherules become often angular in form. As a rule, there is a slight shrinkage in the spherules after fixation. Feulgen's nucleal staining shows usually fairly deeply stained chromatin spherules. Five minutes in fuchsin sulphurous acid is sufficient to bring about a deep coloration. The karyochylema is usually slightly rose-colored, which indicates that the chromatin is soluble in water after fixation with the mixture as in the case of absolute alcohol.

Bouin's fluid. The fluid was made up according to Bouin (1897); i. e., 75 cc. of saturated aqueous solution of picric acid, 25 cc. of 40 per cent formaldehyde and 5 cc. of glacial acetic acid. When treated with this fixative, the chromatin spherules become less refractile and shrink a little, their outlines remaining somewhat indistinct due to decrease in refractivity. Moreover the spherules often undergo deformity. The karyochylema does not seem to shrink through fixation. Feulgen's nucleal staining shows distinctly stained chromatin spherules and unstained karyochylema. The staining is completed within three minutes after being in fuchsin sulphurous acid.

Flemming's fluid. Flemming's fluid which was tried in the present study is the strong formula (Flemming, 1884) which is composed of the following parts: 15 parts of one per cent chromic acid, 4 parts of 2 per cent osmium tetroxide and one part of glacial acetic acid. It fixes the chromatin spherules with little deformity and without any shrinkage. The karyochylema is not precipitated. The entire nucleus does not show any perceptible shrinkage, even after the completion of the preparation. The nuclear membrane is well preserved. Feulgen's nucleal staining shows distinct and fairly deeply stained chromatin spherules and colorless karyochylema. Prolonged treatment in fuchsin sulphurous acid stains the cytoplasm deeply. Therefore, the treatment with the fuchsin solution was limited to five minutes. The permanent preparations show almost true pictures of the chromatin spherules as they appear in life with respect to their form, size and arrangement.

Feulgen's nucleal staining and fixatives.

Feulgen (1926) stated that in order to obtain a positive nucleal staining, fixatives containing oxidizers such as chromic acid must be avoided, since they destroy the "nucleal body" or the thymonucleic acid. He advocated a mixture of mercuric chloride and acetic acid (98 : 2) as a suitable fixative. Feulgen-Brauns (1924) showed that

the nucleus fixed with ZENKER's fluid gave a weaker staining. The majority of workers have used mercuric chloride and acetic acid. However, ZUELZER (1927), JIROVEC (1927, 1932), MARGOLENA (1932) and others obstained positive results by using several other fixatives. BAUER (1932) showed that by changing the time of partial hydrolysis the testis of *Stenobothrus parallelus*, the root of *Allium cepa* and *Vicia faba*, ovarian tubes of *Ascaris megalocephala*, and *Lumbricus*, sp. which were fixed with twenty-four commonly employed cytological fixatives, gave positive nucleal staining. The present study confirms that of BAUER.

FEULGEN's nucleal staining is essentially an aldehyde reaction. In all FEULGEN preparations of sections of host's colon, it is common to find certain plant structures as deeply stained as are the nuclei of *Nyctotherus ovalis* or of the host tissue cell nuclei. MARGOLENA (1932) found that when lignin, suberin and cutin occurring in the structures of the roots and leaves of several plants are subjected to the nucleal staining, all give a typical staining. It appears however probable from the results abtained by previous workers as well as by the present writer that the nucleal staining is a reliable means of determining thymonucleic acid-containing bodies or chromatin substance at least within the confine of the nuclear membrane in a protozoan.

The chromatin spherules and nuclear staining.

HEIDENHAIN's iron haematoxylin and DELAFIELD's haematoxylin which are quite frequently used as nuclear stains by students of protozoology, are, of course, not exclusive nuclear dyes and stain different parts of the nucleus and the cytoplasm in different tones of the same color. Repeated comparison was made between the chromatin spherules stained by these dyes in usual manner and those treated with nucleal staining, which reveals that the haematoxylin staining gives as true pictures of the chromatin spherules as are given by FEULGEN's staining.

It is interesting to notice, however, at least in one instance, the chromatin spherules which were clearly observable both in life and after fixation with SCHAUDINN's fluid, were stained by HEIDENHAIN's iron haematoxylin less deeply than the interchromatin space which was colored deeply. Consequently the stained nucleus presented an entirely different appearance which may have led one to consider it as composed of a chromatin "network", had one not seen

the nucleus in both the living and fixed conditions. It is not probable that the chromatin substance has dissolved and become absorbed by the karyochlylema, as Schaudinn's fluid fixes the spherules and renders them insoluble.

This peculiar staining of the macronucleus has a superficial resemblance to the staining shown by Bělař (1926) in the resting and dividing macronuclei of *Chilodon uncinatus*, which were fixed in Schaudinn's fluid and stained with Heidenhain's iron haematoxylin. Bělař wrote: "Man sieht hier, wie die Färbbarkeit der verschiedenen Strukturelemente des 'Außenkerns' während der Teilung dem Ruhekern gegenüber 'invertiert' ist." By subjecting dividing *Chilodon cucullulus* to the nucleal staining, Reichenow (1928), however, observed: "Die einzelnen (chromatin) Körnchen fließen zusammen, wobei in diesem Falle allerdings keine ausgesprochen fädigen Strukturen entstehen, sondern vielmehr eine spongiöse, mit farblosen Räumen durchsetzte Masse gebildet wird, die rings den Binnenkörper umhüllt." Thus it becomes apparent that in these two species of *Chilodon* there is no "inversion" in staining, as was suggested by Bělař, but that there occur actually changes in the nuclear constituents at the time of division.

Reference might further be made to the findings of Enriques (1912) on the difference in the macronuclei of *Stylonychia pustulata* and *Opercularia coarctata* under well-nourished and starved conditions, observations having been made on permanent preparations fixed with mercuric chloride and stained with Heidenhain's iron haematoxylin. Enriques found that the macronucleus of a normal individual is made up of numerous small chromatin granules and threads forming a network which leaves "achromatic parts" in several places. The smallest chromatin granules are said to be about 0.2 μ in diameter. The macronucleus of a starved organism shows however many vacuoles and the chromatin granules apparently fuse with one another and become transformed into a coarse "homogeneous chromatin" network. Therefore, this observation does not assist in explaining the peculiar staining of the particular macronucleus of *Nyctotherus ovalis* under notice.

Whatever cause of this seemingly true 'reverse' staining of the two main constituents of the macronucleus of *Nyctotherus ovalis* toward Heidenhain's iron haematoxylin may be, it is to be remembered that haematoxylin does not give as sound basis for judging intranuclear chromatinic structures as the nucleal reaction.

General discussion.

A great deal of attention has been given by the students of cytology to the state of existence of the chromatin within a nucleus. HEIDENHAIN (1907) wrote: "Diese Chromatine sind natürlich in der lebenden Substanz nicht als solche (Kerngerüst) enthalten, vielmehr sind sie Zersetzungsprodukte der lebenden Masse, welche bei Gelegenheit der Fixierung zur Ausfällung kommen." Perhaps BĚLAŘ (1926) gave a summary of the more recent state of our conception on how chromatin occurs in the nucleus, when he stated: "Es braucht nicht ausführlich erörtert zu werden, in welchen Strukturelementen das Chromatin im Ruhekern auftritt: als granuläres Aggregat, als ± feines Reticulum, als Alveolarsystem, in Klumpen und Bälkchen. Die Lichtbrechung des Chromatins gestattet es, gelegentlich seine Verteilung am lebenden Kern zu beobachten und festzustellen, inwieweit die im fixierten Kern vorgefundenen Strukturen als Artefakte zu betrachten sind. In all den Fällen, wo eine solche Kontrolle unmöglich ist (sie sind bei Heteroplastiden in starker Majorität), muß man sich mit Analogieschlüssen begnügen, ein Verfahren, welches jedem Cytologen geläufig ist. Es ist jedoch zu betonen, daß zu allermeist das Chromatin im Ruhekern nicht mit Sicherheit von der Kerngrundsubstanz zu unterscheiden ist." In another passage, BĚLAŘ wrote that "das Chromatin kann als dichtes granuläres Aggregat oft im Leben deutlich sichtbar sein und färbt sich dann gut mit Kernfarbstoffen."

Formed chromatin bodies as they occur ordinarily in the resting nuclei of varions cells appear to be invariably minute. With reference to the chromatin bodies of metazoan cell-nuclei, HEIDENHAIN (1907) stated: "Das Chromatinkügelchen oder Chromiolen beiderlei Art (Basichromiolen und Oxychromiolen) sind nach meinen Erfahrungen drehrund und haben etwa die Größe eines mittleren Zentralkörpers (ca. 0.3 bis 0.4 μ). Sie sind in den Strangwerken des Kernes frei suspendiert." The same holds true with the chromatin granules of the macronuclei of the majority of euciliates which contain them in large number. They are present in almost all cases as minute granules.

The unique characteristic of the macronucleus of the members of Nyctotherus, especially of *Nyctotherus ovalis*, is the abundance and distinct form of chromatin substance and the total absence of plastin material. The chromatin substance occurs in a spherical form which in some individuals may reach as large as 20 μ in diameter. Large

spherules are ordinarily alveolated; the substance present in alveoli does not seem to be of plastin nature, since it fails to take light green after the nucleal staining. These spherules are therefors unlike in structure the distributed granules of *Dileptus anser* which, according to CALKINS (1926), are "composed of a plastin core and a chromatin cortex." On the other hand, they are somewhat similar to those occurring in the macronucleus of *Chilodon cucullulus* which were subjected to the nucleal staining by REICHENOW (1928). This author observed that larger chromatin granules located between the endosome and the nuclear membrane appeared "gewöhnlich im Innern heller," when stained with this method.

In some of the alveoli of larger chromatin spherules, there are small spherical bodies which are also made up of chromatin material as judged by their reaction to FEULGEN's staining. How these bodies are formed and what become of them are questions that cannot be answered at present. But it is not improbable that in these alveoli, small chromatin bodies are formed and may later be set free in the karyochylema. Occasionally one finds the alveolus containing the minute granules located near the periphery instead of near the center of the spherule (Pl. 2 Fig. 11), which may be a stage prior to the actual extrusion of these granules. In the paper already referred to, REICHENOW (1928) stated that the lightly FEULGEN-stained (therefore chromatin-containing) endosome in the macronucleus of *Chilodon cucullulus* showed often one, two or three deeply stained chromatin granules and supposed that in the center of the endosome, a nucleic acid-containing substance becomes condensed and that it migrates to the periphery. He considered this "ein klares Beispiel von Chromatinbildung im Innern eines Binnenkörpers."

With regard to whether or not individual chromatin granules divide at the time of nuclear division, there seems to be very little information. CALKINS (1926) writes that at the time of division of the macronuclei of the Infusoria, "each (chromatin) granule elongates and divides into two parts, thus doubling the number of chromomeres," and that "the mass thus formed is passively distributed to the daughter cells by division of the nucleus through the center." Such does not seem to be the case with the chromatin spherules of the macronucleus of *Nyctotherus ovalis* in which no division of chromatin spherules as suggested by CALKINS was so far seen in a number of individuals in which the macronucleus was apparently ready to undergo division. Therefore, in *N. ovalis*, the macronuclear division halves approximately the number of chromatin spherules, and this

loss is probably made up by formation of new minute chromatin bodies within larger spherules as suggested above.

This leads to the question: whether or not these chromatin spherules increase in size. The mitotic figure observed in metazoan cells and numerous protozoans, show the chromosomes of the same dimensions in any generation of a given tissue cell or a protozoan, which would indicate that the chromatin material must increase in size during either interphase or early prophase. But how do the chromatin bodies in the macronuclei or in the cytoplasm of the euciliates behave? In the distributed chromatin granules of *Dileptus anser* to which reference has already been made, CALKINS (1926) noticed that the plastin core increased "enormously in size after treatment of the organism with certain kinds of food such as beef broth." The same author (1909) previously noticed that the normal and starved individuals of this ciliate vary a great deal not only in their body sizes, but also, according to his figures, in the size and number(?) of the distributed chromatin granules, the starved ones containing much smaller granules.

Evidence so far obtained appears to indicate that the chromatin spherules of the macronucleus of *Nyctotherus ovalis* grow in size under certain conditions. The colon of a host insect fed on yeast cakes for three weeks, contained numerous *N. ovalis* when opened and cultivated on May 20, 1935. At that time, ten ciliates were examined separately, all of which showed macronuclei, each containing small chromatin spherules, not exceeding 2 μ in diameter. On May 27, the organisms increased in number. Ten individuals were examined and the chromatin spherules present in their macronuclei were found to have increased in size so that larger forms measured 3 to 4 μ in diameter. Two days later, all the ciliates present in the culture were examined and showed equally large chromatin spherules. Another host insect which had been fed on yeast cakes for three weeks was sacrificed on May 22, 1935. It contained a large number of the ciliate. Twenty individuals examined individually at that time possessed macronuclei, each showing small chromatin spherules, the largest of which did not exceed 2 to 3 μ in diameter. On June 16 to 18, all the ciliates found in the culture, 63 in number, were studied. Of these the macronuclei of 52 specimens showed considerably larger chromatin spherules, some of which measured as large as 8 to 10 μ in diameter, while those of the remaining 11 individuals did not show much change with respect to the size of the spherules. Although these two cultures showed

rapid growth of the chromatin spherules of the macronuclei, the enlargement of the chromatin spherules takes place very slowly in the majority of cultures.

In the macronucleus of *Nyctotherus ovalis*, the chromatin spherules are suspended within an optically homogeneous karyochylema. This condition is clearly noticeable in life, after fixation and in FEULGEN-stained permanent preparations. The karyochylema itself does not contain any chromatin substance as judged by its nucleal reaction. The statement found in a certain number of general works on cytology to the effect that the karyochylema contains dissolved chromatin, does not hold true in the case of the macronucleus or the micronucleus of the present ciliate. This structure is usually in the gel state, but becomes changed, at the time of the nuclear division or when subjected to a strong mechanical pressure, into the sol state, as judged by its streaming movement or by the Brownian movement of chromatin spherules. The whole picture of the macronucleus of *N. ovalis* as seen in life resembles closely the slightly schematized drawing of the protoplasm of the growing starfish egg given by WILSON (1923) with the exception of the relative amount of the ground substance.

The fact that the resting macronucleus and micronucleus, present in one and the same organism, do not give the same color when stained with HEIDENHAIN'S or DELAFIELD'S haematoxylin, has been noticed by every worker of the ciliates. Ordinarily the macronucleus stains much more deeply than the micronucleus. Such is also the case with *Nyctotherus ovalis* when treated with haematoxylin stains. One explanation for this unequal coloration may be that both nuclei stain equally deeply, but the micronucleus is decolorized much more rapidly than the macronucleus, because of its usually much smaller size; hence in finished preparations, the former is stained much less deeply than the latter. This is however invalidated by the following observation. In *N. ovalis*, as was stated before, the micronucleus is extremely small compared with the macronucleus. When the macronucleus is highly flattened before fixation, the peripheral chromatin spherules become widely scattered and the nucleus is as thin as the micronucleus at that point. When such a preparation is stained with haematoxylin and decolorized, the chromatin spherules in the peripheral zone of the macronucleus are still much more deeply stained than those of the micronucleus. Therefore, a true explanation must be looked for elsewhere.

FEULGEN'S nucleal staining does also not give the same intensity of color to the two nuclei of the euciliates. In some species the micronucleus may be more deeply stained than the macronucleus, as for example in many hypotrichous ciliates such as Urostyla (REICHENOW, 1928), Euplotes, Stylonychia, etc. In other species such as *Nyctotherus ovalis*, *Paramecium caudatum*, *P. aurelia*, Chilodon, etc., the macronucleus stains invariably much more deeply than the micronucleus. Certain authors believe that there are two distinct kinds of chromatin (idiochromatin and trophochromatin) in a nucleus and that in the case of a euciliate, the micronucleus and macronucleus contain them separately, while others maintain that there is only one kind of chromatin which under certain conditions manifests either generative or vegetative activities. The evidence on hand shows the following points: The difference in the nucleal staining between the macronucleus and the micronucleus of *Nyctotherus ovalis* is most probably due to the difference of the aldehyde-groupings which become set free in the two nuclei. It appears therefore probable that in the resting stage there is a certain difference in chemical nature between the thymonucleic acid-containing substances which are present in the macronucleus and the micronucleus.

Summary.

The present study deals with the body organization, especially the nuclear structure, of *Nyctotherus ovalis*, a common heterotrichous ciliate inhabiting the colon of *Blatta orientalis*.

There is a single vesicular micronucleus situated closely to the anterior surface of the very large massive macronucleus which maintains its position by means of a suspension system or karyophore.

The macronucleus is composed of numerous chromatin spherules and karyochylema. These spherules vary in size from a fraction of micron up to more than 20 μ in diameter, and are composed exclusively of chromatin substance. Frequently these spherules are alveolated, but aside from the suspended chromatin bodies, the alveoli do not contain plastin material. The karyochylema is homogeneous and does not contain any dissolved chromatin substance.

The chromatin spherules manifest positive FEULGEN'S nucleal reaction after fixation with various fixatives.

That the acidified methyl green is a reliable specific chromatin dye is confirmed, although the stain brings about a slight swelling of the chromatin spherules as well as of the alveoli present within them.

Ten per cent solution of sodium chloride causes a high degree of swelling of individual chromatin spherules and consequently an enlargement of the entire nucleus.

Chromatin spherules are hydrolyzed to a certain extent by 2 per cent pepsin in 0.5 per cent hydrochloric acid solution when kept at 35° to 37° C., but show much stronger resistance to the action of the enzyme than the nuclear membrane or the cytoplasmic structures which become completely digested in 12 to 16 hours.

Observations on the effects of various simple and mixed fixatives upon the chromatin spherules show that Flemming's (strong) fluid or 2 per cent osmium tetroxide gives their true pictures and that absolute alcohol, 0.5 per cent acetic acid, 4 per cent formaldehyde or Carnoy's fluid appears to cause deformity and leave the chromatin spherules partially soluble as judged by the rose coloration of the karyochylema. Mercuric chloride and alcohol, Schaudinn's and Bouin's fluid bring about somewhat unnatural form and size of the chromatin spherules.

Staining by Heidenhain's or Delafield's haematoxylin may be compared favorably with the nucleal staining. However, the occurrence of 'reverse' staining by Heidenhain's stain makes it less reliable than Feulgen's staining.

Possibility of formation of new chromatin spherules within larger alveolated spherules is considered and the growth of chromatin spherules is recorded from two cultures.

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Explanation of photomicrographs.

Plates 2—4.

Plate 2.

Fig. 1. Anterior two-thirds of a living specimen of Nyctotherus ovalis, viewed from its left side, showing the striation of the body surface. × 690.

Fig. 2. Anterior half of another living specimen seen from the left side, showing the macronucleus, the cytopharynx, the karyophore, the frontal lamella and glycogenous bodies. × 690.

Figs. 3 and 4. Oral and right side views of a somewhat compressed living individual. Fig. 3 was obtained first and the ciliate was rolled slightly to lie on its side, during which process part of the cytoplasm was extruded and this loss accounts for the shorter body length shown in Fig. 4 as compared with that of Fig. 3. × 282.

Fig. 5. Stained section of an individual showing the karyophore, frontal lamella, micronucleus and transversely cut macronucleus. × 690.

Fig. 6. A sectioned individual, showing the macronucleus in oral view with several fibrillar connections with the peripheral ectoplasm and cytopharynx. × 690.

Fig. 7. A small sectioned individual showing the karyophore, macronucleus and frontal lamella. × 690.

Fig. 8. A transverse section through the macronuclear region of an individual, showing the fibrillae which connect the macronucleus with the peristome and the peripheral ectoplasm. × 690.

Fig. 9. A transverse section through the frontal lamella. × 690.

Fig. 10. A mature cyst as seen in life. × 650.

Fig. 11. Portion of a macronucleus as seen in life, showing chromatin spherules of various dimensions. × 1380.

Plate 3.

Fig. 12. A dark-field view of a macronucleus in life. × 975.

Fig. 13. Part of the macronucleus of a slightly compressed living specimen, showing the chromatin spherules of various sizes. × 1380.

Fig. 14. Part of the macronucleus of another living specimen, showing smaller chromatin spherules. × 1380.

Fig. 15. The moderately compressed macronucleus of a living specimen in side view. × 1380.

Fig. 16. The same nucleus showing its membrane and enlarged contents after being treated with 10 per cent sodium chloride for three minutes. × 1380.

Fig. 17. The same nucleus after being rolled over several times under the cover glass, showing in surface view numerous folds on the membrane. × 1380.

Fig. 18. The same nucleus as it appears after being subjected to the nucleal staining. × 1380.

Fig. 19. A dark-field view of part of the macronucleus of a highly compressed organism in life, showing the chromatin spherules. × 975.

Fig. 20. A bright field view of the same field. × 975.

Fig. 21. The same field after treatment with the acidified methyl green. × 975.

Plate 4.

Figs. 22—27. Six views of one and the same nucleus, showing the effects of artificial digestion on the macronucleus and the surrounding cytoplasm. × 1380.

Fig. 22. In life before the treatment;

Fig. 23. Five minutes;

Fig. 24. One hour and a half;

Fig. 25. 13 hours and a half;

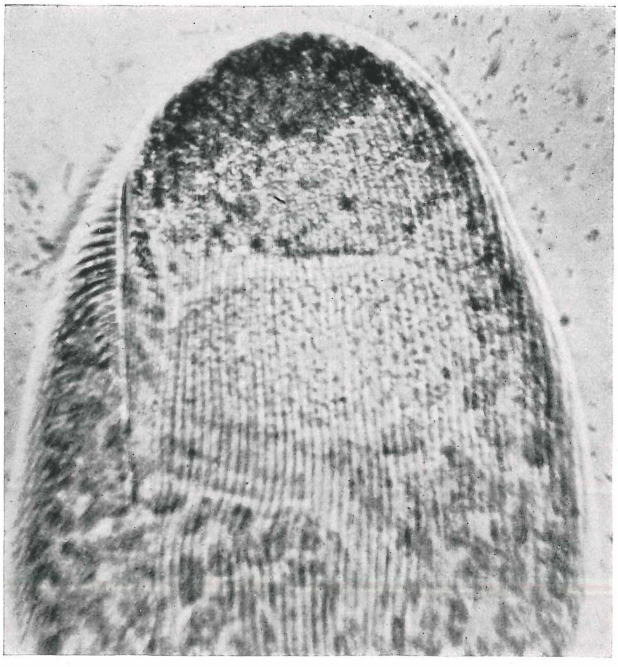
Fig. 26. 38 hours and a half;

Fig. 27. 61 hours after the addition of the mixture of pepsin and hydrochloric acid to the preparation.

Fig. 28. Part of a macronucleus in life. × 1380.

Fig. 29. The same nucleus fixed with SCHAUDINN's fluid. × 1380.

Fig. 30. The same nucleus subjected to FEULGEN's nucleal staining and mounted in toto. × 1380.



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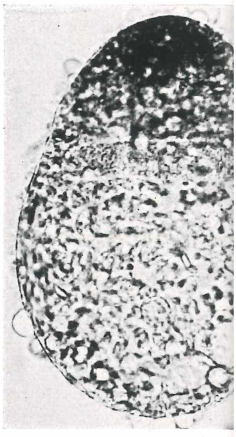

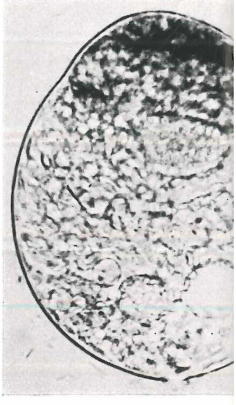

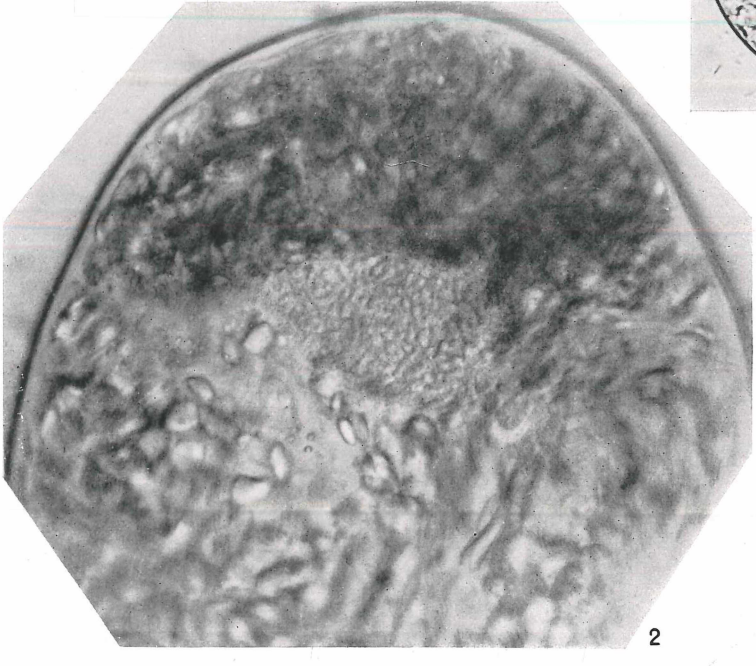

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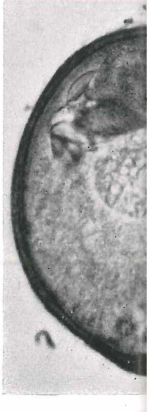

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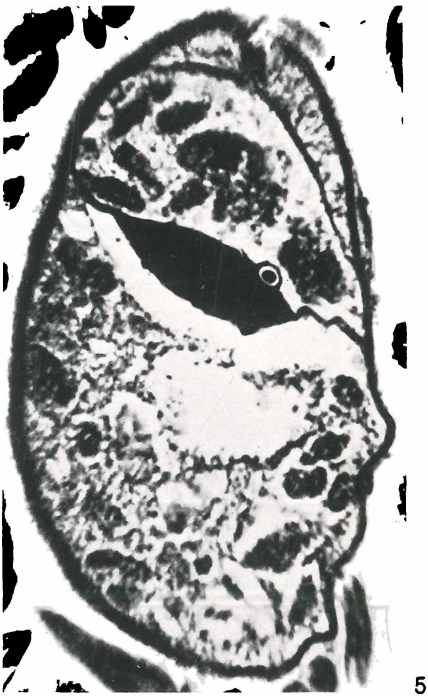

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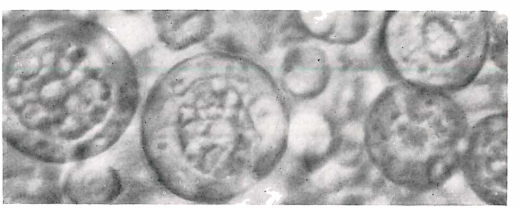

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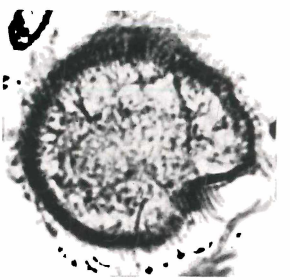

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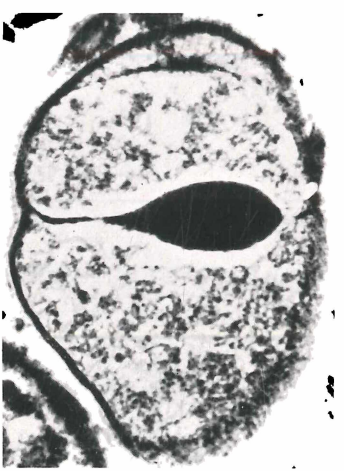

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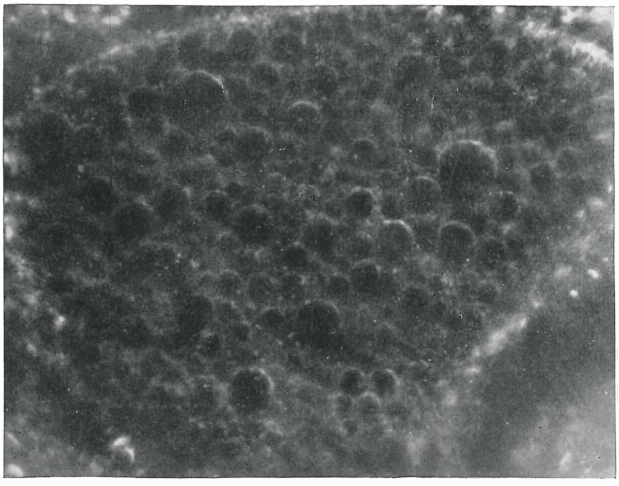

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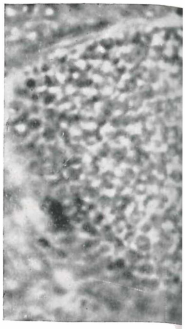

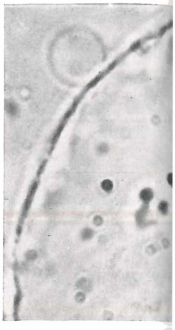

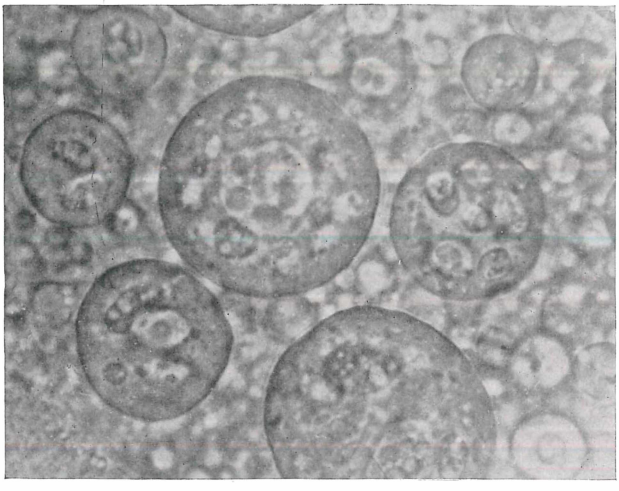

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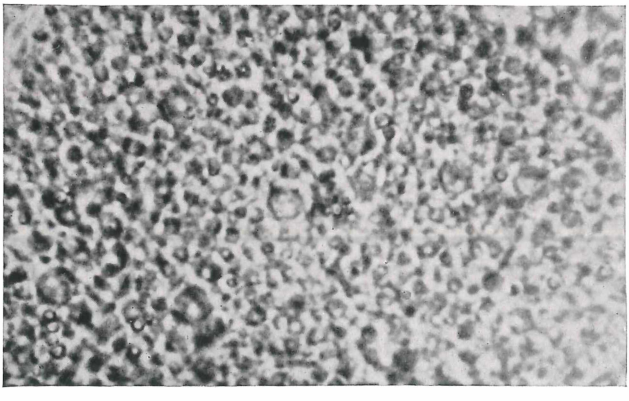

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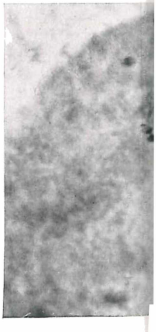

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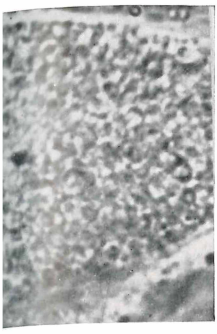

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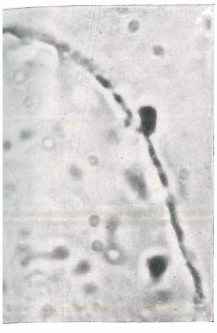

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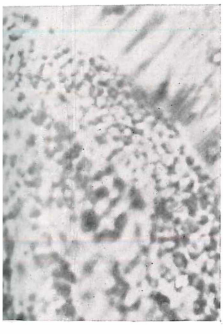

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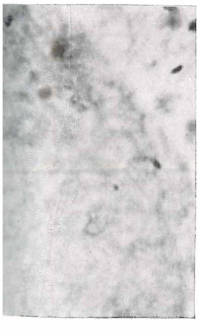

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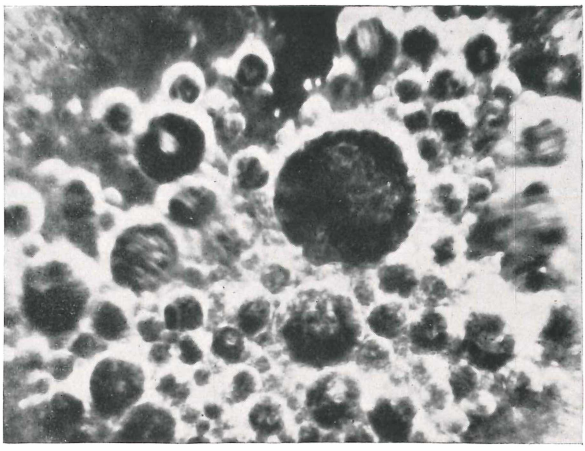

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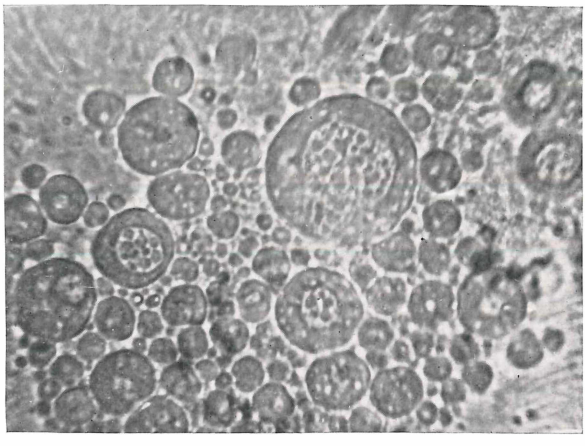

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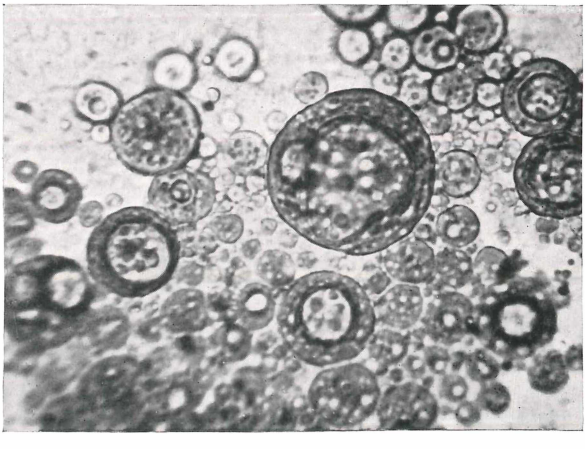
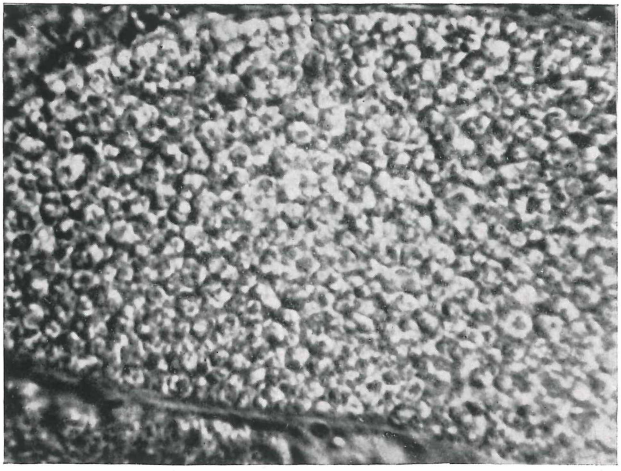


21

RÖHER IN JENA.



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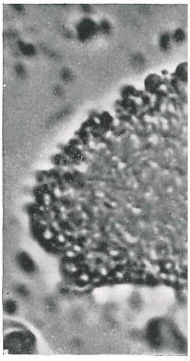

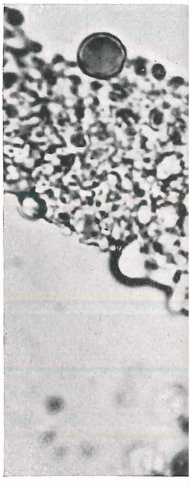

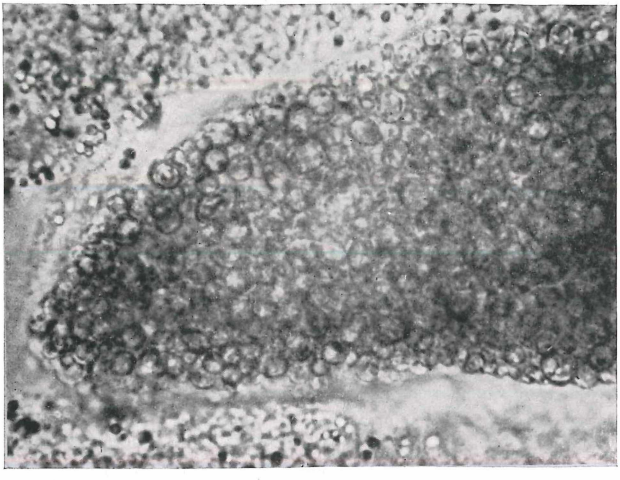

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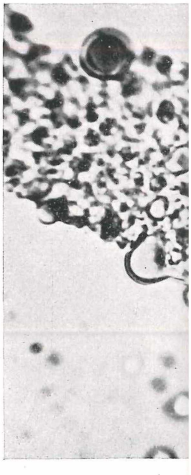

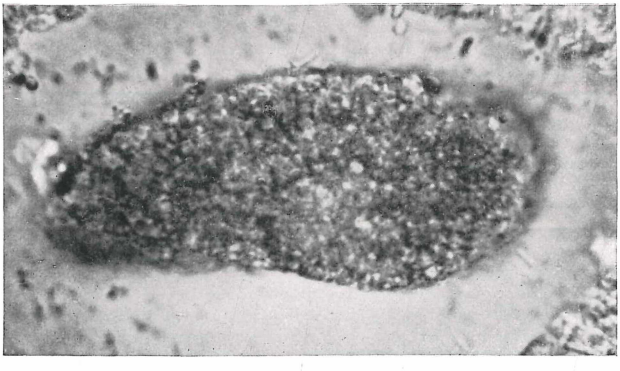

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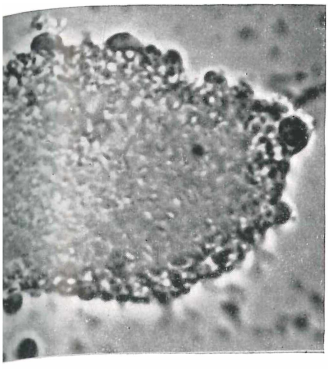

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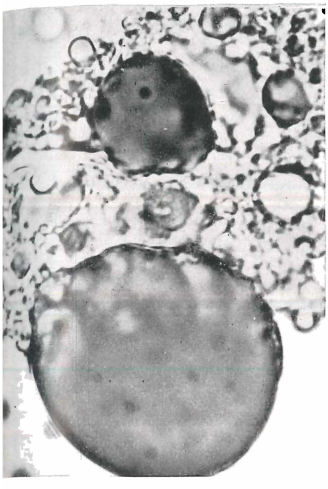

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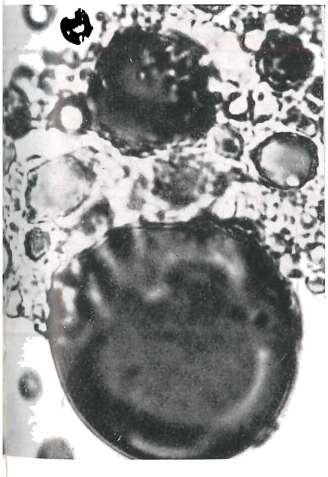

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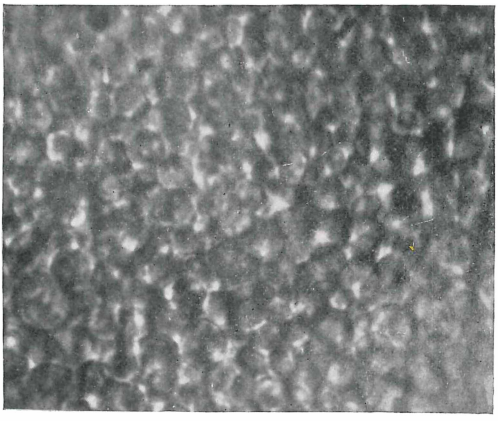

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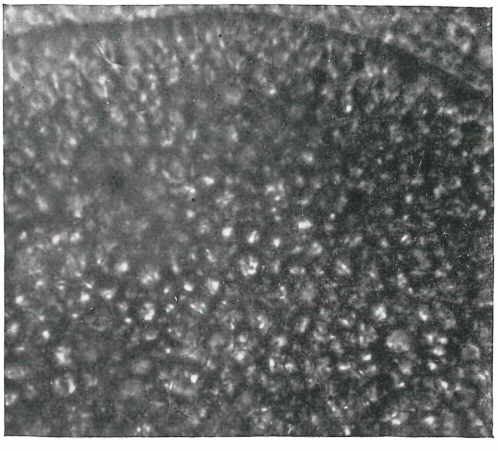

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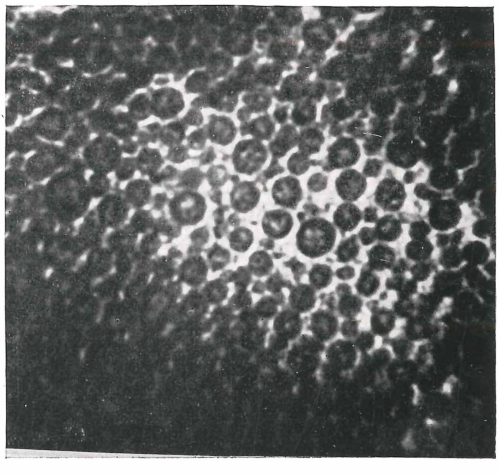

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FISCHER IN JENA