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The parasitic ciliates of *Dreissensia polymorpha* and other *Bivalvia* in the Ohrid Lake

Orzęski pasożytujące w *Dreissensia polymorpha* i innych małżach jeziora Ohrid

In August 1964 I had opportunity of spending a month on the Ohrid Lake in Macedonia (south Yugoslavia). The excellent conditions and the unusually cordial atmosphere I met with in the Hydrobiological Station in Ohrid belonging to the University of Skopie enabled me to collect an abundant material of ciliate parasites of the local molluscs — *Bivalvia* and *Gastropoda*.

I wish to express here my thanks to the patron of the Hydrobiological Station at Ohrid, Prof. Dr Siniša Stanković, to the director of the H. S. Dr Janče Točko, and to the whole scientifical and technical staff of the H. S. for their cordial and fraternal help and their really Slavonic hospitality.

In the present publication the results of my research are presented concerning the parasites of *Bivalvia* of the lake Ohrid. Stanković 1960 reported from this territory: *Dreissensia polymorpha* Pall., *Unio* sp. *Anodonta* sp., and several species of not endemic *Pisidia*. My research concerned *Dreissensia polymorpha* (approx. 500 specimens investigated), *Unio crassus* Retz. (8 specimens) as well as *Pisidium* sp. sp. (over 50 specimens). Some data on the parasites of *Bivalvia* of the lake Ohrid originate from Georgevitch 1950. They concern *Anodonta* sp.

The highly specific fauna of the lake Ohrid has often been compared with the equally specific fauna of the lakes Baikal or Tanganyika. Especially numerous endemic elements occur here among molluscs, chiefly among *Hydrobiidae*. No less endemic elements are represented by *Spongiaria, Turbellaria, Annelida, Crustacea* and also fishes. Besides these so numerous endemic forms, cosmopolitan elements occur in the lake Ohrid as well. These are known also from other fre h waters of Europe or Palaearctics, although they often change here their habits and their ecological properties. This is evoked by the high specifity of the lake itself which is an oligotrophic structure, located on the mountainous territory, on the height of the water mirror of 700 m. over s. l. The lake Ohrid covers the area of 500 km^2, its depth reaches nearly 400 m. It is supplemented mostly by spring waters and represents special thermic conditions (Stanković 1960).

The amazing endemic character and specifity of the Ohrid lake fauna (similarly like that of the Baikal or Tanganyika) has been explained in different way. Three lines of interpretation may be distinguished. The first one explained

the endemism of the lake Ohrid by its marine origin, the second — saw in it the relict of the Tertiary fauna, the third one — rather dominating presently — considered the specifity of this fauna and the special development of some groups as a result of a specific evolutionary process — of the intralacustrine speciation. This theory applied to many animal groups of the lake Ohrid is put forward by Hubendick 1958, Radoman 1955 and first of all — by Stanković 1960.

A characteristic feature of the lake Ohrid fauna is its considerable vertical expansion which is associated with depth and with vertical differentiation of the bottom zones of the lake. The encroachment of single species into these different zones involves its vertical differentiation which is very distinct in some cases. In this process the authors look for the origin of the intralacustrine speciation. The vertical differentiation would evoke the same effect as the geographical differentiation (Hubendick 1960 concerning *Ancylidae*).

Possibly a part of its primitive fauna — especially in our case of *Hydrobiidae* — might be acquired by the lake already in Pliocen or in Miocen when it was a part of the brackish Sarmatic Sea. It may be assumed that the whole abundant fauna of *Gastropoda*, especially of *Hydrobiidae* could be developed by means of radiation from the species which had penetrated about that time. It seems that many species, which are clearly cosmopolitan penetrated into the lake Ohrid at a later period, for all that the water tracts — except Drin discharging into the Adriatic — were cut off. By this way *Unionidae, Pisidia, Theodoxus fluviatilis* and some *Pulmonata* were able to penetrate into the lake Ohrid. The way of penetration of *Dreissensia* is not clear.

Situation of *Dreissensia polymorpha* in the lake Ohrid

Dreissensia polymorpha Pall. is a very recent species in the European fresh-water fauna, and besides, its expansion over the European territory is not yet accomplished. According to Haas 1933—1941, it trespassed at about 1800 its former territory — the basins of Volga, Don and Danube, proceeded westwards, reached at about 1830 Belgium and Holland, initiating subsequently its way southwards and southeastwards. It is known that it penetrated as far as the lake Balaton (Hungary) just in 1932. When and by which way reached *Dreissensia* the lake Ohrid?

The only direct communication of the lake Ohrid leads by its outflow, the river Drin, which discharges into the Adriatic on the territory of Albania. It joins the lake Skadarsko at its mouth. However, in this lake *Dreissensia* has not been reported, neither was it found in the course of my investigations in 1960. Indirect connections across the watersheds between Drin and the basins of Morava and Danube are more probable, but they would indicate the arrival of *Dreissensia* to the lake Ohrid not earlier than at the beginning of the XX century whereas it seems to be a species settled down in the lake since longer time. It also appears in a slightly different form (distinctly broader), as yet not described and not distinguished. It seems not improbable that *Dreissensia* is here a much earlier tertiary acquirement; at any rate it arrived prior to the period of its last European migration.

The present situation of *Dreissensia polymorpha* in the lake Ohrid is no doubt also rather peculiar, owing to the considerable specificity and depth of the lake. On the European lowland *Dreissensia* inhabits rivers, canals, pools

and lakes. In the last case it keeps at the littoral zone, not encroaching down to more considerable depth. It reaches the highest numerousness usually at the depth of 3—8 m (Tab. I). Deviation of these conditions may occur, e.g. in Schalsee (Lundbeck 1926). In the lakes of north Poland (Mazury), *Dreissensia* shows its maximal density at the depth of 2—4 m, and reaches as deeply as about 12 m (Stańczykowska 1964). In the lake Ohrid *Dreissensia* reaches much deeper, down to 60 m, the maximal density of population being at the depth of 5—10 m. Its number is here unusually high.

Table 1

Vertical distribution of *Dreissensia polymorpha* in various lakes

Lake	Maximal density of population		Lower limit of distribution	Author
	depth	no of indiv./m²		
Trammersee	3— 4 m	769	7— 8 m	Lundbeck 1926
Schalsee	7— 8 m	4267	44 m	„ „
Bechler See	3— 4 m	2266	11—12 m	„ „
Eutiner See	3 m	2311	3 m	„ „
Esrom See	8 m	5200	14 m	Berg 1938
Mazury Lakes	2— 4 m	2900	11—12 m	Stańczykowska 1964
Ohrid Lake	5—10 m	12239	60 m	Stanković 1960

The vertical distribution of *Dreissensia* in the lake Ohrid is not uniform being different in low and in steep shores. In general, it occurs in the littoral at the depth of 5—10 m, and deeper (in a lower number) in the sublittoral at the depth of 20—30 m. Both zones are separated more or less distinctly by a zone of empty shells which reaches its maximal thickness at the depth of 20 m. Both zones of *Dreissensia* occurrence are not sharply separated and the migration between them may surely occur. They are also connected by larvae which occur in the plankton of the lake Ohrid all the year round in nearly the same number of about 1000 specimens in 1 m³ (Serafimović-Hadžišče, after Stanković 1962).

Parasitic ciliates in *Dreissensia polymorpha*

The assembly of ciliates living in the mantle cavity of *D. polymorpha* is not abundant and in the majority of European territories covers *Conchophthirus acuminatus* (Clap. et Lachman, 1858/59), *Hypocomagalma dreissenae* Jar. et Raabe, 1932, and *Sphenophrya dreissenae* Dobrzańska, 1958 as species specific to *Dreissensia* and *Ancistrumina limnica* Raabe, 1947 which occurs casually in this mussel (Jarocki, in Jarocki et Raabe 1933; Raabe 1956). All they are species belonging to *Thigmotricha* reported from Poland and know from Germany, Czechoslovakia and Hungary (the lake Balaton). No data from other countries are known, therefore this list may be somewhat broader. However, the list must be recognized at the moment as full, at least for the European lowland.

All the three above mentioned specific species were found in the lake Ohrid. *Ancistrumina limnica* Raabe never occurred here in *Dreissensia* although it

was found in *Hydrobiidae* (R a a b e 1965). Together with the above forms, two new species were found in different intensity and in different zones of the lake Ohrid; they are distinctly similar to their ubiquitous relatives. These are: *Conchophthirus klimentinus* sp.n. resembling *C. acuminatus,* and *Sphenophrya naumiana* sp.n. resembling *S. dreissenae. Ophryoglena* sp. was rather frequently found in the mantle cavity of *Dreissensia.* This species is, however, not discussed in the present considerations which are limited only to *Thigmotricha.*

Conchophthirus acuminatus (Clap. et Lachm., 1858)

The ciliates of this species occurring in Ohrid correspond exactly to the description of R a a b e 1932/33, as reported for their population in Poland and supported by R a a b e 1950 for the lake Balaton.

The body is laterally flattened, of ovoid outline, tapering anteriorly. The left side is deeply concave in its anterior part which is occupied by the thigmotactic area. Peristome is approximately at the middle of the body length, on its right convex side near the ventral margin. Cytopharynx starts at the infundibulum and runs nearly to the dorsal body margin as an arch bent forwards. The ovoid Ma lies beyond the middle of the body length, slightly dorsally. Mi lies in its recess. C. V. lies more ventrally than Ma. Its canal terminates approx. in the centre of the left body surface (Fig. 2 B).

The ciliary system: the number of kineties is about 90. They constitute two systems which correspond more or less to the right and left body sides. The boundary of this system is marked by the anterior suture which is parallel to the anterior body margin and lies slightly on the left side, and by the posterior suture which runs concurrently to the posterior margin on the right side. The boundary is complemented by the stomatogenic kinety. About 27 kineties of the right system — beginning with the dorsal margin — run from the posterior suture forwards, bend in front of the peristome and run towards the antero-ventral margin. The next 12 kineties run at first concurrently to the former ones, enter the infundibulum, circumscribe its walls with gradually deeper arches and form a ciliated peristomal funnel. Their ends reach

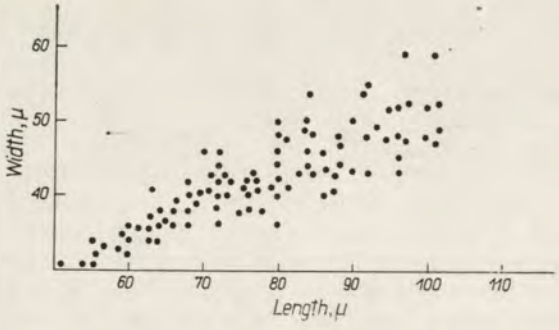

Fig. 1. *Conchophthirus acuminatus* Clap. Lachm. — results of measuring of length and width of individuals in populations in the Ohrid Lake

the surface producing a fan over the peristome. The mouth of C. V. lies on the first of the 12 kineties which fails to reach the posterior suture. The last of these kineties is the stomatogenic one (the kinety No. 1 in the concept of Chatton et Lwoff). Several next kineties (approx. 8), which already belong to the left side system (kinety n, n−1, n−2 etc), break off before joining the fan of the peristome, the next ones pass as arches upon the left body side. These few kineties, which belong already to the left system since they lie on the left side of the stomatogenic kinety but break off on the right side past the peristome, constitute an essential character of *C. acuminatus*. This character is absent in the other representatives of the genus *Conchophthirus* described more precisely (e. g. *C. unionis, C. anodontae, C. curtus* — Raabe 1933) but is more distinctly marked in *C. klimentinus* sp. n.

Dimensions of the body in *C. acuminatus* from the Ohrid lake approach those known from Poland and show a great variability. In individual populations (originating from one individual of *Dreissensia*) a considerable dispersion of size from 50—100 μ in length and 30—60 μ in width is manifested. A general impression is that populations from more shallow places — from the actual littoral, 1—10 m of depth — contain slightly bigger individuals than those from deeper places — 20 or 40 m (Table 2). The numerousness and frequency of occurrence of *C. acuminatus* in *Dreissensia* in the lake Ohrid depends rather distinctly upon the biotope of the host. The infection is not numerous and not frequent (approx. 50%) on stones near steep shores in the surge zone especially near cliffs. In such conditions mussels are also not very large, not eceeding 1.5 cm in length and are already mature. A common and high infection occurs in the zone at the depth of 10 m or of 20 m. Its intensity and numerousness diminishes with the increase in depth so that in the zone 40—45 m deep only 20% of mussels are infected and populations of *C. acuminatus* are very scarce.

Table 2

Dimensions of *C. acuminatus* and *C. klimentinus* sp. n. in various depths of the Ohrid Lake

Depth	*C. acuminatus* Clap. Lachm.			*C. klimentinus* sp. n.		
	length	width	index	length	width	index
0.5 m	84 μ	44 μ	37	82 μ	52 μ	43
10 m	84 μ	43 μ	36			
20 m	73 μ	40.5 μ	30	85 μ	56 μ	48
40 m	72 μ	40 μ	29	100 μ	71 μ	71
average	78 μ	42 μ	33	89 μ	60 μ	54

Consequently it may be concluded that, except the unfavorable for infection surge zone, the best conditions for *C. acuminatus* are presented by the *Dreissensia* colonies living on the depth of 0.5 to 10 or 20 m. The same depth is reached by the *Dreissensia* colonies in the lakes of Central Europe and in some of its marine brackish bays (e.g. Szczecin Firth, Vistula Firth). In the lake Ohrid the littoral is also the zone of the most dense occurrence of *Dreissensia*.

Conchophthirus klimentinus sp. n. [1]

Body strongly flattened, of an ovoid outline, slightly rounded on both ends. The right side is uniformly convex, the left side is distinctly and uniformly concave. Peristome in the posterior body part, on its right side, shifted towards the ventral margin which is in this place incised like in the majority of the representatives of the genus *Conchophthirus*. Cytopharynx initiates at the deep and narrow peristomal funnel and runs forwards to the dorsal body part. Ma is ovoid, lies approx. in the middle of the body length, near its dorsal margin. Mi — 1 or 2 — lies in its close vicinity. C. V. in the posterior body part.

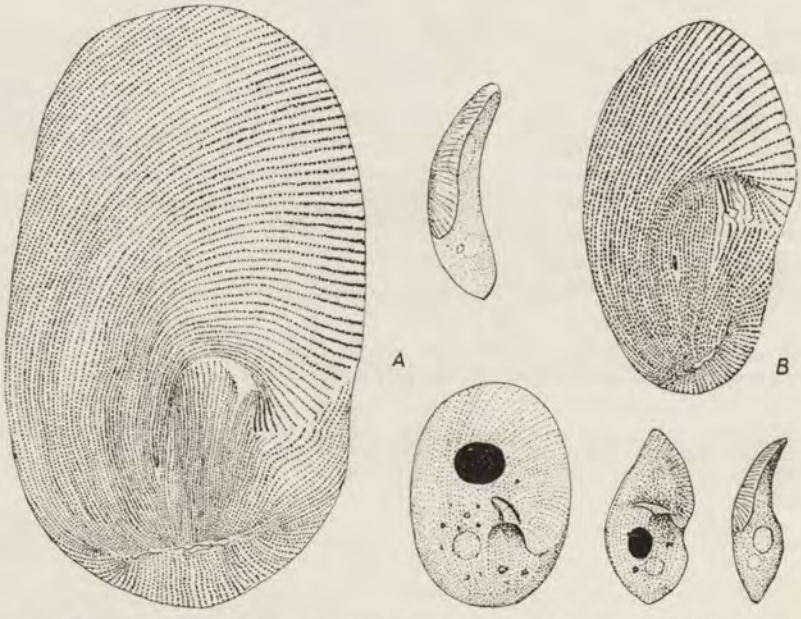

Fig. 2. A. *Conchophthirus klimentinus* sp. n. B. *C. acuminatus* Clap. Lachm. After silver impregnated and haematoxylin preparations and infravital sketches

The ciliary system: about 160 kineties constitute two systems corresponding more or less to both body sides — the right and the left one. Similarly as in *C. acuminatus*, the boundary of the systems is marked by the anterior suture which is slightly shifted to the left body side by its anterior and antero-lateral margins, by the posterior suture which lies on the right body side near the posterior margin, and by the stomatogenic kinety. About 50 kineties of the right system — beginning from the dorsal margin — run forwards and towards the anterior part of the ventral margin. Their course is parallel to the dorsal

[1] The name *C. klimentinus* has been introduced in honor of the role performed by Sv. Kliment (St. Clement) at the turn of the IX and X century, for the development of the Makedonian — as well as general Slavonic — language and culture. He had his abode in the ancient town Ohrid for a long time.

margin. The next 15 kineties enter the peristome, run round the narrow peristomial funnel, and leave the peristome backwards as a broad fan constituted of about 10 kineties. The first of them does not reach the posterior suture breaking off in the place of the opening of C. V. This is the 15th kinety counting from the stomatogenic kinety. The last of the 15 kineties is the stomatogenic kinety (kinety no. 1). This may be easily followed in division stages because the anlage of the opisthe adoral kineties arises just on it. The next 10 kineties belong already to the left system. They run forwards to the posterior suture, partly penetrate into the infundibulum, and partly break off when they have attained the fan over the peristome. These are the kineties: n, n−1, n−2, etc. Farther kineties (about 10) of the right body side clearly manifest their belonging to the left system: they pass to the left body side and run together with its kineties towards the anterior body margin and to the anterior suture.

The naked peristomal area, occurring in the other representatives of the genus, fails to differentiate into the trophic stage of *C. klimentinus* sp. n. because the first kineties (1, 2, 3, etc.) and the last ones (n, n−1, n−2, etc.) fail to diverge sideways but reach or penetrate the peristome. The adoral kineties, so characteristic of other *Conchophthirus* sp. sp., are neither seen on the surface. The observation of living ciliates seems to indicate that they are pushed inside the peristome. Their existence is also proved by the division stages.

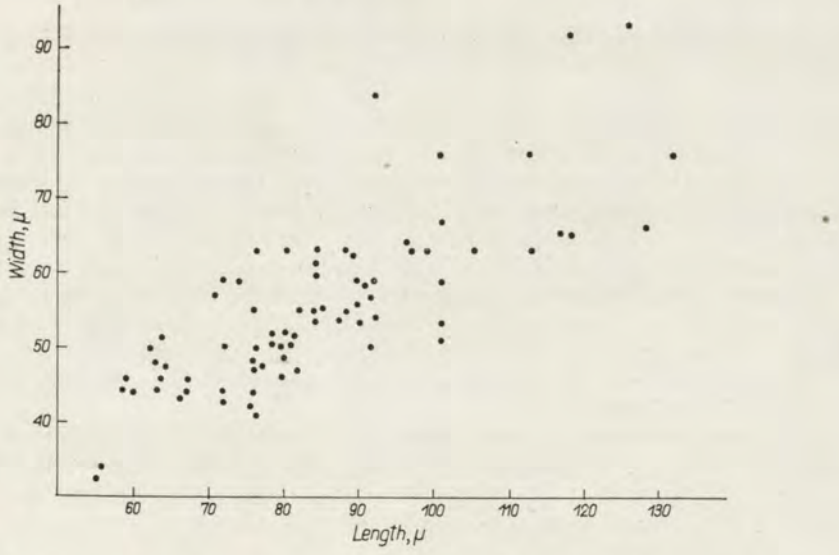

Fig. 3. *Conchophthirus klimentinus* sp. n. — results of measuring of length and width of individuals in populations in the Ohrid Lake

Modifications of the ciliary system in *C. klimentinus* connected with the peristome seem to be a further realization of the tendencies marked already in *C. acuminatus*: kineties of the left system running from the posterior suture break off before they reach the peristome.

The body dimensions of *C. klimentinus* are various and fluctuate within very large limits. The body length attains 60—130 μ, its width is 40—100 μ. The distribution and frequency of individuals of these different dimensions is rather peculiar (Fig. 3).

The individuals belonging to more numerous populations from shallow places (0.5—2 m in depth) do not generally exceed the size of 90 μ, in those of deeper sites (20—40 m) their size amounts to 97 μ. However *Dreissensia* specimens with very scarce population of *C. klimentinus* sp.n. occur at any depth. In such conditions the parasite attains considerable dimensions, 110—130 μ. The post-division forms of these giants measure 60—90 μ in length so they fit to the dimension limits of individuals of numerous populations. Consequently it may be assumed that in scarce populations the individuals grow beyond the norm in the inter-division periods. Generally it follows from the statistic count that the maximum mean value is attained by *C. klimentinus* sp.n. in mussels originating from considerable depth (about 40 m); smaller forms come from 20 m, and the smallest from the zone of a shallow littoral (0.5—2 m). These data cocerning *C. klimentinus* are just inverse to those for *C. acuminatus,* but are more distinctly expressed (Table 2).

C. klimentinus sp.n. differs also from *C. acuminatus* in another respect; both species occur sometimes simultaneously, but the population of *C. acuminatus* is very numerous in a given host individual if *C. klimentinus* sp.n. fails to occur at all or is not numerous. Inversely, the mussels with numerous population of *C. klimentinus* are usually free of *C. acuminatus.*

Conchophthirus klimentinus is surely a "good" species. It differs from the all accurately described species of the genus *Conchophthirus* (*C. anodontae, C. unionis, C. curtus, C. discophorus, C. acuminatus;* Raabe 1933, 1934) by lack of the naked external peristomial are with two adoral pseudomembranelles (Raabe 1962). The buccal apparatus is distinct, especially in large forms, and shifted backwards; the fan over the peristome is exceptionally strong, a considerable number of kineties of the first right and last left system break off before they reach the peristome. The majority of species of the genus *Conchophthirus* fails to have such breaking off kineties at all; only *C. acuminatus* has several of them. Despite the great difference in general appearance and in details of the structure, *C. klimentinus* sp.n. approaches mostly *C. acuminatus*! The strong fan of *C. acuminatus,* the small naked peristomial area, the kineties n, n−1, etc. which break off, constitute features which in *C. klimentinus* have been strengthened.

This large and conspicuous species has not been found in the European lowland territory. Although such a negative argument cannot be fully convincing, it seems that *C. klimentinus* sp.n. can be recognized as a species specific to *Dreissensia polymorpha* in the lake Ohrid.

Hypocomagalma dreissenae Jarocki et Raabe, 1932

The body, like in the majority of *Ancistrocomidae,* is elongated, banana-shaped, and in contrast to the representatives of this family — entirely covered with cilia. Dimensions: length 30—50 μ, width — approx. 17 μ. The anterior part of the body with suctorial tentacle, the posterior end rounded. Ma is ovoid or fusiform, 10—20×7 μ, and lies in the middle body length, rather shifted

backwards. Small Mi lies close to it. C. V. is situated centrally. The "Konkrementvacuolen" sometimes occur in the posterior body part (Fig. 4 C).

The ciliary system: the concave body side is occupied by thigmotactic zone part of the body with suctorial tentacle, the posterior end rounded. Ma is ovoid of kineties. Starting from the first of them which lies on the right boundary of the thigmotactic area, 3 kineties diminish gradually reaching from the base of the suctorial tentacle — as far as approx. the middle of the body length. The next five kineties keep this length. The thigmotactic area is closed by the kinety 10 which is bent symmetrically with 1st one. The kinety 10 is followed by 14 kineties of a more or less meridional course; they gradually reach further backwards and originate further from the base of the suctorial tentacle. In this manner the kineties 15—24 approach obliquely the kinety 1 producing a one-side "système secant".

The structure and dimensions of *Hypocomagalma dreissenae* Jar. et Raabe from *Dreissensia polymorpha* of the Ohrid lake strictly correspond to the data of the original description (from the surroundings of Warszawa and from the Wigry lake in NE Poland) and to the characteristics of the species found by me in the Vistula Firth (R a a b e 1956) and in the lake Balaton in Hungary (R a a b e 1950). In all these places, *H. dreissenae* was not numerous and occurred in a low percentage of hosts. A slightly higher extensiveness of infection was revealed in the brackish waters of the Vistula Firth. In the lake Ohrid, *H. dreissenae* is not frequent either (10—20% of infection), and no differences associated with the depth of occurrence of its host were observed. Several or over ten individuals were found in individual mussels.

Hypocomagalma dreissenae Jar. et Raabe is undoubtedly a species specific to *Dreissensia polymorpha* and accompanies it, as it seems, on the whole area of its occurrence. It is interesting that another species of this genus, *H. pholadidis* Kozloff, 1946, has been described in the marine mussel *Pholadidea penita* from California.

Sphenophrya dreissenae Dobrzańska, 1958

S. dreissenae is a species which distinctly deviates from the other representatives of the genus *Sphenophrya* by the shape of its body being not elongated and canoe-shaped. However, it may be included in the genus *Sphenophrya* with no doubt, because it corresponds to all its other characters (Fig. 4 B).

S. dreissenae Dobrz. has the shape of a helmet (30×30 μ), slightly flattened laterally, with one or two distinctly protruding processes on the body margin. The processes are not always equally distinct which seems to be associated with the position of the ciliate on the margin of the mussel gill. The body adheres to the substrate by its clinging surface, the foot. The system of kineties (with no cilia) is really limited to one body side and is constituted by two assemblies which converge on the apical body convexity. From this place one assembly consisting of 4—5 kineties runs towards the base, turns right and sometimes passes in the other body side. Another assembly composed of two pairs of kineties (2+2) diverges from the former one and deviates left. Ma is irregular, usually ovoid, it measures ca. 10×8 μ. Mi lies on its side and measures 3—4 μ in diameter. After silver impregnation following Klein, the whole body surface, except the sole, is covered with an irregular network.

The characteristic of *S. dreissenae* Dobrz. from the Ohrid lake is consistent with the original diagnosis (Dobrzańska 1958, 1961) which was based on the material from the environment of Warszawa and with the characters of *Dreissensia* populations from other regions of Poland. In the specimens from the Ohrid lake, the spiralization of kineties, as described by Dobrzańska 1958, 1961, is less distinctly marked. *S. dreissenae* Dobrz. occurred in *Dreissensia polymorpha* of the lake Ohrid rather irregularly and most frequently in low numbers. The mass occurrence was rather rare (1% of mussels). I found it in *Dreissensia* from different depth: 0.5 m, 10 m, 20 m, and even at 50 m. It occurred together with *Conchophthirus acuminatus, C. klimentinus, Hypocomagalma dreissenae,* but was never found together with *Sphenophrya naumiana* sp.n. In a mass occurrence of *S. dreissenae,* other ciliates were found in very small numbers or were absent at all.

Sphenophrya naumiana sp.n. [2]

Much elongated body has a shape of a reversed canoe, clinging by its "deck" to the gill of the host; one of its ends is beak-shaped. Body dimensions: length 60—80 μ, width 12—18 μ. The sole of this species is broad (8 μ). Ma is elongated, its dimensions amount to 50×10 μ. Mi lies near Ma and measures approx. 3—4 μ. The system of the non-ciliated kineties is really limited to one of the body sides and constitutes two assemblies. The right assembly consists of 6—7, sometimes even 9 kineties, runs turning right from the peak point towards the sole (sometimes by a sharp bent), pointing concurrently to the sole and reaching as far as the body end. The left assembly consists of two pairs of kineties (2+2, or sometimes 2+3 and even 2+4) and runs diverging towards another body end. After silver impregnation, a dense network covers the whole body except the sole. The bud measures ca. 17×10 μ. Budding occurs at night (Fig. 4 A).

S. naumiana sp.n. was found rather irregularly in the lake Ohrid, both at the depth of 0.5 m (on rocks) and of 10 or 40 m. It occurred sometimes separately, sometimes in mass (1% of mussels), similarly as *S. dreissenae* Dobrz. In the last case, other ciliates were very few or absent at all (single specimens of *Conchophthirus*). A simultaneous occurrence of *S. dreissenae* Dobrz. and *S. naumiana* sp.n. in the same host individual was never stated.

S. naumiana sp.n. is undoubtedly a "good" species, being distinct from *S. dreissenae* Dobrz. Its shape approaches it to the other species of the genus *Sphenophrya,* especially to *S. sphaerii* Miassn., 1930 (Raabe 1949). It cannot, however, be identified with any of these species.

As suggested by Raabe 1949 and documented by Dobrzańska 1961, numerous species of the genus *Sphenophrya,* and even other genera of the fam. *Sphenophryidae* (as *Gargarius, Lwoffia, Pelecyophrya*) constitute assembly of forms which retain a very similar system of kineties. Moreover this system, according to these authors resembles the system of ciliated kineties occurring

[2] The name *S. naumiana* has been introduced here for stressing the role of Sv. Naum for the development of the language and culture of Makedonia as well as of the general Slavonic culture at the turn of the IX and X century. He resided in Ohrid for a long time.

in some *Ancistrocomidae* and, first of all, in *Hypocomatidium sphaerii* Jar. et Raabe. In all these cases 2+2 kineties occur here in the left system (with deviation: 2+3, 2+4), and several ones (5—7) in the right system. For this reason, Dobrzańska postulates the monophyletism of *Sphenophryidae* or, at any rate, of the representatives of the genus *Sphenophrya*, and their descendence from the forms having the structure of *Hypocomatidium*.

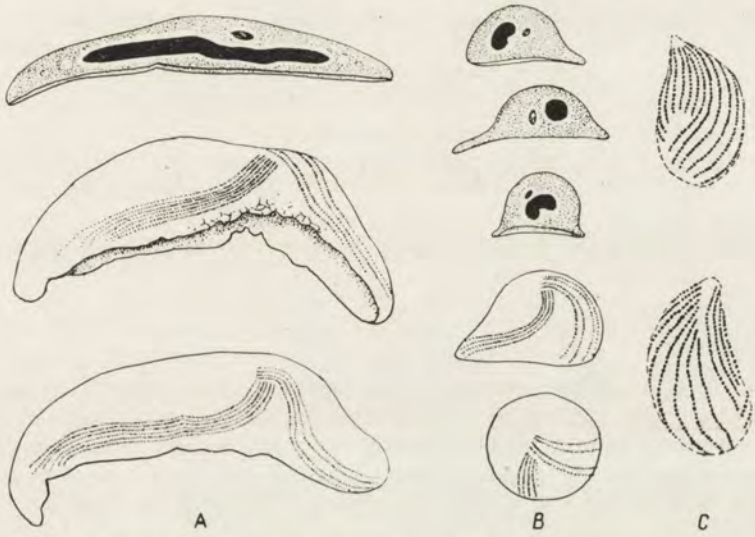

Fig. 4. A. *Sphenophrya naumiana* sp. n. B. *S. dreissenae* Dobrz. C. *Hypocomagalma dreissenae* Jar. Raabe. After silver impregnated and haematoxylin preparations

S. dreissenae Dobrz. occupies a specific position among the forms included in the genus *Sphenophrya* with a mostly elongated body which involves a rather complicated process of the transformation of the bud (tomit) into the trophic form (Raabe 1949, Dobrzańska 1961). It represents a plesiomorphic ancestral form in relation to other species which also had assumed this form in the course of ontogenesis. It cannot be excluded — in my opinion — that *S. naumiana* sp.n. is an endemic form derived from a more cosmopolitan but primitive species *S. dreissenae* Dobrz. by way of evolution similar to that of other elongated species.

Ciliate parasites of other *Bivalvia* of the lake Ohrid

Unio crassus Retz.

The list of ciliate parasites of *Unio* sp.sp., as stated for Europe, is as follows: *Conchophthirus unionis* Raabe 1933, *C. curtus* Englm. 1862, *Conchoscutum inversum* Raabe 1947, the rare *Hypocomatophora unionidarum* Jar. et Raabe 1933 (=*Heterocineta anodontae* Mawr., 1913), *Trichodina unionis* Hampl., 1955, and — in a very irregular degree — *Ancistrumina limnica* (Raabe 1947).

All these parasites or some of them have been found in Poland, Germany and Hungary (lake Balaton — Raabe 1950), and seem to occur together in all the European species of the genus *Unio*.

I succeeded to examine only 8 specimens of *Unio crassus* from the lake Ohrid. Six of them originated from shallow littoral territories from the depth of 0.5 m and two — from *Dreissensia* shoals from the depth of 20 m. All parasites of *Unio* specific to this genus had been found, namely:
Conchophthirus unionis — in great number especially on the vela;
Conchophthirus curtus — in moderate numbers, but generally occurring;
Conchoscutum inversum — not numerous, as always — and only in *Unio* from the depth of 20 m;
Trichodina unionis — numerous and common.

Consequently, *Unio crassus* preserved in the lake Ohrid all its most characteristic parasitic ciliates. The occurrence of *Conchoscutum inversum* Raabe should be stressed. It is specific, as I could ascertain, to large lakes (Żarnowieckie Lake, Pomerania; Balaton Lake, Hungary; Skadarsko Lake, Crna Gora, Yugoslavia — J. et Z. Raabe 1961).

Anodonta sp.

I failed to examine *Anodonta* from the lake Ohrid. Judging from the results of Georgevitch 1950, I have the impression that *Anodonta* has maintained its species in the lake Ohrid. Georgevitch sygnalized from it a new species, *Conchophthirus ochridensis*. Despite his unsufficient description, poor drowings and lack of adequate bibliographical references, everything indicates that he dealt with two species well known and characteristic to *Anodonta: Conchophthirus anodontae* Ehrbg. and *C. unionis* Raabe which were jointly described by him in one diagnosis.

Pisidium sp.

Pisidium sp. was investigated on 12 specimens from the shore of the lake Ohrid from a depth of approx. 1 m, and about 50 specimens from the territory of the outflow of the river Drin, from the depth of 10 m. Only in one specimen of *Pisidium* sp. a mass occurrence of *Cepedella hepatica* Poyarkoff, 1903 was found with distinctive characters consistent with those which were reported by Dobrzańska 1959. I failed to find in *Pisidium* of the lake Ohrid neither *Hypocomatidium sphaerii* Jar. et Raabe, 1932 nor *Conchophthirus discophorus* Mermod, 1914. These ciliate species occur very frequently in Poland (Dobrzańska 1958).

Conclusions

In contrast to *Gastropoda, Bivalvia* are not represented in the lake Ohrid by endemic forms. As it seems only cosmopolitan forms occur in this lake: *Anodonta* sp., *Unio* sp., *Dreissensia polymorpha* and numerous *Pisidia* sp.sp.

Bivalvia in the lake Ohrid maintain, as it seems, their ciliate fauna which is characteristic of them in other territories. The appearence of different forms among their parasitic ciliates seems little probable. An exception is presented by *Dreissensia polymorpha* which occupies in the lake Ohrid specific habitats and whose origin in this lake has not been elucidated.

Dreissensia has maintained also its ciliate species which are known from the Central European Lowland, namely: *Hypocomagalma dreissenae* Jar. et Raabe, *Sphenophrya dreissenae* Dobrz. and *Conchophthirus acuminatus* (Clap. Lachm.). Besides, it acquired two new species: *Sphenophrya naumiana* sp.n. and *Conchophthirus klimentinus* sp. n. Both species resemble by their characters the species of their genera known from *Dreissensia* so that it may be concluded that *C. klimentinus* derives from *C. acuminatus* and *S. naumiana* from *S. dreissenae*.

It seems probable that both new species arose in the lake Ohrid under the influence of specific conditions found here by *Dreissensia*, first of all as a result of encroachment into more deep zones of this lake. This would be the case of intralacustrine speciation which is a phenomenon stressed also for other animal groups of the lake Ohrid.

There is no vertical differentiation in the occurrence of both species of the genus *Sphenophrya*. *C. klimentinus* seems to be associated with rather deep waters. The lack of a strict regionalization of both pairs of species may be accounted for by the lack of isolation of *Dreissensia* populations in the lake Ohrid which makes possible both spreading of molluscs and their parasites.

Summary

Bivalves (*Anodonta, Unio, Pisidium* sp.sp.) preserved in the lake Ohrid their specific fauna of parasitic *Ciliata*. An exception is represented by *Dreissensia polymorpha* which preserved its ubiquitous ciliates, *Hypocomagalma dreissenae* Jar. et Raabe, *Sphenophrya dreissenae* Dobrz. and *Conchophthirus acuminatus* (Clap. Lachm.), but obtained new ciliates, *Sphenophrya naumiana* sp. n. and *Conchophthirus klimentinus* sp. n. The last two species clearly resemble known species of their genera from *Dreissensia*. The author is of the opinion that new species could originate in the lake Ohrid under the influence of its specific conditions by means of intralacustrine speciation.

STRESZCZENIE

Małże jeziora Ohrid reprezentowane są w nim (w przeciwieństwie do endemicznych ślimaków) przez gatunki kosmopolityczne: *Anodonta* sp., *Unio crassus, Pisidium* sp. sp. i *Dreissensia polymorpha*. Małże te zachowały w jeziorze Ohrid na ogół swą faunę *Ciliata*, która charakteryzuje je w innych terenach. Wyjątek stanowi *Dreissensia polymorpha*, która w jeziorze Ohrid zajmuje swoiste środowiska i której pochodzenie tu jest niejasne.

Dreissensia zachowała również swe gatunki orzęsków, znane z niżu europejskiego, a mianowicie: *Hypocomagalma dreissenae*. Jar. et Raabe, *Sphenophrya dreissenae* Dobrzańska i *Conchophthirus acuminatus* (Clap. Lachm.), ale zyskała dwa nowe gatunki: *Sphenophrya naumiana* sp.n. i *Conchophthirus klimentinus* sp.n. Oba te gatunki nawiązują swymi cechami do znanych gatunków swych rodzajów z *Dreissensia*. Wydaje się dosyć prawdopodobne, że oba nowe gatunki powstały w jeziorze Ohrid pod wpływem swoistych warunków, w jakich znalazła się w nim *Dreissensia*, a przede wszystkim wobec wkroczenia tego małża do głębszych stref jeziora. Byłby to więc przypadek wewnątrzjeziornej specjacji — zjawiska sugerowanego w odniesieniu do innych grup zwierzęcych jeziora Ohrid przez wielu autorów.

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Division morphogenesis in the genus *Didesmis* Fior. of the family *Buetschliidae (Ciliata, Gymnostomata)*

Morfogeneza podziałowa w rodzaju *Didesmis* Fior. z rodziny *Buetschliidae* (*Ciliata, Gymnostomata*)

The ciliature of the representatives of the genus *Didesmis* Fior. is reduced to the anterior and posterior zones of short and closely disposed ciliary rows surrounding the cytostome and cytopyge. The infraciliature however is not limited to the above zones. Several rows of non-ciliated big kinetosomes are grouped on the "Konkrementenvacuole" (Wolska 1964). Rare rows of non-ciliated kinetosomes run along the body between the ciliated zones, as reported formerly (Wolska 1964), and this view seems to be supported by the present examination (Pl. I 2). Restitution of the posterior zone of the prother and the anterior one of the opisthe in the transverse fission occurs therefore in association with the parental infraciliature existing, i. e. in the manner which is typical for *Gymnostomata-Rhabdophorina*. Nevertheless some peculiar details of formation of these zones deserve to be mentioned.

The study was performed on ciliates of the horse coecum from the district of Łódź. Material was fixed in 10% formalin, impregnated after Rio-Hortega or Bielszowski solution after being previously covered with a gelatin layer according Chatton.

Illustrations represent the genus *Didesmis ovalis* Fior. In another species of this genus — *Didesmis quadrata* Fior. the course of morphogenesis is the same.

The first sight of division is the appearence of two primordia of short closely disposed kineties in the equatorial region on both sides of the flattened ciliate body between the existing bipolar kineties (Fig. 1 A). At this most early stage, cilia are present on primordia. Initially one (Fig. 1 A), then several kineties of the right side primordium, considerably elongate. Those elongated non-ciliated segment pass over to the territory on which the "Konkrementenvacuole" of the opisthe arises (Pl. I 4) and shift to a certain distance of the ciliated kineties.

The lateral primordia increase gradually in size i. e. they grow so as to cover the ventral and dorsal sides and they elongate (Fig. 1 B). In *Didesmis quadrata*, Hsiung 1930 calls the "dorsal side" this one on which the furrow is present. The "Konkrementvacuole" lies near the right margin, rather near the ventral side. A similar determination, related to the site of the "Konkrementvacuole", is applied by Hsiung for *Didesmis ovalis*.

In the subsequent stages observed, with the division furrows incised into the cytoplasm on the body sides (Fig. 1 C) splitting of the ciliated primordia into the anterior narrow zone (the future posterior ciliature of the prother), and a posterior broader one (future anterior ciliature of the opisthe) is seen. In the Pl. I 2 (the left side view) the split of the primordium is seen. In the Pl. I 1 (the right dorsal side view) the posterior zone of the prother is not seen, the lens being focussed on the anterior zone of the opisthe. It could be stated — on a few but distinct preparations — that the broader zone i. e. the anterior zone of the opisthe is differentiated into the anterior composed of rather short kineties, and the posterior one of much longer kineties. In Pl. I 3 this differentiation is quite distinct. In Pl. I 2 this differentiation is scarcely perceptible because the photogram is not sufficiently sharp, but the preparation proves it clearly.

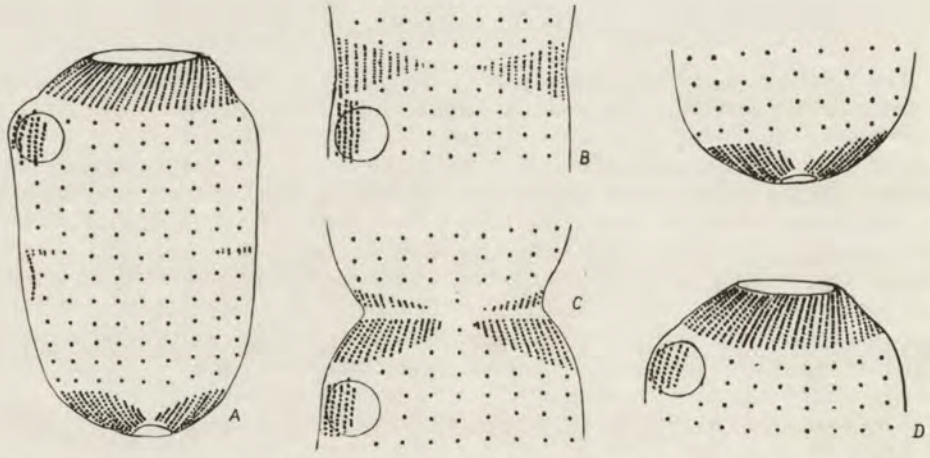

Fig. 1 *Didesmis ovalis*. A. View from the ventral side. The earliest stage of division observed. One kinety of the right primordium is elongated. B. Primordia of the ciliated zones are grown. Several kineties on the territory of the "Konkrementvacuole". C. Primordia of ciliated zones are split. Kineties on the "Konkrementvacuole" are at a distance from the ciliated zone. D. Posterior pole of the prother and anterior of the opisthe

What is the sequence of formation of those two ciliary rows in the opisthe? Exists only the anterior stripe initially and the posterior broader one arises later on, or is the differentiation a secondary process, could not be ascertained. Probably the first case takes place. As it seems, the posterior broader stripe arises later, and joins the first one, since in fully formed individuals the split is not distinctly discernible.

The left and right primordia of the ciliated zones of prother and of opisthe elongate and gradually approach each other on the dorsal and ventral sides. Finally the left and right primordia of the anterior zone of the opisthe touch each other and produce one uniform zone of slightly spiralized kineties of equal length. I failed to state in which moment occurs the twist of the anterior zone of kineties.

The primordia of the posterior zone of the prother meet on the dorsal and ventral sides but never form a compact unit, a gap between them remains always on both dorsal and ventral sides. Kineties of the left and right side converge at a certain angle (Fig. 1 D).

Two facts are here worth being stressed: formation of the anterior ciliated zone from two lateral primordia, similarly as in *Ophryoscolecidae* (Noirot-Timothée 1960), and differentiation of this zone in transversal direction, at least in ontogenesis, similarly as in *Cycloposthidae* (Wolska 1965).

No doubt, those are not sufficient evidences for drawing conclusions of a phylogenetic character but they suggest some possibilities.

The established view about the close relationship between *Entodiniomorpha* and *Spirotricha* seems to be not adequate and not proved by facts. On the contrary, the study of Fernandez-Galiano 1959 and of Noirot-Timothée 1960 seem to indicate that the development of *Entodiniomorpha* is not consistent with that of *Spirotricha* although the authors express another view.

According to Noirot-Timothée 1960 and according to my observations the adoral zone of *Entodiniomorpha* is not the zone of membranelles characteristic of *Spirotricha* but is a zone composed of parallel closely disposed kineties; their cilia are grouped into syncilia. This statement seems to be of importance. The similitude of texture of ciliated zones in *Entodiniomorpha* and in some *Gymnostomata* of the horse coecum — especially in *Didesmis* — appears striking.

It seems worth to consider the possibility of deriving *Entodiniomorpha* from *Gymnostomata*. Possibly the genus *Didesmis* might serve as a model of transition to this order, on account of its rudimentary general infraciliature, and with its reduced zones of cilia which are formed in a similar manner as in the order *Entodiniomorpha*.

The family *Buetschliidae*, so common but scarcely investigated, may supply interesting facts.

Summary

The course of morphogenesis in the genus *Didesmis* Fior. is followed in silver impregnated material. Attention is payed to characteristic formation of ciliated zones which arise of paired primordia. The similitude of development of ciliated zones in *Didesmis* Fior. to that of adoral zones in *Entodiniomorpha* is put forward.

STRESZCZENIE

Autorka opisuje, na podstawie preparatów srebrzonych, przebieg morfogenezy w rodzaju *Didesmis* Fior. i zwraca uwagę na charakterystyczne powstawanie stref orzęsionych z parzystych zawiązków. Autorka podkreśla pewne podobieństwo tworzenia się stref orzęsionych *Didesmis* Fior. i stref adoralnych *Entodiniomorpha*.

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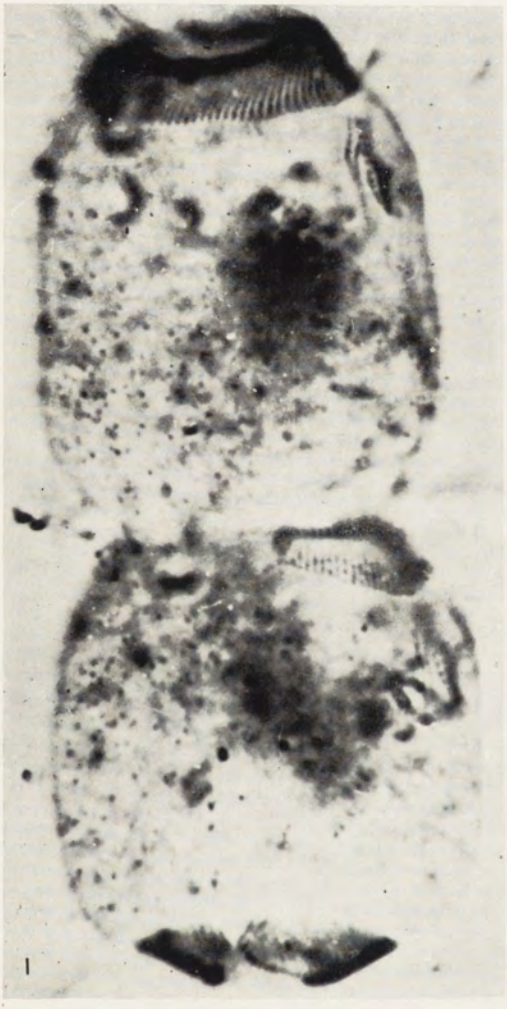
EXPLANATION OF THE PLATE I

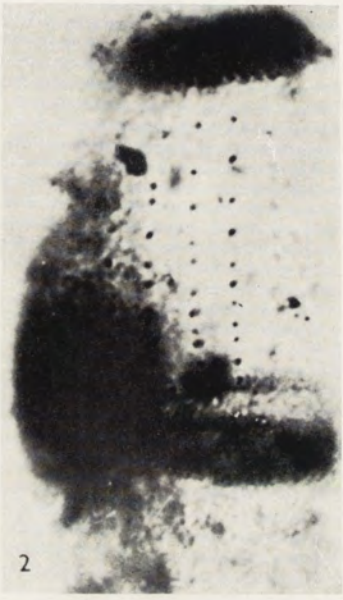
1: *Didesmis ovalis*. Right dorsal view. Right primordium of the anterior zone of the opisthe (× 2000)

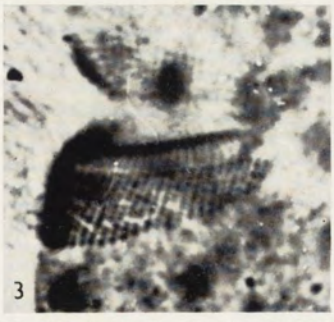
2: Non-ciliated kineties (?) are seen on the territory of the prother. An anlage of the posterior zone of the prother is seen as a nerrow stripe, anterior zone of the opisthe is broad (× 2000)

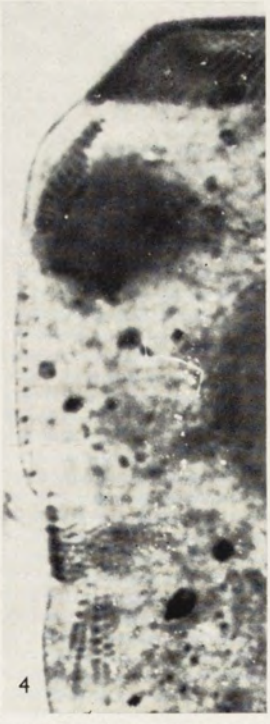
3: Left primordium of the anterior zone of opisthe, stratification is seen (× 2400)

4: Kineties on the territory of "Konkrementvacuole" of opisthe (× 2400)









M. Wolska auctor phot.

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Blastocrithidia raabei sp. n., a flagellate parasite of *Mesocerus marginatus* L. *(Hemiptera: Coreidae)*[1]

Blastocrithidia raabei sp. n., wiciowiec pasożytujący w *Mesocerus marginatus* L. (*Hemiptera: Coreidae*)

While conducting studies on infectious processes caused by protozoans in insects a number of insects, belonging to various orders, were collected and examined on protozoan infection. The hemipteran *Mesocerus marginatus* L.[2] was one of the studied in ects that have been found to be parasitized by flagellates.

Adult bugs of *Mesocerus marginatus* L. were collected with a sweeping net on various plants in Białowieża National Park. The collected insects were dissected on a dissecting pan and fresh preparations of gut, hemolymph, and salivary glands were first microscopically examined. Then, smears were prepared fixed in absolute methyl alcohol and stained with 0.25% Giemsa's solution for 16—24 hours.

The morphology and life cycle of flagellates were studied on fresh and stained preparations. Flagellates were measured at a 1000th magnification with the possible error 0.1 micron.

Blastocrithidia raabei sp. n.

Host insect: *Mesocerus marginatus* L.
Locality record: Białowieża National Park, July 11, 1964
Infection level: Out of three collected adults of *Mesocerus marginatus* two of them were infected with the parasitic flagellates.

A number of morphological forms are recognized in the development of flagellates. In the studied species blastocrithidial, crithidial, leptomonad, leishmanial and incysted forms have been easily recognized and are described. Herpetomonads and the so called heptomonads have not been observed.

The blastocrithidial form.

This is the predominant form in the insect gut and in the hemolymph. Blastocrithidial forms vary in size from 21.3×3.0 to 31.7×3.1 μ (Table 1). The

[1] This investigation was supported by the research grant FG-Po-112 from the United States Department of Agriculture.

[2] The author's identification of this species was confirmed by Prof. Dr A. Wróblewski, Institute of Zoology, Polish Academy of Sciences, Poznań, what is kindly acknowledged.

mean measurement of eleven flagellates is 26.56 μ. The body of the flagellate is elongated and the anterior and posterior ends are pointed. The nucleus is located in the posterior part, approximately in $^1/_3$—$^1/_4$ of the overall length. The diameter of the nucleus is from 2 to 3 μ.

The kinetoplast is located close to the nucleus, always in the anterior part of the body. The kinetoplast is round or bean shaped and it measures 0.5—0.9 by 0.5 μ.

The undulating membrane is well visible (Pl. I 1—2). The flagellum after passing a short reservoir goes along the membrane. The length of the free flagellum varies from 4.1 to 13.1 μ.

The cytoplasm of blastocrithidial forms is not homogeneous. In the anterior part it is dense and in the posterior part it is vacuolized (Pl. III 13). The number and size of vacuoles in the cytoplasm show a great variation.

Table 1

Results of measurements of 11 flagellates of each form in microns

Form		1	2	3	4	5	6	7	8	9	10	11	Average
Blastocrithidial	pe-mn	6.1	8.1	5.1	4.1	6.1	8.1	8.0	6.1	7.1	8.1	8.1	6.82
	mn-k	3.0	2.5	1.0	1.5	4.0	3.1	3.2	2.0	3.5	1.8	1.9	2.50
	k-tff	12.2	13.3	20.4	19.5	17.1	19.2	17.2	20.4	21.1	20.1	16.2	17.83
	lff	4.1	5.1	8.2	9.2	9.1	5.0	8.1	11.1	13.1	8.1	5.1	7.84
	gb	3.0	4.0	4.0	4.0	4.1	3.0	3.1	3.1	3.1	3.5	3.0	3.44
	oal	21.3	23.9	26.5	25.1	27.2	30.4	28.4	28.5	31.7	23.0	26.2	26.56
Crithidial	pe-mn	6.1	3.0	2.1	3.0	2.2	4.1	3.1	2.4	3.5	4.2	3.5	3.38
	mn-k	1.8	3.0	2.0	1.5	2.1	2.2	2.0	1.8	1.5	3.0	2.0	2.08
	k-tff	17.3	19.7	4.1	10.2	17.1	16.1	21.4	17.3	18.3	19.4	15.3	16.02
	lff	6.1	11.1	13.2	5.1	6.1	11.1	18.4	10.3	10.3	10.1	8.2	10.00
	gb	5.1	5.1	4.1	4.1	5.1	4.2	5.1	5.1	4.5	6.1	4.5	4.82
	oal	25.2	25.7	8.2	14.7	21.4	22.4	26.5	21.5	23.3	26.6	20.8	21.48
Leptomonad	pe-mn	6.1	7.1	8.3	7.1	10.2	6.1	10.6	9.2	8.2	7.1	8.2	8.02
	mn-k	3.0	3.0	3.1	3.0	4.1	4.1	3.7	3.5	4.1	3.0	3.0	3.42
	k-tff	15.3	16.3	11.2	16.3	20.4	23.5	23.1	18.4	22.5	20.4	30.1	19.77
	lff	7.1	9.1	6.0	10.2	13.4	6.1	7.1	8.2	6.1	7.1	23.2	9.42
	gb	2.5	2.2	2.0	2.6	2.0	2.5	2.5	3.0	3.0	2.0	2.0	2.39
	oal	24.4	26.5	22.6	26.4	34.7	33.7	37.4	31.1	34.8	30.5	41.3	31.22

pe-mn — posterior extremity to middle of nucleus
mn-k — middle of nucleus to kinetoplast
k-tff — kinetoplast to tip of free flagellum
lff — length of free flagellum
gb — greatest breadth
oal — over-all length

The crithidial form

This form is quite frequently observed. The length of the body with flagellum varies from 8.2 to 26.6 μ. The mean length of ten flagellates is 21.11 μ.

The body of crithidial forms is oval or round (Pl. I 3). The posterior end is mostly pointed while the anterior end is oval. The body is much wider than that of other forms, and varies from 4.1 to 6.1 μ.

The nucleus is located in the center of the body or in other positions. It is mostly oval and has 2—2.5 μ in diameter.

The kinetoplast is mostly bean shaped and on an average 1.2 μ long and 0.5 μ wide.

The flagellum goes through a short and widely opened reservoir. Some typical crithidial forms have the undulating membrane.

The leptomonad form.

These forms are very common in the foregut and midgut; in the hemocoel they are not so frequent as blastocrithidial forms. They vary from 24.4 to 41.3 μ in length and from 2.0 to 3.0 μ in width. The mean length of ten flagellates is 31.22 μ.

Leptomonad forms are elongated and very slender, frequently with a twist about midway of the body (Pl. I 4—6 and III 14). Both ends are sharply pointed. The nucleus has on the average 1.5 μ in diameter and is located in the middle of the body.

The kinetoplast is small and oval. It is located midway between the nucleus and the anterior end or closer to the nucleus. The flagellum passes through a short, sometimes longer, reservoir. The length of the free flagellum varies from 11.3 to 23.5 μ.

The leishmanial and incysted forms.

These forms are predominant in the rectum and have very rarely been observed in the hemocoel. No attempts have been made to differentiate them as they look much alike. They are about 5 microns in diameter. A small flagellum may be seen inside the cytoplasm. The position of the nucleus and the kinetoplast show a great variation.

Development

The life cycle of the flagellate was not completely worked out, some data, on this subject, however, have been collected.

Binary fission seems to be the main type multiplication. Multiple fission was also observed. In case of binary fission the sequence of division of various organellas varies among individual flagellates (Pl. II 7—14). In some individuals the kinetoplast is divided first while in others the nucleus and later the kinetoplast.

Clusters of flagellates are frequently observed (Pl. IV 15). In case of the multiple division at first plasmodium is formed which later turns into a rosette-like structure. The diameter of such clusters on an average is about 60—75 μ.

Site of infection

In both studied infected insects the flagellates inhabited the intestine and hemocoel. As it is based on two cases only it cannot be concluded whether general infection is typical for this flagellate.

Taxonomic position

According to present taxonomic criterions in the family *Trypanosomatidae*, the species found in *Mesocerus marginatus* should be placed among the genus *Blastocrithidia* Laird, which is characterized by a short undulating membrane and is parasitic in insect (Laird 1959, Lipa 1963, Wallace 1963).

There are no previous records of flagellate infections in *Mesocerus marginatus*. There is also no evidence that the studied flagellate can be identified with *Herpetomonas tortum* Poisson known from related coreid *Camptopus lateralis* (Germ.) (Poisson 1930). Plant flagellates belonging to the genus *Phytomonas* Donovan cannot be taken into consideration as in members of that genus developmental forms possessing the undulating membrane are never met.

For the reasons just outlined it is assumed that the flagellate from *Mesocerus marginatus* is a new species. Accordingly, the name *Blastocrithidia raabei* sp. n. is proposed. The specific name of that flagellate is in honour of my distinguished teacher Professor Dr. Zdzisław Raabe.

The holotype slide, stained with Giemsa, from an adult host collected on July 11, 1964, in the Białowieża National Park, is in the author's collection. Paratype slide material is deposited in the collection of Protozoological Laboratory, Zoological Institute, University of Warszawa and in the collection of Dr. F. G. Wallace, Department of Zoology, University of Minnesota, Minneapolis, Minnesota, U.S.A.

Discussion

Until very recently there has been much confusion concerning the taxonomy of insect flagellates of the family *Trypanosomatidae*. A new genus created by Laird 1959 and new taxonomic criteria proposed by Wallace 1963 corrected the situation. Lipa 1963 has made the first attempt to revise some previously described species and has proposed a new combination of generic names according to present taxonomic criteria.

The difficulties in taxonomy of *Trypanosomatidae* are mostly connected with the fact that in the life cycle of flagellates belonging to all genera metacyclic forms can be observed. Four morphological forms are recognized and named after the generic names of those genera which they morphologically resemble. These are: leishmanial, leptomonas, blastocrithidial and trypanosomidal. Although in a recent treatise (Lipa 1963) on insect flagellates I proposed only these four morphological forms, in the course of further studies on insect flagellates, I have found that such a classification is not sufficient. In a recent paper (Lipa 1965) I have made the first attempt to clarify this problem and the following forms have been recognized: leishmanial, leptomonad, crithidial, herpetomonad, blastocrithidial, trypanosomidal and encysted.

Beside the forms mentioned above some authors recognize still others e. g. haptomonad, nectomonad and others. Due to that fact the state of confusion is quite serious and should be urgently corrected.

In the life cycle of *Blastocrithidia raabei* sp. n. five morphological forms have been observed: incysted, leishmanial, leptomonad, crithidial and blastocrithidial. Other forms have not been observed. The blastocrithidial form has been prevelent in all studied preparations.

In all examined insects symbionts were observed (Pl. IV 15).

Summary

Out of three adult *Mesocerus marginatus* L. collected in Białowieża National Park two insects were found to be infected with a new flagellate parasite *Blastocrithidia raabei* sp. n. The parasite inhabited hemocoel and intestine of

the host. Morphology of various developmental stages was described; blastocrithidial forms are most predominant. Morphological forms occuring in the life cycle of the insect trypanosomatids are listed and the need of correction of present situation in this matter is emphasized.

STRESZCZENIE

Z trzech dorosłych pluskwiaków *Mesocerus marginatus* L. zebranych w Białowieskim Parku Narodowym dwa owady zarażone były przez nowy gatunek wiciowco *Blastocrithidia raabei* sp. n. Pasożyt występował w jelicie i hemolimfie żywiciela. Opisano morfologię poszczególnych stadiów rozwojowych wiciowca; dominującą postacią jest forma blastokritidialna. Wymieniono morfologiczne formy występujące w rozwoju wiciowców owadów i podkreślono potrzebę uporządkowania tego zagadnienia.

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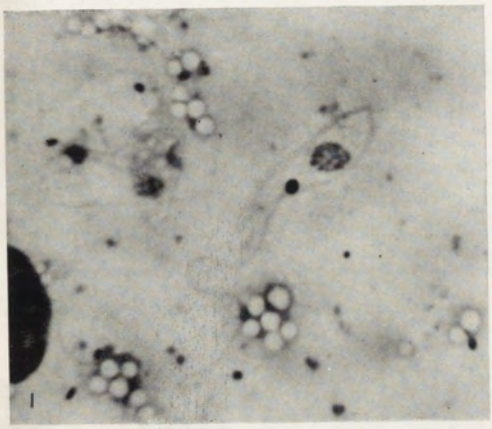
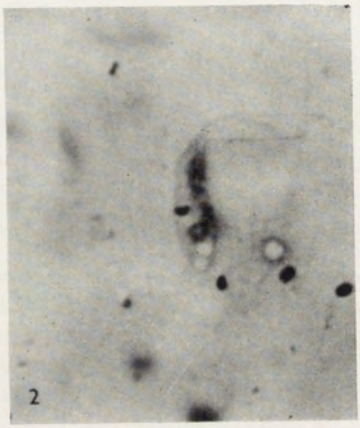
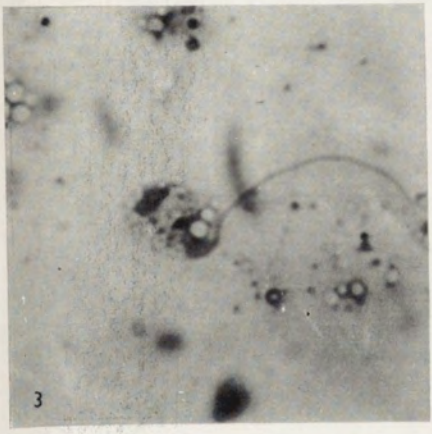
EXPLANATION OF PLATES I—IV

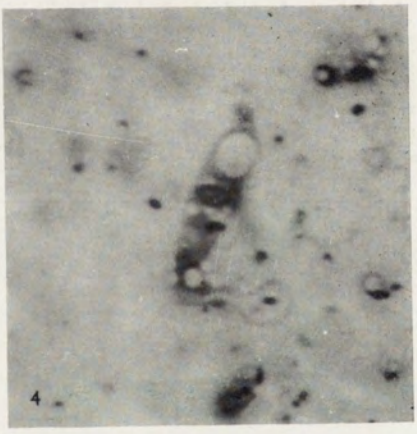
Blastocrithidia raabei sp. n.

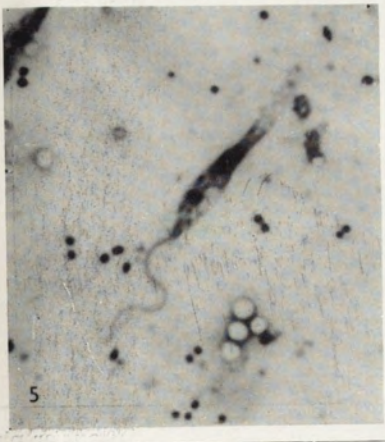
1—2: Blastocrithidial forms
3: Crithidial forms
4—6: Leptomonad forms
7—12: Various types and stages of binary fission
13: Blastocrithidial forms, notice the vacuolized cytoplasm in the posterior part of the body
14: Dead leptomonads in the host's intestine
15: Rosette-like cluster of leptomonads in hemolymph of *Mesocerus marginatus* L.
16: Symbionts in the smeared tissue of *M. marginatus* L.

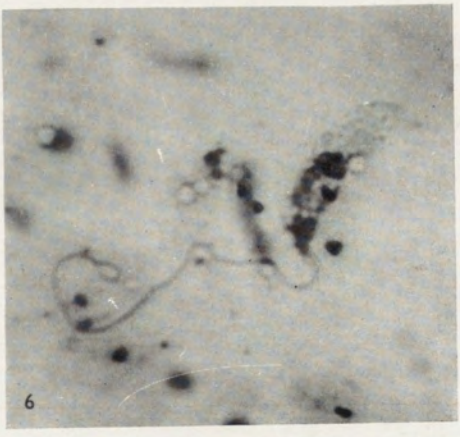












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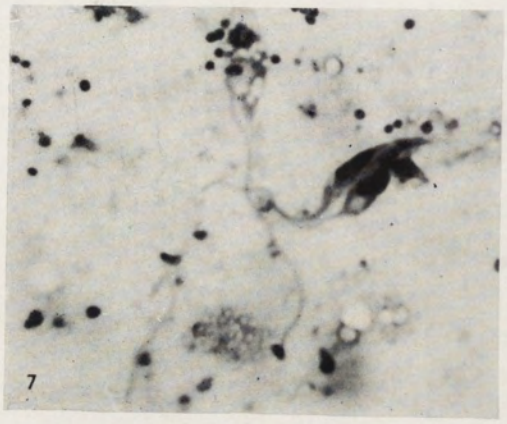

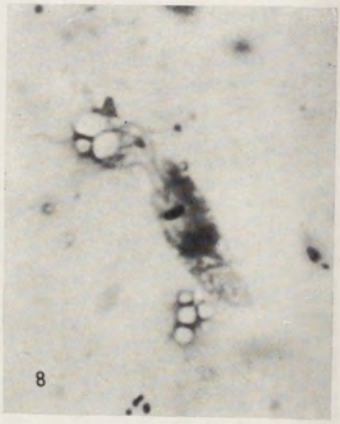

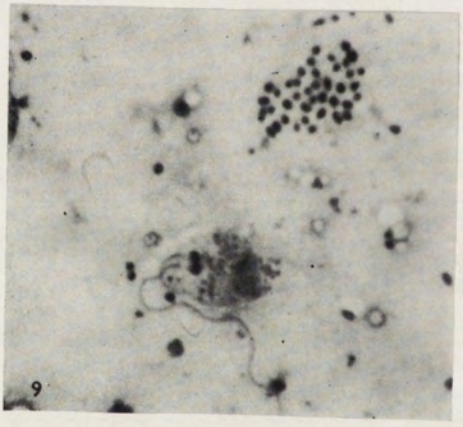

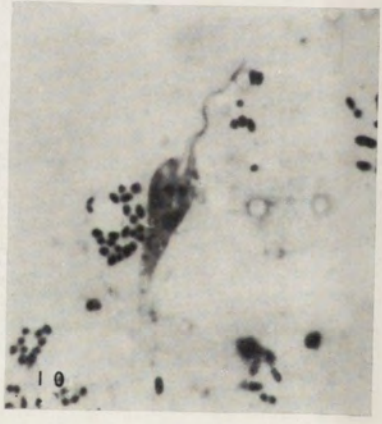

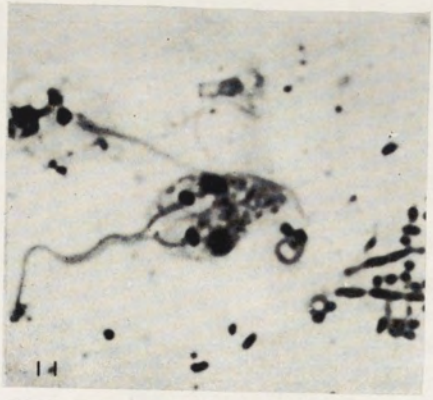

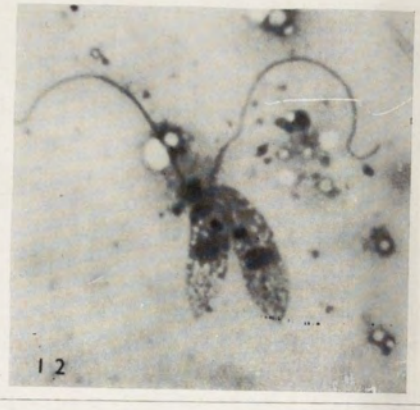

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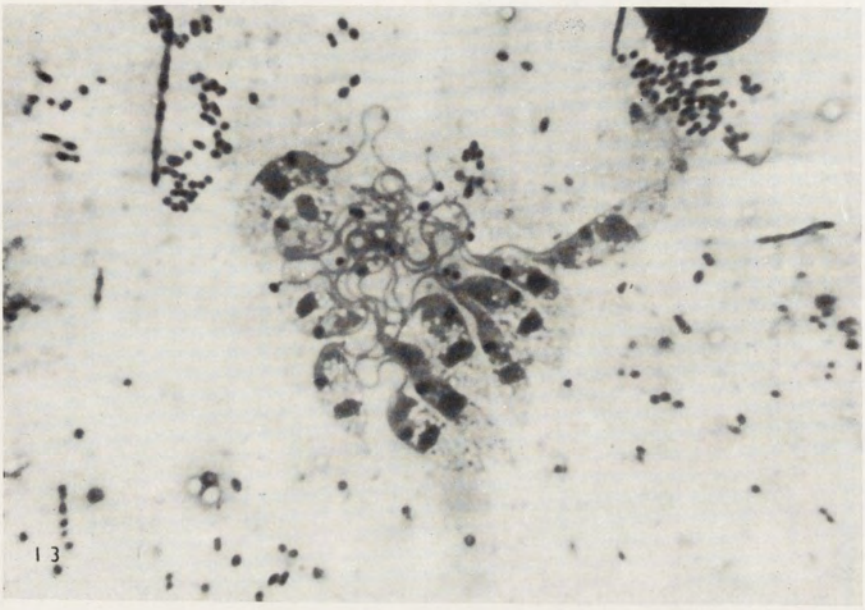

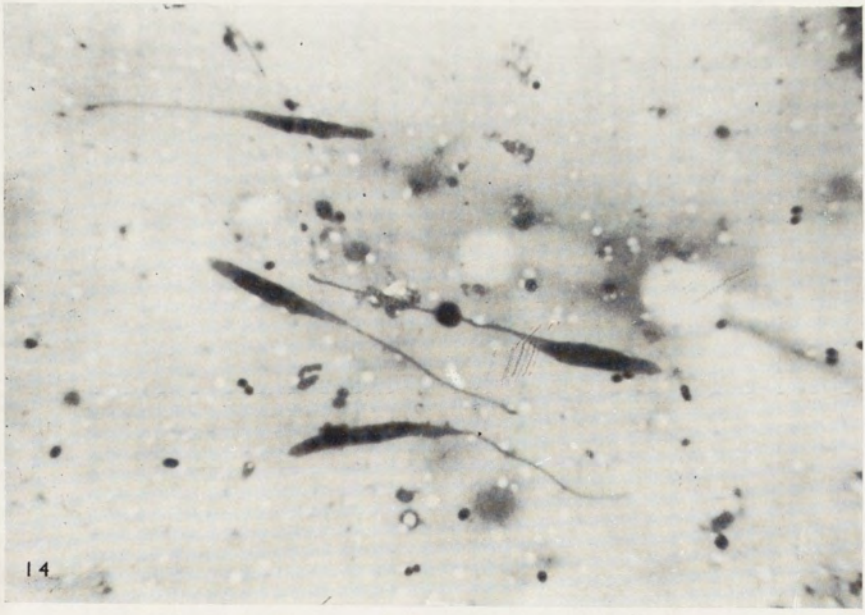

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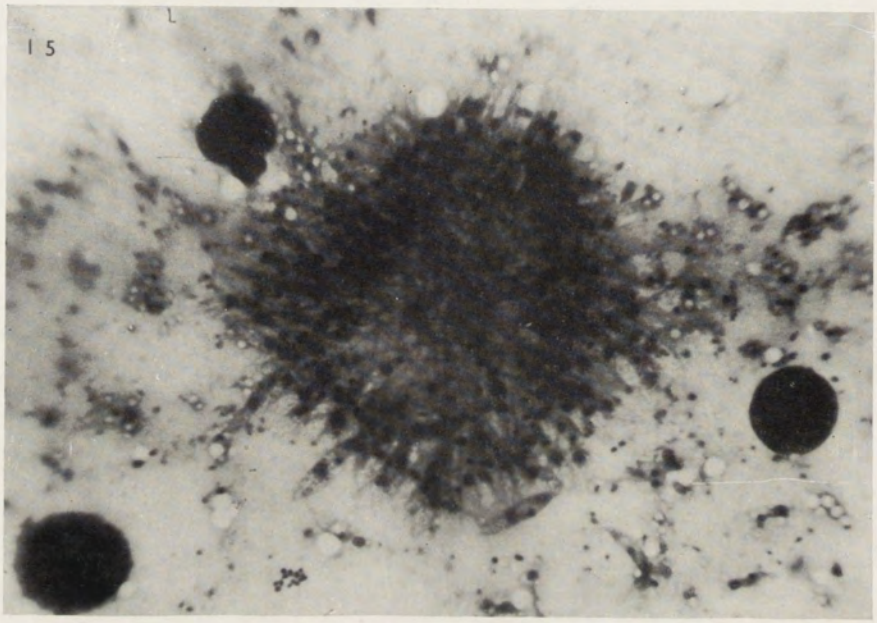

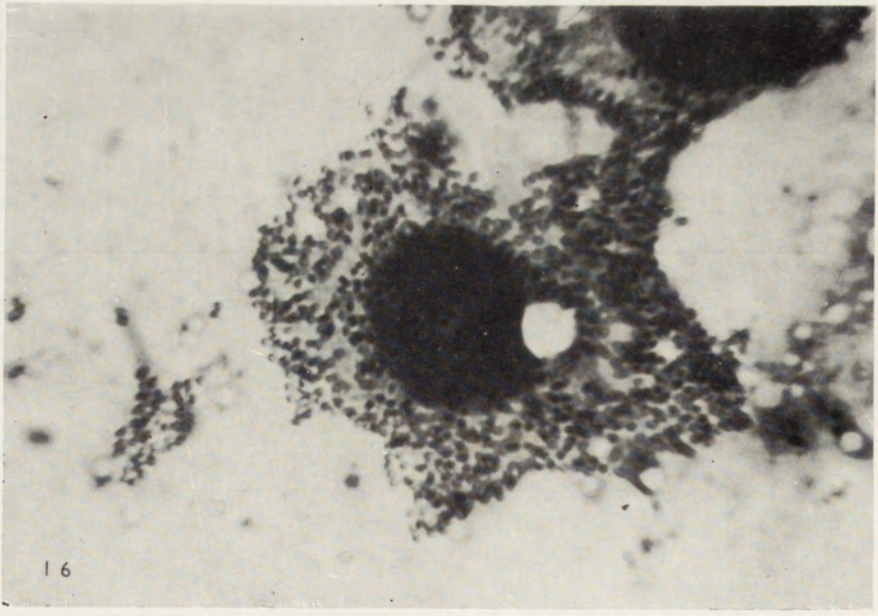

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Влияние ионизирующего излучения на процессы ядерной реорганизации *Paramecium putrinum*. II. Ядерные процессы у эксконъюгантов

Effect of ionizing radiation on the processes of nuclear reorganization in *Paramecium putrinum*. II. Nuclear processes in exconjugants

В предыдущем сообщении (Ковалева и Янковский 1965) обсуждался вопрос о влиянии ионизирующей радиации на ядерные процессы у конъюгантов *Paramecium putrinum*. Было показано, что облучение конъюгантов разовой дозой, равной 75 кр, вызывает значительные нарушения делений созревания микронуклеуса (Ми) на всех стадиях. Что касается макронуклеуса (Ма), то существенных различий в картинах его распада у необлученных и облученных конъюгантов обнаружить не удалось

Цель настоящего исследования заключается в изучении последующих стадий ядерных процессов, происходящих после расхождения конъюгантов (у эксконъюгантов).

Материал и методика

В опыте были использованы 2 линии инфузорий *Paramecium putrinum*, относящиеся к разным типам спаривания (линия *M* 6 — т. сп. I, линия *M* 12 — т. сп. II сингена 4, миксотип А). Методика культивирования парамеций и условия их облучения были подробно изложены в I части нашего исследования (Ковалева и Янковский 1965). Здесь мы лишь укажем, что парамеции облучались дозой, равной 75 кр, данной им в течение двухминутной экспозиции. Основная масса конъюгантов была облучена на стадии поздней профазы I деления созревания Ми. Инфузорий фиксировали смесью Буэна в солонках, приклеивали к предметным стеклам по методу Чена (Chen 1944). Препараты окрашивались на ДНК по Фельгену, на РНК по Унна-Паппенгейму и гематоксилином Бемера.

Результаты

К моменту расхождения необлученных конъюгантов *P. putrinum* их синкарион претерпевает три последовательных деления. Синкарион же облученных конъюгантов, как правило, не делится ни до, ни после расхождения конъюгантов. В нашем первом сообщении синкарион облученных конъюгантов назван „комплексным" синкарионом. Дело в том, что синкарион облученных конъюгантов является сложным образованием, в состав которого входят, помимо стационарного и мигрирующего ядра, еще и другие ядра — недегенерировавшие после II деления созревания Ми. Следует указать, что синкарионы облученных конъюгантов отличаются большим разнообразием в отношении количества составляющих их компонентов и степени слияния этих компонентов между собой (Рис. 1 A — D). На первых стадиях формирования комплексного синкариона во всех его ядерных компонентах четко различаются хромосомы в виде нитей, соединенных между собой одним своим концом, в то время как другой конец нити остается свободным. При таком объединении хромосом образуются щетковидные фигуры, характерные для ядер облученных конъюгантов парамеций. В дальнейшем, когда хромосомы укорачиваются и приобретают вид глыбок, комплексный синкарион, в одних случаях, превращается в плотное массивное тело, с хорошо различимыми крупными хроматиновыми комками (Рис. 2 A —D). Такое ядерное образование очень интенсивно окрашивается по Фельгену и, судя по увеличению его размеров и укрупнению хроматиновых комков, можно заключить, что в нем происходит синтез ДНК. Увеличение размеров клеточных ядер и слипание хроматинового вещества в комки наблюдается как в клетках многоклеточных, так и у одноклеточных организмов после воздействия на них ионизирующей радиацией (Фриц-Ниггли 1961, Ковалева 1963). В других случаях, когда дополнительные ядра не сливаются с синкарионом, в каждом из компонентов наблюдается образование хроматиновых комков и их укрепнение (Рис. 2 B, D).

Как было указано, комплексный синкарион, как правило, не делится. При делении эксконъюганта он остается в одной из дочерних клеток, которая вскоре погибает. Одной из главных причин неспособности синкариона облученных конъюгантов к последующему развитию является, как мы предполагаем, его комплексность. Даже в тех случаях, когда ядра — компоненты синкариона — сливаются в морфологически единое образование, оно не является таковым с точки зрения функциональной, так как его компоненты сохраняют свою индивидуальность и специфическим образом проявляют себя. Нам удалось наблюдать единственный случай деления комплексного синкариона в облученном конъюганте, который хорошо иллюстрирует проявление функциональной активности ядер — компонентов синкариона. Как показано на Рис. 3 C, эксконъюгант находится в состоянии деления, его синкарион делится амитотическим путем (вытянутая форма ядра, начинающаяся перетяжка). По одной стороне синкариона расположена лентовидная нуклеола. Следует заметить, что как амитотический способ деления, так и наличие нуклеолы совершенно нетипичны для нормального синкариона. Появление в синкарионе нуклеолы связано с функциональной активностью его компонентов. Как нам удалось наблюдать, в так называемых „дегенерирующих" ядрах — продуктах II деления созревания Ми — образуется пиронинофильная полоска, которая имеется и у облученных конъюгантов в аналогичных ядрах. Если такие ядра не дегенерируют, они могут присоединиться к комплексному синкариону и сохраняют в нем свою индивидуальность.

Рис. 1 Морфология ядерного аппарата у эксконъюгантов (конъюганты были облучены на стадии профазы I деления созревания микронуклеуса). Окраска по Фёльгену; рис. апп., об. 90х, ок. 20х. Обозначения: m — фрагменты старого макронуклеуса; s — синкарион; видно разное количество компонентов, входящих в состав синкариона; n — ядра, не присоединившиеся к синкариону.

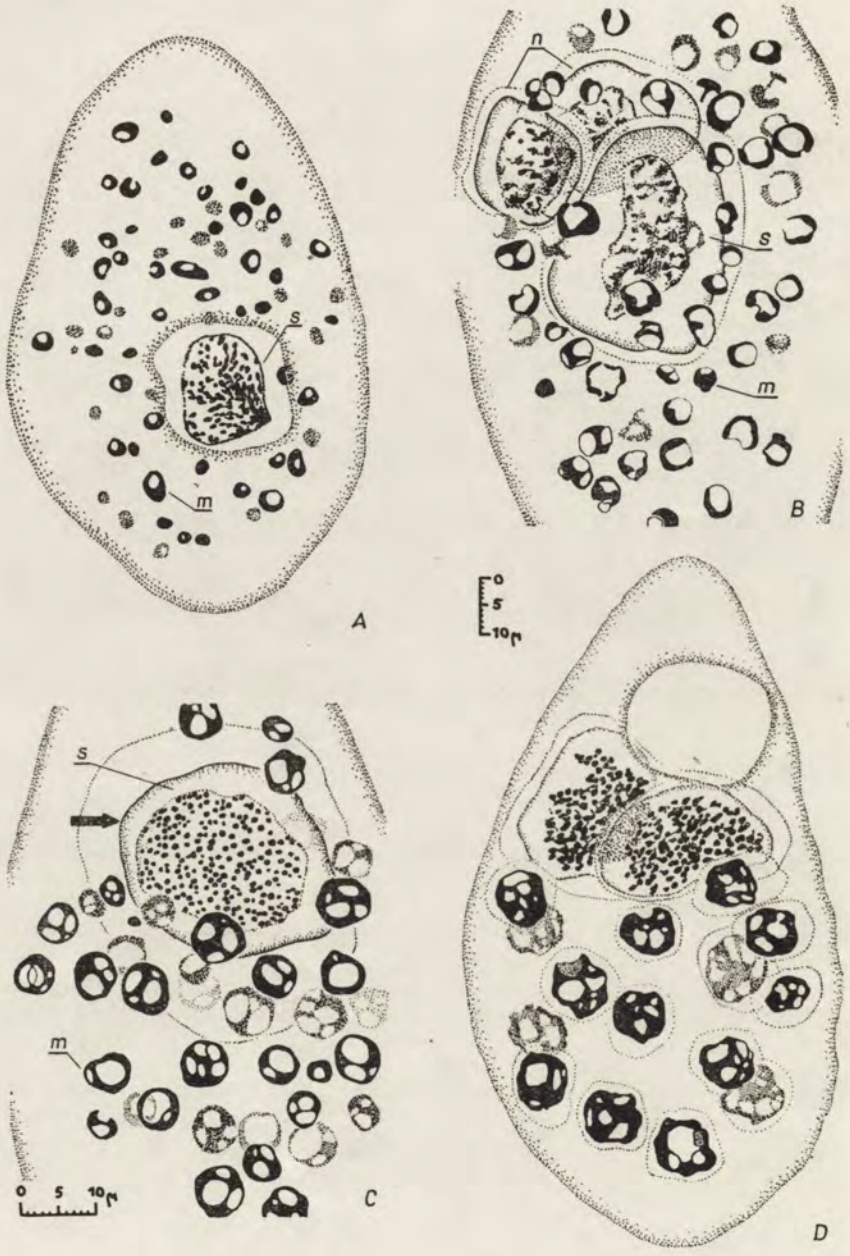

Рис. 2. Картины изменения структуры синкариона у экскончюгантов. Рис. апп., об. 60х, ок. 20х. Окраска по Фёльгену. А. Следующая стадия изменения хромосом; видны слипшиеся хроматиновые глыбки; фрагменты старого Ма мелкие. В. Вблизи синкариона находятся 2 ядра — продукты II деления созревания Ми. С. Размеры синкариона увеличены; в фрагментах старого Ма увеличилось количество нуклеол; стрелкой обозначена лихтгрюновая зона. D. Видны 2 ядра с укрупненными хроматиновыми глыбками; эти ядра образовались в результате слияния пронуклеусов с недегенерировавшими ядрами. Количество фрагментов старого Ма уменьшилось

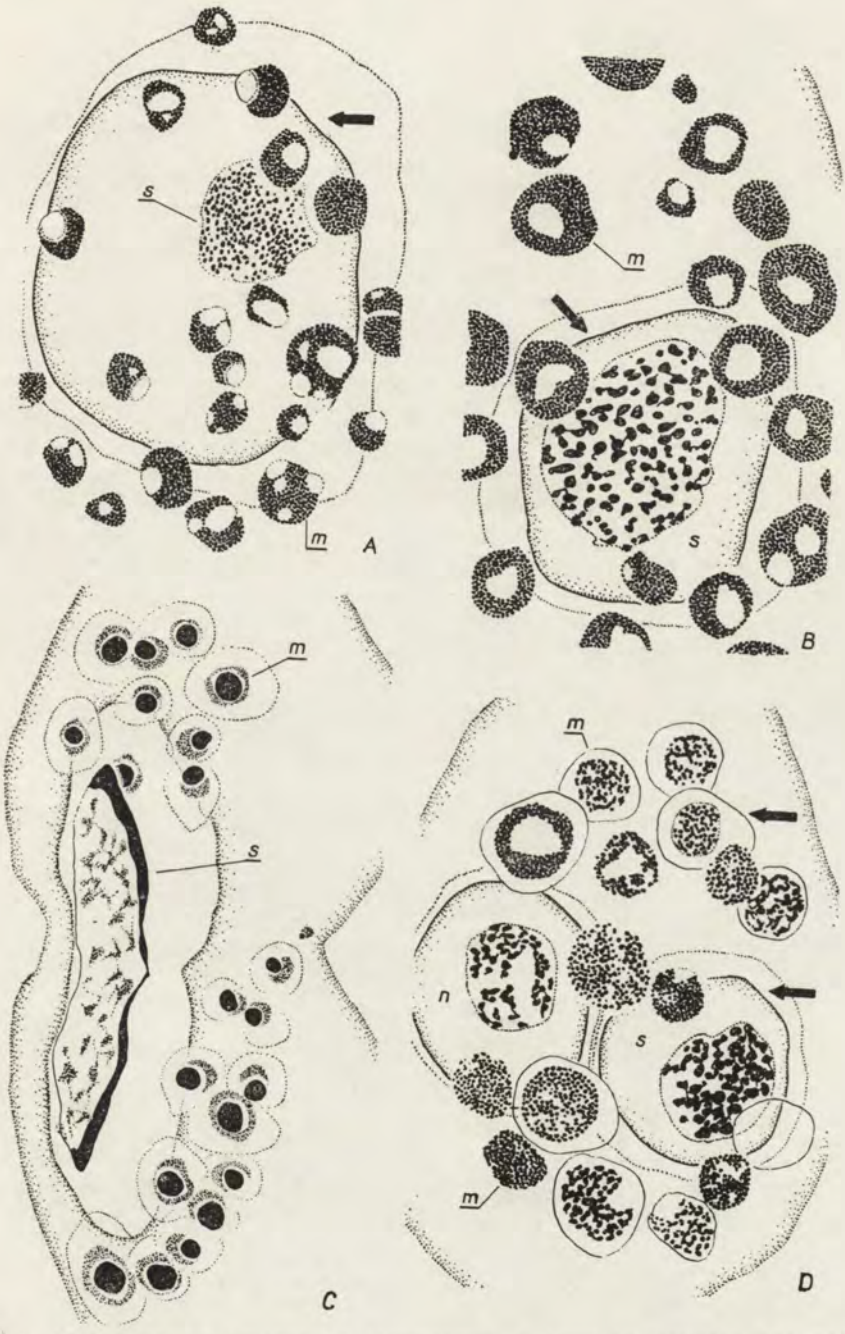

Рис. 3. Последующие изменения синкариона и один из случаев его деления у эксконъюганта. Окраска по Фёльгену, об. 90х, ок. 15х (A, B, D); окраска по Браше, об. 60х, ок. 20х. (C). Рис. апп. A. Лихтгрюновая зона достигает максимальных размеров (36 μ). B. Синкарион имеет вид компактного тела с крупными хроматиновыми глыбками. C. Деление синкариона; нуклеолы закрашены черным цветом. Видна лентовидная нуклеола на одной стороне ядра. D. Видны изменения в фрагментах старого Ma: слипание хроматиновых зерен и появление полостей в фрагментах. Обозначения те же, что и на рис. 1; стрелки указывают на лихтгрюновую зону.

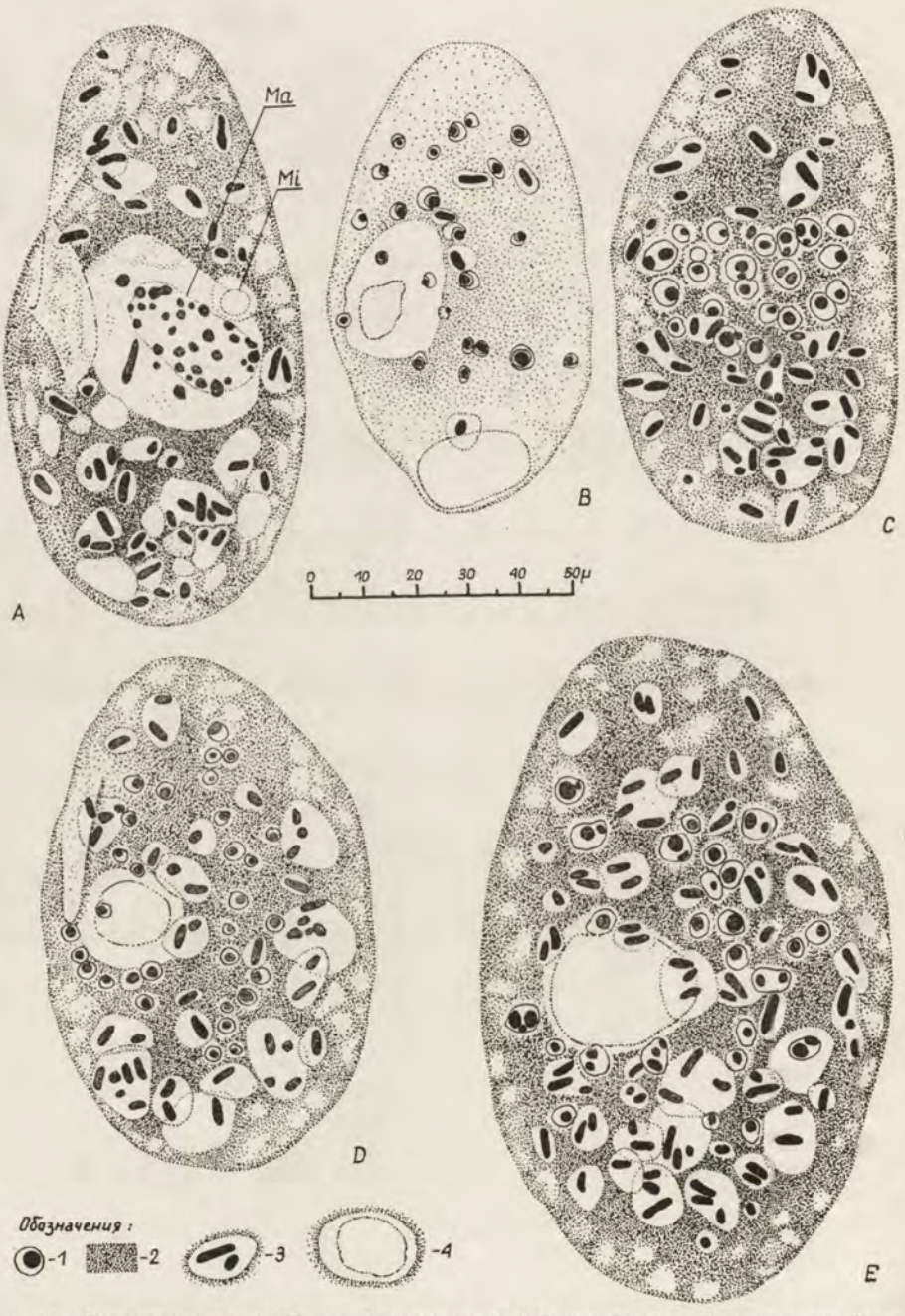

Рис. 4. Вегетативная особь и категории эксконъюгантов. Окраска по Браше, схематизировано. Рис. апп., об. 60х, ок. 20х. Обозначения: 1 — фрагменты старого макронуклеуса с нуклеолами; 2 — цитоплазменная РНК; 3 — дрожжевые клетки в пищеварительных вакуолях; 4 — контуры синкариона. А. Вегетативная особь. В — Е. Категории эксконъюгантов. В. Мелкая светлая клетка (4-я категория). С. Общий вид клетки эксконъюганта (из 3-й категории). D. Общий вид клетки эксконъюганта (из 2-й категории). Е. Общий вид клетки эксконъюганта (из 1-й категории)

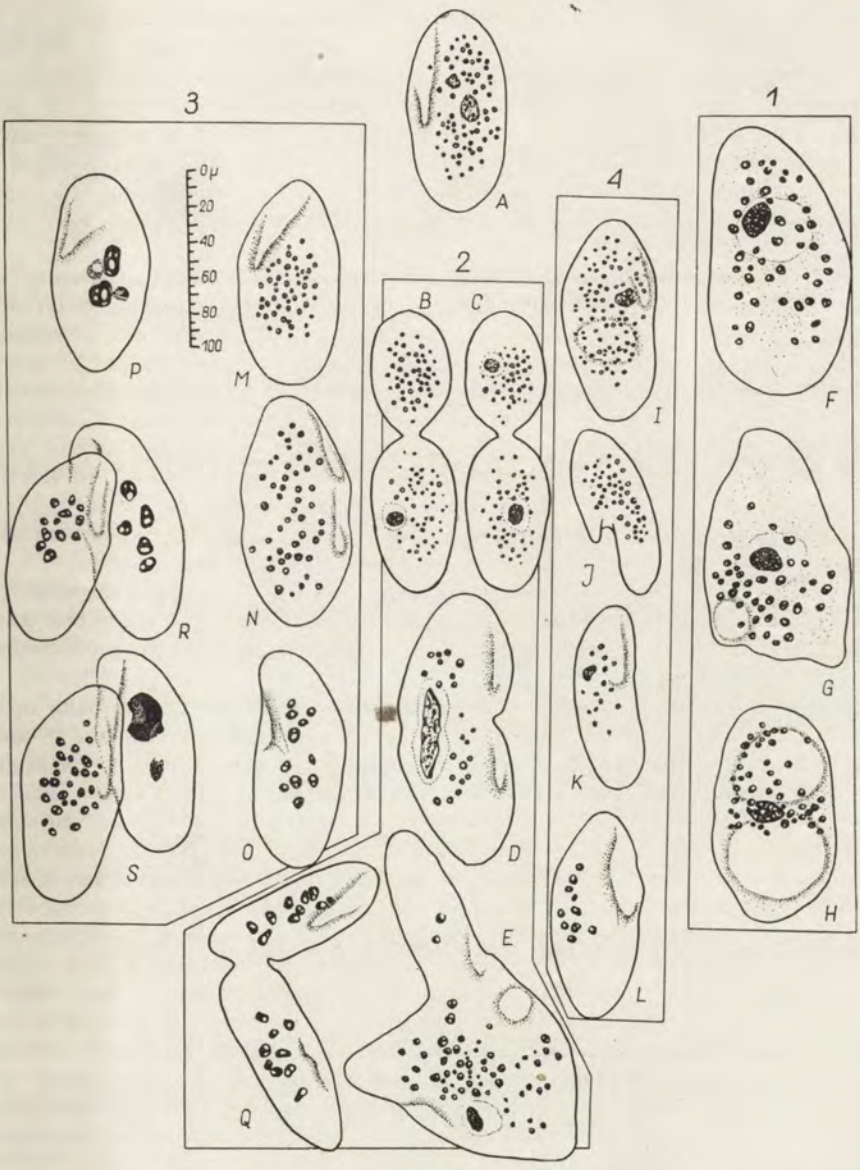

Рис. 5. Дивергенция в потомстве облученных партнеров *P. putrinum*. 1 — категория крупных клеток; 2 — варианты деления экскончюгантов; 3 — категория клеток с регенерирующим макронуклеусом; 4 — категория мелких клеток. С препаратов, рис. апп., об. 40х, ок. 7х. A. Клетка из 2-й категории; виден синкарион, недегенерировавшее ядро и фрагменты Ma. B. I вариант деления. C. II вариант деления. D. III вариант деления. E. IV вариант деления (незавершенное деление). F — H. Изменение состояния крупной клетки — уродливая форма тела, вакуолизация цитоплазмы. I — L. Разновидности клеток из 4-й категории. M — P. Последовательные стадии регенорации Ma. R — S. Коньюгация жизнеспособных особей.

У облученных конъюгантов имеется еще дна особенность, которая, по всей вероятности, оказывает влияние на дальнейшую судьбу синкариона. Мы имеем в виду изменения в так называемой „лихтгрюновой зоне" (ЛЗ), окружающей синкарион. Эта зона окружает Ми у необлученных конъюгантов. В работе Пенна (Penn 1936) приведены микрофотографии, на которых видна сходная зона у конъюгантов иного вида парамеций — *P. caudatum*. Зона хорошо выявляется в анафазе и профазе делений созревания Ми *P. putrinum*, при окраске ЛЗ на ДНК и РНК всегда получается отрицательный результат; зону можно окрасить лишь лихтгрюном.

У облученных конъюгантов и эксконъюгантов *P. putrinum* комплексный синкарион окружен зоной, которая, судя по избирательной окраске лихтгрюном, соответствует ЛЗ у необлученных конъюгантов. У последних она сохраняется только до III деления синкариона и далее перестает различаться. У облученных конъюгантов и эксконъюгантов ЛЗ достигает значительно больших размеров, чем у необлученных; максимальная ширина ЛЗ вокруг синкариона эксконъюганта достигает 36 μ, причем эта зона сохраняется все время у всех эксконъюгантов. В первой части нашего сообщения (Ковалева и Янковский 1965) были описаны нарушения в формировании веретена при делениях созревания Ми. Эти нарушения имеют, по-видимому, какую-то связь с повреждением вещества ЛЗ действием ионизирующей радиации.

Наблюдения над облученными эксконъюгантами в культуре и изучение цитологических картин на препаратах позволили нам выделить 4 категории клеток в соответствии с их морфологическими и физиологическими особенностями (Рис. 4 B — E; ср. Рис. 4 A).

Первую категорию составляют эксконъюганты, выделяющиеся среди прочих клеток в культуре мешковидной формой тела, крупными размерами и темной цитоплазмой (Рис. 4 E). Поведение этих особей в культуре отличает их чрезвычайной вялостью: они либо неподвижно лежат на дне чашки Петри, либо едва передвигаются, не поднимаясь в верхние слои воды. На препаратах эти клетки выделяются сильно выраженной пиронинофилией цитоплазмы, множеством пищеварительных вакуолей, буквально забитых пищевыми частицами и огромным Фёльген-положительным образованием, которое, очевидно, соответствует комплексному синкариону (Рис. 4 E). Фрагменты Ма крупные, с 2—3 нуклеолами. Все это указывает на то, что особи данной категории способны к росту, однако нам никогда не приходилось видеть, чтобы эти особи делились. Они погибают на 5—6-й день после разъединения конъюгантов, изредка при явлениях сильнейшей вакуолизации цитоплазмы (Рис. 5 H). В фрагментах Ма у гибнущих особей может наблюдаться слипание хроматиновых зерен и образование полостей (Рис. 3 D, 5 F — H). Способность облученных клеток к росту, приводящему к увеличению ее размеров до гигантских — это одно из характерных проявлений радиационного воздействия (Фриц-Ниггли 1961, Кузин 1962).

Ко второй категории относятся клетки, которые составляют большую часть эксконъюгантов (Рис. 5 A). По количеству пищеварительных вакуолей, окраске цитоплазмы на РНК (Рис. 4 D), состоянию фрагментов Ма (крупные нуклеолы, картины выхода РНК в цитоплазму), внешнему виду и поведению их в культуре, они стоят ближе, чем другие категории клеток, к нормальным потомкам необлученных эксконъюгантов. Однако наличие комплексного синкариона, а также ядер — продуктов делений созревания Ми — отличает эти клетки от нормальных. Все же, данная категория эксконъюгантов является наиболее жизнеспособной, на что указывает размножение клеток данной категории. Сле-

дует отметить, что как эксконъюганты, так и их потомки обладают разной жизнеспособностью, что по нашим наблюдениям в значительной степени связано со строением их синкариона. Особи, обладающие синкарионом в виде массивного плотного тела, в котором произошло наиболее полное слияние всех его ядерных компонентов, нежизнеспособны; они погибают, ни разу не разделившись (клетки I категории). Особи, у которых синкарион имеет вид рыхлого ядра и помимо синкариона в клетке содержатся 1—2 дополнительных ядра (продукты II деления созревания Ми) оказываются способными делиться. Создается впечатление, что степень угнетающего действия комплексного синкариона на жизнеспособность эксконъюганта определяется количеством присоединившихся к нему дополнительных ядер.

Распределение ядерного материала между дочерними особями при делении клеток II категории осуществляется в трех вариантах. Дочерняя особь может получить синкарион, который сбычно не делится (Рис. 5 B); в другом варианте она может получить 1—2 ядра, являющиеся продуктами делений созревания Ми (Рис. 5 C); наконец, в третьем варианте дочерняя особь может получить только фрагменты старого Ма (Рис. 5 B). Среди потомков эксконъюгантов, содержащих только фрагменты старого Ма, находятся особи нежизнеспособные (о них речь пойдет далее) и особи, у которых обнаруживаются явные признаки регенерации Ма. Они и составляют III категорию клеток (Рис. 4 C). О наличии регенераторного процесса в фрагментах старого Ма свидетельствуют такие признаки, как последовательное укрупнение фрагментов, увеличение количества и размеров нуклеол в этих фрагментах и уменьшение числа фрагментов в результате их распределения между дочерними особями при делении эксконъюганта (Рис. 5 M — P; 6 A — C). Описанные картины можно было бы трактовать и не связывая их с происходящими регенераторными процессами; скажем, укрупнение фрагментов Ма и уменьшение их числа можно было бы отнести за счёт слияния фрагментов. Но нам ни разу не приходилось встретить картин слияния фрагментов Ма; к тому же функциональное состояние особей с регенерирующими Ма свидетельствует об их жизнеспособности. Регенерирующие клетки содержат нормальное количество пищеварительных вакуолей, их цитоплазма проявляет нормальную пиронинофилию. Кроме того, данные особи часто вступают в конъюгацию с вегетативными клетками — потомками тех вегетативных особей, которые находились в культуре в момент облучения (Рис. 5 S, 6 D), изредка с клетками, относящимися к той же категории (Рис. 5 R). Разумеется, мы не можем утверждать, что фрагменты старого Ма могут дать полноценный Ма, тем более, что нам удалось проследить уменьшение числа фрагментов только до 4 (Рис. 5 R). Однако, если исходить из представления о том, что повышение плоидности ядра является одним из способов защиты против радиационных повреждений (Малиновский 1960), то можно ожидать, что в Ма, как высокополиплоидном ядре, разрушенные радиацией участки могут быть возмещены, что и обеспечит восстановление полноценного ядра.

Четвертую категорию клеток составляют мелкие светлые клетки, имеющие в длину до 90 μ (Рис. 4 B, 5 I — L). В культуре эти клетки выделяются не только мелкими размерами, но и необычной прозрачностью цитоплазмы, а также весьма слабой подвижностью. При окраске по Унна-Паппенгейму выявляется очень слабая пиронинофильная реакция цитоплазмы мелких клеток; фрагменты Ма мелкие и без признаков регенерации. В каждом из них содержится одна нуклеола. Пищеварительные вакуоли немногочисленны (1—5), содержат по 1—2 дрожжевых клетки (дрожжевые клетки в смеси с бактериями *Bac. subtilis* до-

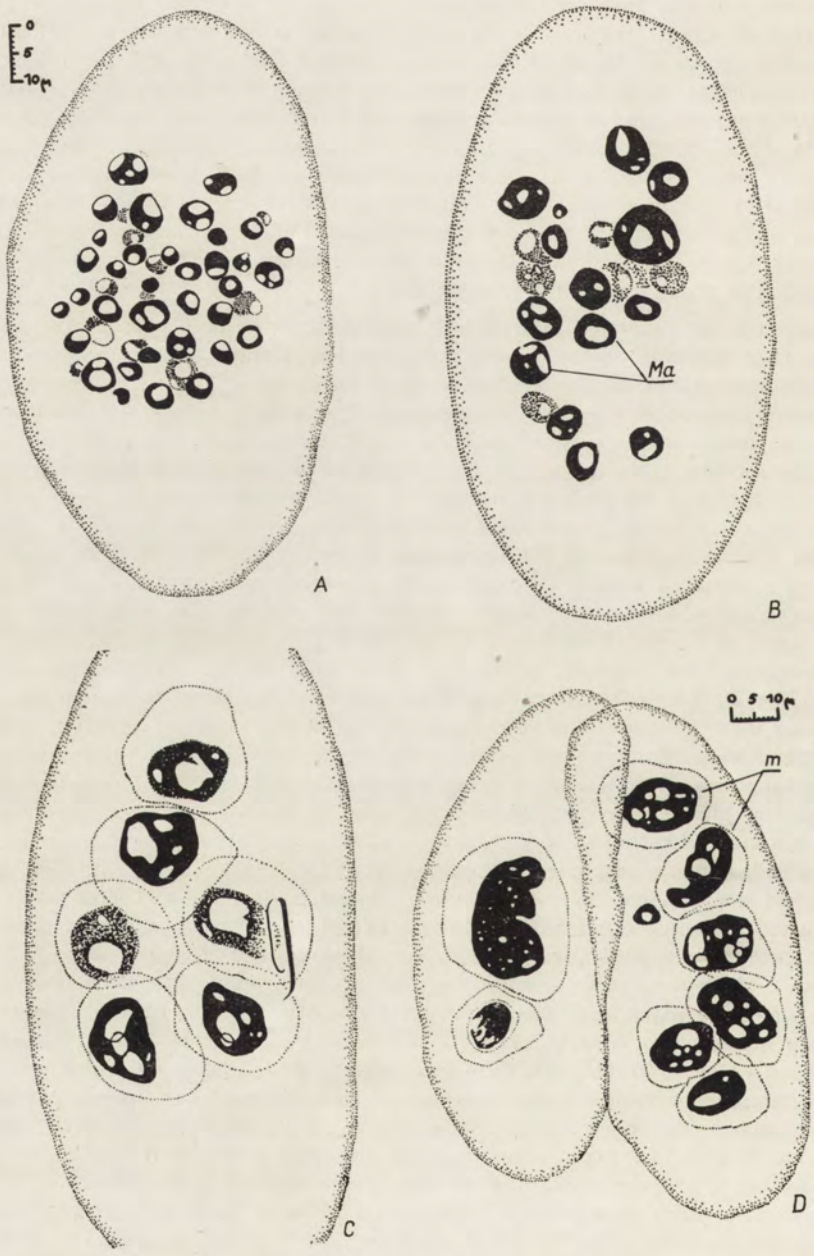

Рис. 6. Картины регенерации макронуклеуса. Окраска по Фельгену, рис. апп., об. 60х, ок. 15х (A — C); об. 60х, ок. 10х (D). A — C. Укрупнение фрагментов макронуклеуса. D. конъюгация клетки 3-й категории и вегетативной особи.

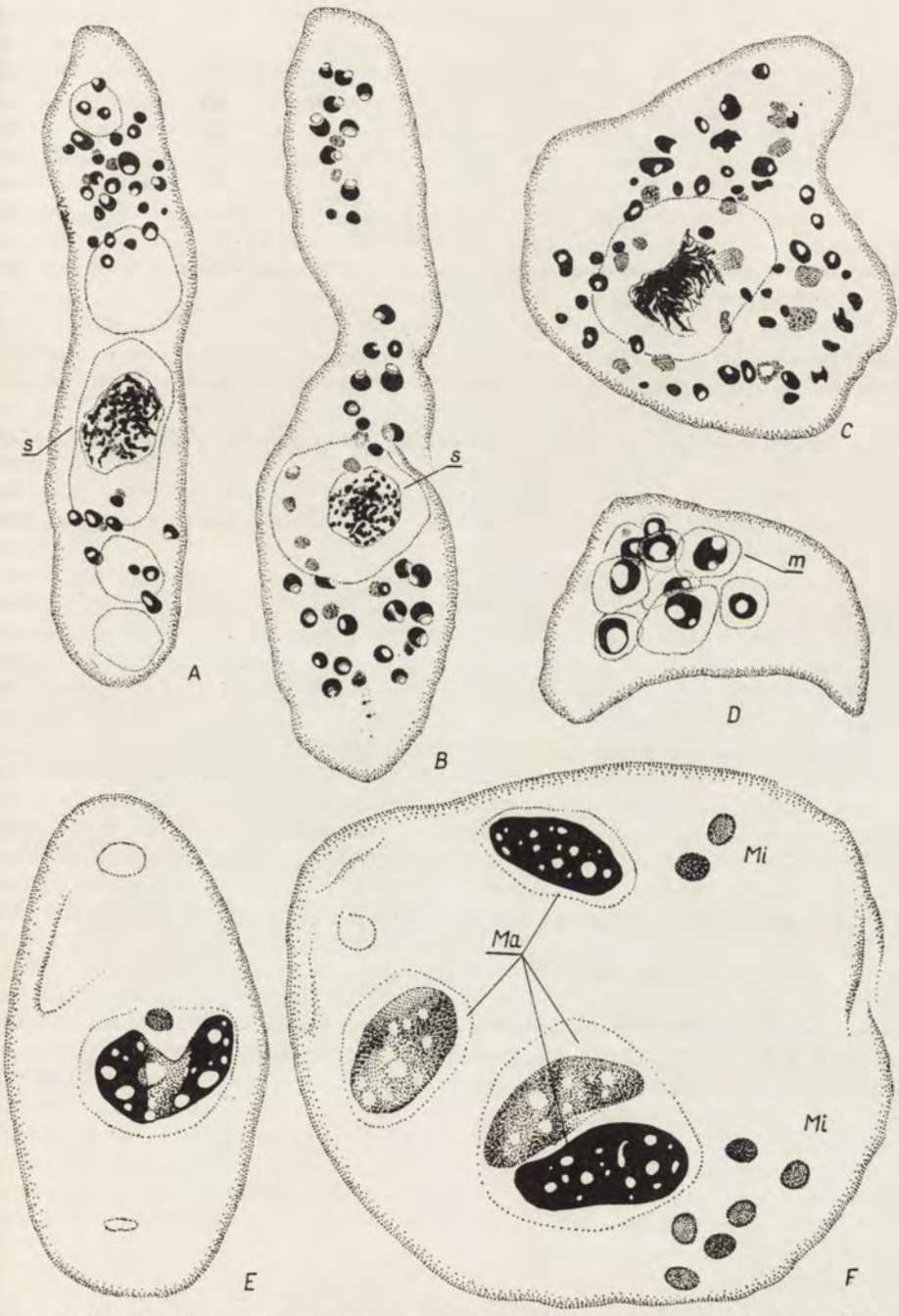

Рис. 7. Морфологические уродства среди эксконъюгантов (A — D) и среди вегетативных особей (F). E. Вегетативная особь (для сравнения с рис. F). Окраска по Фельгену. Рис. апп., об. 60х, ок. 7х (A — D); об. 60х, ок. 10х (E — F). Обозначения те же, что и на Рис. I)

бавлялись в качестве пищи в культуральную среду). Часть мелких клеток имеет только фрагменты Ма (Рис. 5 I, L), другие особи кроме фрагментов старого Ма содержат либо синкарион, либо 1—2 ядерных образования, которые имеют сходство с продуктами делении созревания Ми, находящимися в состоянии рассасывания. Мелкие клетки являются, по-видимому, потомками клеток II категории, у которых, как указывалось выше, кроме синкариона имеются еще 1—2 ядра, не присоединившихся к синкариону и являющихся продуктами делений созревания Ми; в меньшем количестве это потомки клеток III категории. Описанные особенности клеток четвертой категории свидетельствуют об их пониженной жизнеспособности. Из культуры эти клетки исчезают довольно скоро (на 3—4-й день).

При анализе процесса деления облученных эксконъюгантов и их потомков были сбнаружены 4 варианта поведения синкариона и цитоплазмы в процессе деления. К первому варианту мы относим те случаи, когда синкарион, не делясь, остается в одной из дочерних клеток, вторая получает только фрагменты Ма (Рис. 5 B). При втором варианте деления дочерние клетки получают ядра, не вошедшие в состав синкариона (Рис. 5 C); эти ядра являются продуктами делений созревания Ми и у облученных конъюгантов не рассасываются, как это происходит у необлученных. Помимо этих ядер клетка может получить весь неразделившийся синкарион.

Третий вариант деления наблюдался в одном случае; он заключается в том, что в синкарионе образуется лентовидная нуклеола; внешне это ядерное образование напоминает Ма, но форма и размеры нуклеолы, а также распределение хроматинового вещества (беспорядочно рассеянные по ядру скопления хроматиновых глыбок) совершенно нетипичны для Ма, а также и для нормального синкариона. Такой синкарион способен к делению (Рис. 3 C, 5 D). Как указывалось выше, этот синкарион делится амитотически.

Четвертый вариант деления эксконъюгантов включает те случаи, когда клетка не завершает деления и останавливается на какой-то стадии. Эти случаи весьма характерны для действия ионизирующей радиации (Фрац-Ниггли 1961). Как показано на Рис. 5 E, эксконъюгант содержит один синкарион и 2 перистома; уродливая форма тела парамеции является результатом незавершенного деления клетки (Рис. 5 E, Q; см. также Рис. 7 A — D). Случаи незавершенного деления клеток мы встречали и среди облученных вегетативных особей парамеций, которые обычно находятся в культуре среди конъюгирующих форм. Как видно на Рис. 7 F, клетка содержит 4 Ма, 8 Ми и 4 перистома. В литературе описаны случаи нетипичного деления эксконъюгантов парамеции, не подвергавшихся действию ионизующей радиации. По данным Соннеборна (Sonneborn 1954), при 1 делении эксконъюгантов *P. aurelia* в некоторых случаях наблюдается неравномерное распределение ядерного материала между дочерними особями. Нобили (Nobili 1959) наблюдал сходные случаи нарушенного деления эксконъюгантов в некоторых линиях *P. aurelia*. Под влиянием ионизирующей радиации происходят, как мы видели, значительные нарушения процесса деления эксконъюгантов, причем эти нарушения характеризуются как разнообразием, так и массовостью.

Обсуждение

Синкарион, образующийся у облученных конъюгантов, обладает признаками, которые резко отличают его от нормального синкариона. „Облученный" син-

карион представляет собой сложное ядерное тело, состоящее из разных компонентв. Он неспособен к дальнейшим преобразованиям, конечным результатом которых является формирование нового Ми и зачатков новых Ма; большая часть эксконъюгантов, обладающая комплексным синкарионом, погибает. Создается впечатление, что он является основной причиной гибели эксконъюгантов, как в тех случаях, когда клетка при делении освобождается от синкариона, она сохраняет жизнеспособность, дочерняя же клетка, получившая синкарион, погибает.

Угнетающее влияние комплексного синкариона на жизнеспособность эксконъюгантов связано, по-видимому, с нарушением качественной стороны синтеза нуклеопротеидов. Имеются многочисленные данные, указывающие на глубокие изменения метаболизма нуклеопротеидов под влиянием ионизирующей радиации (Mandel and Chambon 1960, Фриц-Иггли 1961, Кузин 1964). Что касается количественной стороны синтеза нуклеопротеидов, то, как указывалось выше, синтез ДНК в синкарионе не прекращается и ядро интенсивно обогащается хроматином. Отмеченные нами аномальные фигуры митозов при делениях созревания Ми у конъюгантов (Ковалева и Янковский 1965), образование комплексных синкарионов, неспособных к дальнейшим преобразованиям, незавершенность клеточного деления, нарушения лихтгрюновой зоны обусловлены, по-видимому, как структурными, так и биохимическими сдвигами; эти сдвиги возникают в клетке под влиянием ионизирующей радиации (Бак и Александер 1963).

В реакциях живых клеток на облучение существуют общие закономерности, вытекающие из общности их структуры и химического состава, и парамеции не стоят особняком в этом отношении. Однако они обладают высокой радиорезистентностью, в основе которой лежат, как мы думаем, некоторые особенности, отличающие их от других клеток как одноклеточных, так и многоклеточных организмов. К ним относится наличие двух ядер — макро- и микронуклеуса. Как следует из наших данных, облучение конъюгирующих особей парамеций нелетальной дозой рентгеновских лучей приводит к необратимым повреждениям структуры и функции Ми, в то время как Ма, являющийся высокополиплоидным ядром, сохраняет способность к репарации. Синтез ДНК и РНК в Ма парамеций не прекращается и после облучения, хотя он и бывает временно угнетен (Ковалева 1963, 1964). У облученных эксконъюгантов уже на ранних стадиях развития репарационных процессов наблюдается увеличение количества и размеров нуклеол в фрагментах старого Ма и картины выхода нуклеол в цитоплазму, что указывает на активное функционирование Ма. Это ядро считается ответсвенным за вегетативные функции клетки парамеции. Эксконъюгант, освободившийся от комплексного синкариона при делении и получивший только фрагменты старого Ма, обнаруживает все признаки восстановления нормальной жизнедеятельности.

Резюме

Облучение конъюгирующих особей парамеций нелетальной дозой рентгеновских лучей (75 кр) приводит к необратимым повреждениям структуры и функции микронуклеуса, в результате чего образуется синкарион, неспособный к дальнейшим преобразованиям.

Часть эксконъюгантов, перенесших облучение, оказывается способной к делению; при этом одна из дочерних особей может освободиться от синкариона

и получить только часть фрагментов старого Ма. У таких особей имеет место регенерация Ма: укрупнение фрагментов, увеличение размеров и числа нуклеол, выход РНК и цитоплазму, уменьшение числа фрагментов при последовательных делениях эксконъюгантов.

Высказывается предположение о том, что под влиянием ионизирующей радиации у конъюгантов возникают патологические изменения (структурные и биохимические) не только в ядерном веществе но и в других клеточных компонентах, на что указывают аномалии при делении эксконъюгантов и нарушения лихтгрюновой зоны.

SUMMARY

Conjugants of *Paramecium putrinum* belonging to two complementary mating types — m. t. I and II of syngen 4, of mixotype A, were irradiated with a single dose 75 kR on the stage of a late prophase of the I Mi division. The nuclear processes in conjugant till the moment of fission have been described in the preceding article (K o v a l e v a i J a n k o w s k i 1965).

Synkaryon presents a complex structure comprising — besides the stationary and migrating nuclei — still another nuclei which failed to degenerate after the II maturation division. As a rule synkaryon does not divide either before or after fission of conjugants; if the cell divides, synkaryon remains in one of the daughter individuals which usually perishes.

Among exconjugants and their offsprings, 4 categories of cells were distinguished according their morphological and physiological properties. The majority of cells are not viable. In the category of viable cells, symptoms of degeneration of Ma appear; subsequently occurs the increase of the old Ma fragments, as well as that of the number and size of nucleoli in those fragments, and reduction of the number of Ma fragments as result of their distribution in division.

The cells of the I category are of large dimensions, a bag-shaped body form, a strong pyroninophilia of cytoplasm, numerous food vacuoles and a big compact synkaryon. They perish before they divide. Cells of the II category approach more than the others to the normal offsprings of non-irradiated exconjugants (control) in respect of the number of their food vacuoles, of result of their RNA test and of condition of their old Ma. Cells of the III category are the offsprings of the II one. They are able to normal feeding, multiplication and synthesis of DNA and RNA in fragments of the old Ma, which show symptoms of regeneration. Cells of the IV category are distinguished by small dimensions, a very faint pyroninophilia of cytoplasm, low mobility and a weak phagocytotic activity. Fragments of their old Ma are small and deprived of regeneration symptoms.

Ability of division appears only in the II and II category. Three division variants may be found which are characterized by different distribution of the nuclear material between the daughter cells.

Death of the majority of exconjugants and their offsprings is in considerable degree involved by presence of synkaryon. The depressing action of synkaryon is associated with its structure and — evidently — with impairing the synthesis of nucleoproteids. The irradiation with the dose — as indicated above — applied to conjugants of *P. putrinum*, evokes an irreversible injury of the structure and function of Mi whereas Ma being a highly polyploidal nucleus, keeps its ability of regeneration.

EXPLANATION OF TEXT-FIGURES 1—7

Fig. 1. Morphology of the nuclear apparatus in exconjugants (conjugants have been irradiated in prophase of the I division of micronucleus maturation). Feulgen staining; camera lucida drawing, magnification: lens 90×, eye piece 20×. Lettering: m — fragment of old macronucleus, s — synkaryon, different number of synkaryon components are seen, n — nuclei which failed to join synkaryon

Fig. 2. Pattern of changes in synkarion structure in exconjugants; Feulgen staining; camera lucida drawing, magn. 60×20. A. Next stage of changes in chromosomes; fused chromatin clumps are seen, fragments of old Ma are small. B. Near synkaryon, 2 nuclei — products of the II division of Mi maturation are present. C. Synkaryon dimensions augmented; in fragments of old Ma the number of nucleoli increased; arrow indicates the light green zone. D. 2 nuclei with increased chromatin clumps are seen; those nuclei arose as result of fusion of pronuclei with the non-degenerated nuclei. Number of fragments of old Ma decreased

Fig. 3. Subsequent changes of synkaryon and one of cases of its division in exconjugants. A, B, D — Feulgen staining, magn. 90×15. C — Brachet method, magn. 60×20. A. Light green zone reaches its maximal dimensions (36 μ). B. Synkaryon is a compact body with big chromatin clumps. C. Division of synkaryon; nucleoli stained black. On one side of nucleus, a ribbon-shaped nucleolus is seen. D. Changes in the old Ma are seen: adhesion of chromatin grains and formation of cavities in the fragments. Marking as in the Fig. 1; arrows indicate the light green zone

Fig. 4. Vegetative individual and the exconjugants categories. Staining after Brachet, schematized. Lettering: 1 — fragment of the old macronucleus with nucleoli; 2 — cytoplasmic RNA; 3 — yeast cells in food vacuoles; 4 — outline of synkaryon. A. Vegetative individual. B—E. exconjugants categories. B. Small light cell (4th category). C. General view of exconjugant cell (3rd category). D. General view of exconjugant cell (2nd category). E. General view of exconjugant cell (1st category)

Fig. 5. Divergence of offspings of irradiated partners of *P. putrinum*. 1 — category of big cells; 2 — variants of division of exconjugants; 3 — cell category with regenerating macronucleus; 4 — category of small cells. Fixed preparations. Camera lucida drawing, magn. 40×7. A. Cell of the 2nd category; synkarion, non-degenerated nucleus and fragments of Ma are seen. B. I variant of division. C. II variant of division. D. III variant of division. E. IV variant of division (non completed division). F—H. Change of characters of the big cell — abnormal body shape, vacuolization of cytoplasm. I—L. Variety of cells of the 4th category. M—P. Consecutive stages of regeneration of Ma. R—S. Conjugation of viable individuals

Fig. 6. Pattern of macronucleus regeneration. Feulgen staining, camera lucida drawing, magnif. 60×15 (A—C); 60×10 (D). A—C. Increase of size of the macronucleus fragments. D. Conjugation of the 3rd category cell and of vegetative individual

Fig. 7. Morphological monsters among exconjugants (A—D) and among vegetative individuals (F). E. Vegetative individual (for comparison with F). Feulgen staining. Camera lucida drawing, magn. 60×7 (A—D); 60×10 (E—F). Lettering — same as in Fig. 1

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Regeneration of anuclear fragments in *Dileptus cygnus* Clap. et Lachm.

Regeneracja fragmentów bezjądrowych u *Dileptus cygnus* Clap. et Lachm.

The regeneration of anuclear fragments in ciliates has been reported in a number of publications. They concerned mostly the action of special conditions on this process e.g. raised temperature (Prowazek 1904, Sokoloff 1924) or regeneration of cellular organelles which are not concerned with oral structures. Examples of such a process is the regeneration of contractile vacuoles in *Stentor* (Balbiani 1893, Schwartz 1935) and in *Amoeba* (Höfer 1890) or regeneration of holdfast in *Stentor* (Tartar 1956). Nearly all the authors studying the regeneration in ciliates have reported the capability of the anuclear fragments to the form regulation (Dembowska 1924, Sokoloff 1924, Yagiu 1951).

The remarkable and most convincing case of regeneration of unicellular organism in absence of nucleus is the regeneration of the cap in *Acetabularia mediterranea* discovered by Hämmerling 1934.

In ciliates, Weisz 1942, Tartar 1956, de Terra 1960 reported the growth possibility of buccal rudiments in absence of Ma, as well in division as in regeneration, in *Stentor*. Tartar 1956 proved that only the rudiments of advanced stages of the division cycle, after completion of Ma condensation, are endowed with this capability. This fact suggests that the stimulus to the formation of rudiments or even their separate elements, came from the nuclear apparatus which is a moniliform Ma, similarly as in the interphase. Moreover, the regeneration process in the forms with a chain of Ma segments — as in *Stentor, Blepharisma, Spirostomum* may lead to condensation of Ma after formation of the rudiments. On the other hand, the time necessary for regeneration is in different ciliates — as *Stentor* (Tartar 1961), *Urostyla* (Jerka-Dziadosz 1963) more prolonged than the time of the division process. This seems to prove the existence of a stimulus coming from cytoplasm for excitation of the morphogenetic activity of the nuclear apparatus.

The review of the study on regeneration in ciliates (Balamuth 1940) indicates that in a great majority of cases the presence of the nuclear apparatus is a condition of regeneration of the oral structures. An exception present the findings of Sokoloff 1924 who stated that in *Bursaria truncatella* (cultivated in raised temperature) and in *Dileptus anser* (in normal conditions) a partial regeneration of anuclear fragments occurs. As follows from the drawings of this author, the form studied was rather *Dileptus monilatus* Stokes i.e. a form with a moniliform Ma. The fragments studied were very small, and

the details of the structure of nuclear apparatus are not mentioned. In consequence, it can scarcely be stated whether the described case was really regeneration.

The study of the problem whether the pre ence of the nuclear apparatus is really indispensable for occurrence of regeneration processes in *Dileptus cygnus* seems to be of special interest on account of the specific structure of this ciliate and its manner of feeding. The very long and thin proboscis of this animal may be injured very easily either by the changes in the chemical composition of the medium or by mechanical factors e.g. breaking off in attacking a too big prey (observations in our laboratory). The way of killing the prey requires a great number of trichocysts which involves their permanent regeneration. It is now commonly accepted that regeneration of species specific cellular structures is controlled by the nuclear apparatus (review in Brachet 1961). In favour of this view seems to speak the fact that the related species as *D. anser, D. visscheri, D. cygnus* kill themselves mutually by means of trichocysts whereas this phenomenon fails to occur within the species. Necessity of a permanent regeneration of trichocysts when feeding, suggests that the substances indispensable for regeneration and deriving from the nucleus should be in *Dileptus* permanently present in cytoplasm. The aim of the present study is to prove this possibility.

Material and methods

The cultures of *D. cygnus* were fed everyday with mixed cultures of *Tetrahymena pyriformis* Ehrbg. and *Colpidium colpoda* Ehrbg. Ciliates applied as food were fed on suspension of dried yolk. As culture medium, the Pringsheim's fluid was used.

Fixed preparations were executed for controling the presence of the nuclear apparatus (stained after Feulgen's method or with methyl green) or of the buccal organelles. The ciliary apparatus was visualized by staining with haematoxylin following the method of Párducz or with nigrosine. Nigrosine staining was executed precipitating the stain by drying or by simultaneous fixation and staining. This latter rapid method proved to be especially useful for preparation of single individuals. The fixation fluid contained 50 ml of saturated sublimate and 50 ml of 0.2% aqueous solution of nigrosine. The ciliate with a slight quantity of water was placed into approx. 1 ml of fluid, rinsed in distilled water after 3 min., and stained with methyl green, then dehydrated beginning with the 96% alcohol. The simultaneous control of ciliary structures and of presence of the nuclear apparatus was especially helpful in this study.

Operations were carried out free-hand with a steel needle, under a microscope magnification of 100 times. Ciliates were placed on a concave slide in a small flat drop covered by a layer of paraffin oil. The amount of water in the drop was regulated with a pipette so that the ciliate was slightly pressed to the slide in course of operation which faciliated the exact observation of the nuclear apparatus. The medium of the culture was used during operation and was added after operation, for avoiding the shock after the change.

The majority of operations performed consisted in the cross section followed by destruction of the promer; then the regeneration of the mouth of the opimer was observed. As regeneration criterion formation of the cytopharyngeal complex and of proboscis armament — feeding cilia and trichocysts — was accepted.

Results

Comparison of division and regeneration time

The time of division duration was measured in 30 individuals. The time given between the I and II stage was the longest of all the determined, all the other periods represent the mean of the cases studied. Similarly was measured the duration time of separate regeneration phases in 30 individuals. The ciliate was always cut in the middle line of its body and the opimer was examined.

The stages concerning as well division as regeneration have been established in the preceding article (Golińska and Doroszewski 1964). It should be reminded that the I stage is the appearence of the rudiment of proboscis armature, the II — formation of the rudiment of cytopharyngeal complex and beginning of elongation of the proboscis rudiment with simultaneous onset of Ma condensation (Golińska 1965). The III stage is fusion of both rudiments, the dividing Ma is condensed, all the parts of the oral apparatus are really formed. In the IV stage, the new proboscis is formed as a process of the anterior body part. In regeneration this stage is not clearly perceptible but in division at this stage, occurs the fission of individuals. After fission follows the stage of functional maturation of oral apparatus.

The results of measurements of the duration time of separate stages of division and regeneration are presented in Fig. 1. In analysis of results, a striking fact is the reduction of the period between the stage I and II in regeneration when compared with the same time in division. It should be accepted that the full length of this period in division has not been determined, consequently the difference is rather more considerable. Nevertheless the regeneration process "catches up" in time the division. The blackened segments at the ends of diagrams indicate the onset of functioning of the oral apparatus, the capability as well of killing as of engulfing the prey.

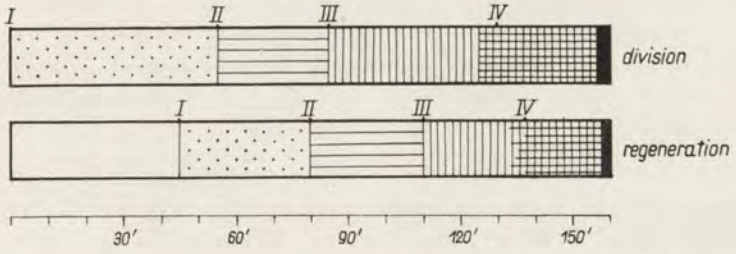

Fig. 1. Comparison of division and regeneration rate in *D. cygnus*. I—IV development stages of oral rudiments

It seems to follow from the comparison of rate of both processes that *Dileptus cygnus* is always ready for regeneration and a preparatory phase fails to exist. Such a phase is obligatory in *Urostyla* and in *Stentor* and amounts 3—4 hrs till the moment of appearence of rudiments. The simultaneous prolongation of the first stage of division, when compared with regeneration, suggests the possibility that *D. cygnus* may be able to regenerate during the division process as well.

Regeneration in course of division

In all the cases, the same operation was executed: transverse section of the proter beneath the cone of the pharyngeal trichites. Only those cases were considered when Ma has not been injured. Operations were performed on ciliates at different stages of division. Observations were carried out mostly up till the moment of fission of daughter individuals. After fission they were stained with haematoxylin or nigrosine. At every division stage, 10 individuals were operated, the results are presented in Fig. 2. It seems to follow from the above data that the potency of regeneration is preserved in all the division stages, the earliest stages however, as the least perceptible, may be an exception. Nevertheless, individuals operated at the earliest stages of division separate in nearly equivalent phases of development of the buccal apparatus in the proter and opisthe. Stages of development of the newly formed mouth of proter in the moment of fission of the individuals, seem to depend only on the length of the period after operation (Pl. I). It was proved in several cases that the regenerated proter undergoes the Ma segmentation in the time which is normal for division. The subsequent regeneration stages and the functional maturation of the buccal apparatus occur in the time which is normal for regeneration.

Consequently, in the case of *D. cygnus*, the process of regeneration of the buccal apparatus in proter is fully independent of the division processes which occur simultaneously in the nuclear apparatus. The further investigation concerned the problem whether the regeneration processes may occur in the anuclear fragment as well.

Regeneration of the anuclear fragments

By means of operation 344 anuclear fragments were obtained. Out of this number 309 were opimers and 35 promers. The anuclear fragments originated of different stages of the life cycle of *D. cygnus*, as well of interphase as of different division stages. The process of regeneration of the buccal apparatus occurred in 260 out of 309 opimers studied i.e. in approx. 84%. Investigation was carried out on: degree of regeneration of the buccal apparatus, dependence of the regeneration process on the size of the fragment and on its origin, and the life length of different anuclear fragments.

Degree of regeneration

Regeneration of anuclear fragments lasted in average 2 hrs.; after this period any signs of further development of the rudiments failed to manifest. The separate regeneration stages of the buccal apparatus correspond to separate stages of regeneration of normal fragments. So the rudiment of the proboscis armature arises in average 40 min. after operation, the rudiment of the cytopharyngeal complex — 1 h. 10 min. and fusion of both rudiments occurs 1 h. 45 min. after operation. The development stops when nearly the IV stage has been reached. The anuclear fragments observed never reached the stage of functional maturation of the buccal apparatus; killing the prey or taking food was not observed either. In the fixed material it is seen (Pl. II) that in the proboscis armature, as well trichocysts as feeding cilia regenerate. The number of trichocysts is low, similarly as in the opisthe of the IV division stage. The cytopharyngeal complex is also only slightly marked, the cones of trichites are

scarcely visible, there is no distinct cytostome or pharynx. Pl. II 10—11 show that in the anuclear fragment, the mouth is marked but not well developed 3 hrs. after operation, which is especially striking when compared with the fragment of the same age containing the nucleus.

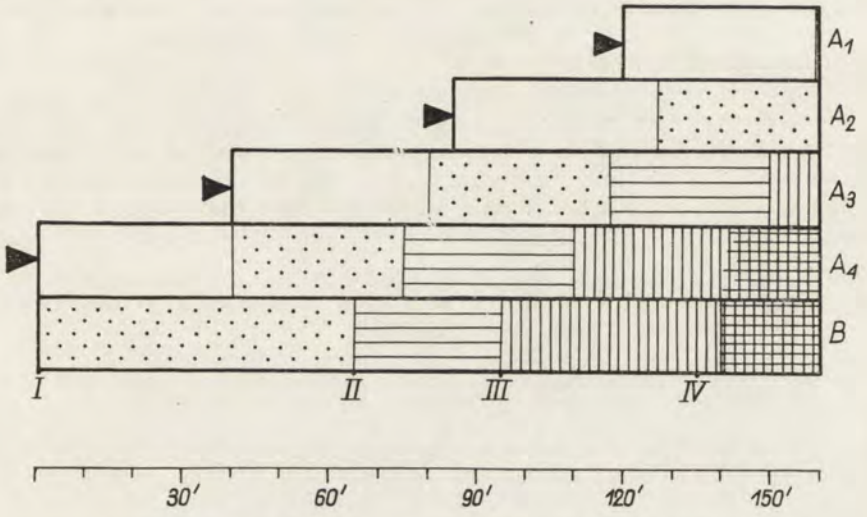

Fig. 2. Regeneration of mouth in proter in course of division. A_1—A_4. Stages of development of the regenerating mouth in proter, sections at different division stages. B. Course of development of oral rudiments in opisthe

The above experiments indicate that the initial regeneration phases seem to occur normally, however after 2 hrs. the development is hampered.

Mouth regeneration and the size of fragment.

It was noticet that the regeneration capability of the anuclear fragment depends on its size. Big fragments regenerate in most cases. The size of fragments was evaluated by the number of contractile vacuoles remaining in them. A row of contractile vacuoles runs along the dorsal body side. Although their number is in certain limits variable in *D. cygnus,* however no other reliable criterion for determining the size of the fragment was found. Its absolute length is elusive because of the great variation in size of individuals in cultures. Calculation of the size of the fragment related to the ciliate body becomes also hard sinc fragments are taken from dividing individuals. Another difficulty in tracing an exact limit between the regenerating and nonregenerating fragments is the fact that outflow of cytoplasm often occurs in operation.

In measurements, no regeneration gradient was found which would correspond to the size of the fragment: simply the fragment of sufficient size regenerates whereas in small fragments no signs of regeneration were stated. This critical size coincides with the number of 2 or 3 contractile vacuoles. Fragments with less than 3 vacuoles regenerated in 23.8% whereas those containing 3 or more vacuoles regenerated in 93.6%.

Regeneration of anuclear fragments of different stages of the life cycle

In subsequent experiments it was proved whether the frequency off regeneration in the anuclear fragments is associated with the phase of the life cycle of the individual from which the fragment derives. It was stated however that fragments comprising 3 or more contractile vacuoles regenerated in a high percentage independently of their origine of interphase or of different phases of division. Big fragments of division individuals regenerated in 94.1% and those of interphase in 93.6%.

The time of regeneration of anuclear fragments related to the stages of their life cycle was also measured, however distinct differences were not found either. So e. g. the time of regeneration (mean of 20 cases) of interphase fragments amounts 2 hrs. 7 min., and of that from the III division stage (Ma condensed) was found to be 2 hrs. 7 min. which is within the limits of the observation error.

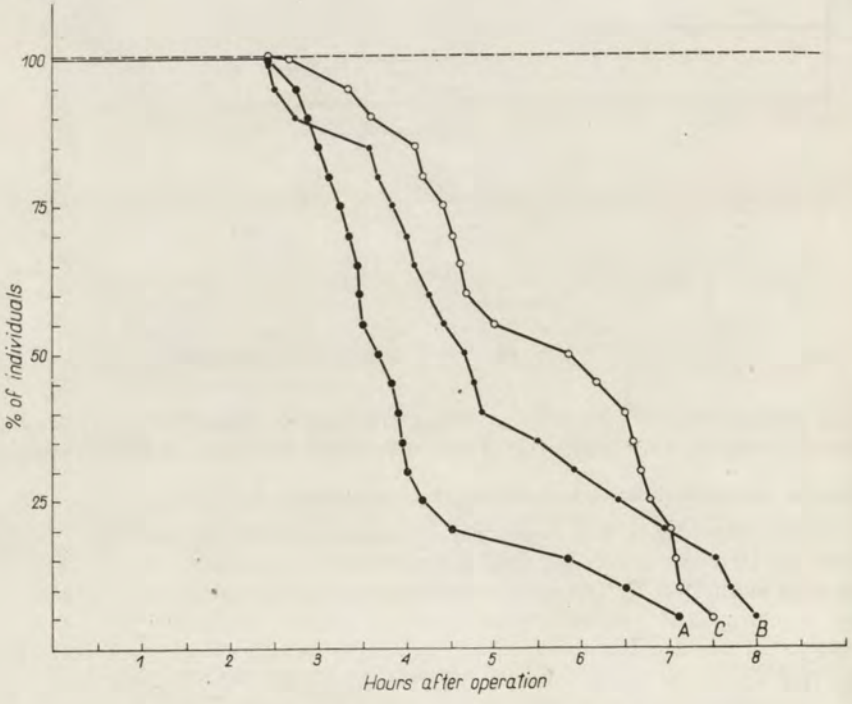

Fig. 3. Life length of different anuclear fragments

Life length of different anuclear fragments

Since a difference was found in the frequency of regeneration in the anuclear fragments of different size, measurements of life length of small non-regenerating fragments were carried out as well as of big opimers which regenerated. Simultaneously the life length of promers was measured in which regeneration — if it may be called so — is limited to formation of tail. The results are represented in the Fig. 3; the curve is a mean obtained of 20 cases.

The high rate at which the anuclear fragments perish is striking. Comparing with other protozoa as *Stentor* or *Amoeba* in which the anuclear fragments live several days or even as long as the normal starving individuals — this span of time is very short being limited to 9 hrs. only. The control fragments of *D. cygnus*, containing the nucleus, lived under the same conditions 3—4 days and then perished probably from starvation. No essential differences were stated in the life length of the regenerating and non-regenerating anuclear opimers. Anuclear promers live for a similarly short time as well.

Another surprising point in those measurements is that the curve of surviving fragments of all sorts declines already after 2 hrs. It should be reminded that at the same time the regeneration of the oral apparatus stops in big opimers. Before elapse of 2 hrs. however, the anuclear fragments did not perish even in separate cases. Differences in the life length of anuclear fragments may also depend e. g. on relative quantity of reserve material but this was not proved.

Discussion

In first place a question arises: at what degree the regeneration of the anuclear fragments in *Dileptus cygnus* is a real one. No doubt, new structures arise — essentially all the elements of the oral apparatus. Nevertheless the restitution of their proper function fails to occur. It is not known either whether other functions of the regenerated proboscis are checked also, whether it acts e. g. as a receptor of external stimuli (Doroszewski 1961).

Dileptus is essentially different from other ciliates in two respects. Those are: the unusual rate of regeneration process and its independance of the physiological condition of Ma. The experiments proved lack of a preliminary period in regeneration (when compared with division), and the possibility of regeneration of the oral apparatus with a simultaneous normal functioning of the division mechanism. It follows from the above facts that regeneration should be logically expected in anuclear fragments as well, and this was actually stated by experiments. Those fact support also the postulation that the substances which are responsible for morphogenesis exist permanently in the cytoplasm of *D. cygnus*. The prolongation of the initial division phases may also speak in favour of the view that those are substances originating from the nuclear apparatus, and that this initial period is the time of supplementation of their deficiency in cytoplasm caused by formation of the division rudiments. In this period, in the condensing Ma, big nucleoli arise in the border segments (Golińska 1965) which content presumably pass into cytoplasm. Consequently, the first division stages in *D. cygnus* would be the only phase reminding the preliminary stage in regeneration of other ciliates as *Stentor* or *Urostyla*. In this regard, especially interesting is the research of Ishikawa 1913 who obtained the regeneration of anuclear fragments of *Stylonychia* which, after a deep incision, were left linked by a cytoplasmic bridge with the remaining body part of the ciliate containing the nucleus. This problem requires — no doubt — further study. As well a study of reiterated regeneration in *Dileptus cygnus* seems to be of a special interest.

D. cygnus may be an especially convenient object for cytochemical investigation. Presently the central object for the study of substances responsible for cell regeneration is *Acetabularia*. Possibly in the case of *Dileptus* the study

would be less complicated on account of the absence of chloroplast which in *Acetabularia* is the center of synthesis of RNA as well as of many enzymes.

Some remote analogies may be found in the works of Danielli. This author found in his study on interspecies transplantation of nucleus in *Amoeba* (Danielli 1955) that the serological test is always characteristic of this species from which the nucleus originates. He stated in his theoretical consideration (Danielli 1959) that: "Nuclear controll is manifest at the macromolecular level, whereas cytoplasmic control is manifest at a higher organizational level, that of multimacromolecular structures". Perhaps the reaction of outshot of trichocysts in *Dileptus* may be compared to the serological test. This phenomenon occurs — as mentioned in the introduction — when the ciliate encounters an individual of another species even within the genus *Dileptus*. It should be reminded that killing the anuclear fragments by twin fragments containing the nucleus, was observed several times after elapse of 2 hrs. since operation. This might prove the nuclear origin of substances which are responsible for regeneration of trichocysts, permanently present and supplemented in cytoplasm. Simultaneously the cytoplasmic control found for the structures on the macromolecular level seems to hold true for morphological structures in the case of *D. cygnus*.

Following conclusions may be drawn from the studies presented in this article:

1. Substances of nuclear origin perform a role of stimulus for regeneration, they are permanently present and supplemented in cytoplasm in *Dileptus cygnus*.
2. Organization of oral structures may occur in absence of the nuclear apparatus.

Summary

The initial stages of regeneration in *Dileptus cygnus* last shorter than the corresponding period in division. The regeneration processes may occur simultaneously with the division processes and are independent of division changes in the nuclear apparatus.

Regeneration of anuclear fragments was stated. Appearence and development of oral rudiments was ascertained as well as a subsequent inhibition of this process after formation of all the structural elements of the cytopharyngeal complex and of proboscis. Anuclear fragments however live very shortly irrespectivley of the occurrence of the regeneration process.

The experiments indicate the possibility of organization of the oral structures in absence of the nuclear apparatus and possibly of permanent presence of morphogenetic substances in the cytoplasm of *Dileptus cygnus*.

STRESZCZENIE

Początkowe stadia regeneracji *Dileptus cygnus* są krótsze od odpowiadającego im okresu w podziale. Procesy regeneracyjne mogą występować jednocześnie z procesami podziałowymi i są niezależne od zmian podziałowych w aparacie jądrowym.

Wykryto regenerację fragmentów bezjądrowych. Stwierdzono powstawanie i rozwój zawiązków oralnych, a następnie zahamowanie tego procesu po utwo-

rzeniu w zasadzie wszystkich elementów strukturalnych kompleksu cytofaryngealnego i proboscis. Fragmenty bezjądrowe żyją jednak bardzo krótko, niezależnie od tego czy wystąpił proces regeneracji.

Doświadczenia wskazują na możliwość organizowania struktur oralnych w nieobecności aparatu jądrowego, a więc prawdopodobnie na stałą obecność substancji morfogenetycznych w cytoplazmie *Dileptus cygnus*.

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EXPLANATION OF PLATES I—III

Regeneration of mouth in proter in course of division

1: Separation of individuals operated between the III and IV stage of division; living material
2: Separation of individuals operated between the II and IV stage of division; living material
3: Separation of individuals operated between the I and II stage of division; living material
4: Separation of individuals operated at the I stage of division; living material
5: Stage immediately after division, the formed rudiment of proboscis with feeding cilia and the not yet segmented Ma are seen; stained after the method of Párducz, and methyl green.

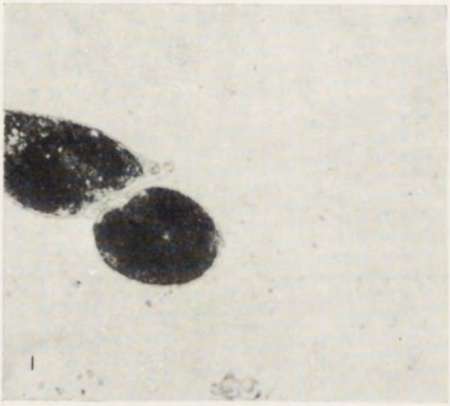
Regeneration of anuclear fragments; nigrosine + methyl green staining

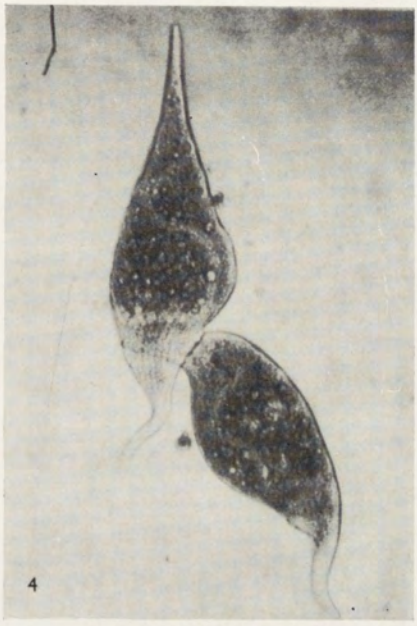
6: Anuclear fragment 1 hr. after operation. The rudiment of new proboscis is seen
7: Same 1.5 hr. later. Both rudiments are seen
8—9: Same after 3 hrs. Armature of proboscis is seen
10: Cytopharyngeal complex 3 hrs. after operation in a fragment containing the nuclear apparatus
11: Cytopharyngeal complex 3 hrs. after operation in an anuclear fragment

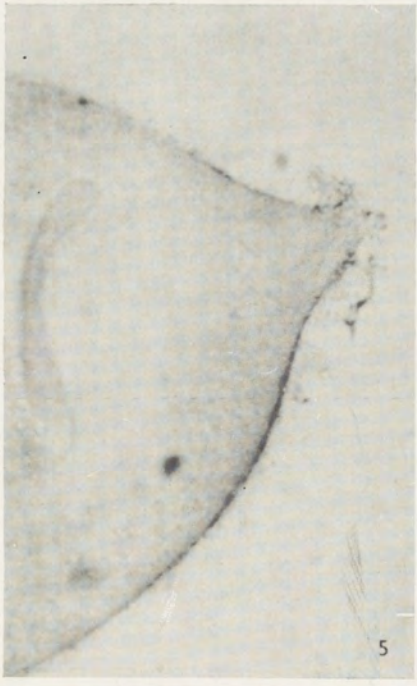
Regeneration of anuclear fragments; living individuals

12: I stage of regeneration
13: II stage of regeneration; rudiment of the cytopharyngeal complex and the advanced rudiment of proboscis are seen
14: III stage of regeneration; both rudiments are fused together
15: Beginning of the IV stage of regeneration
16: IV stage of regeneration; anuclear fragment is cut of an individual at the III stage of division; condensed Ma is seen

[Abbreviations: c — cytopharyngeal complex, p — proboscis, f — feeding cilia, t — trichocysts, Ma — macronucleus]







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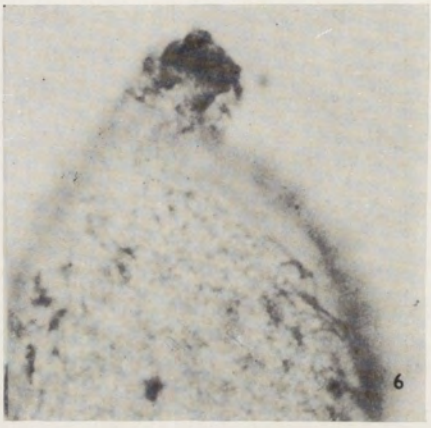

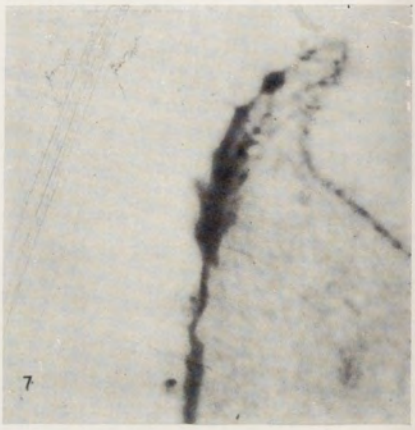

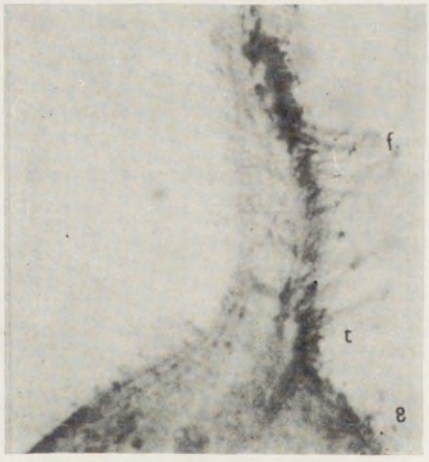

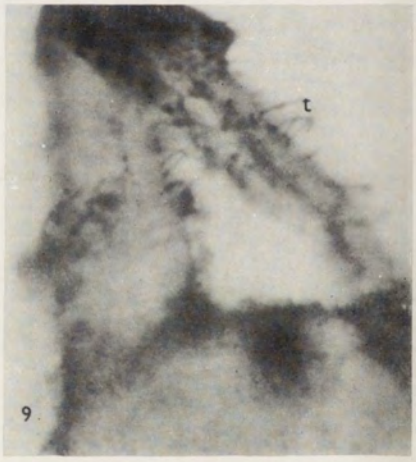

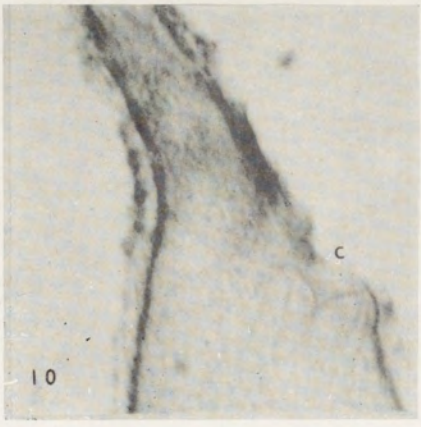

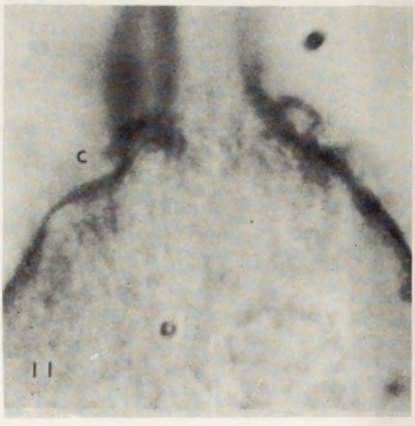

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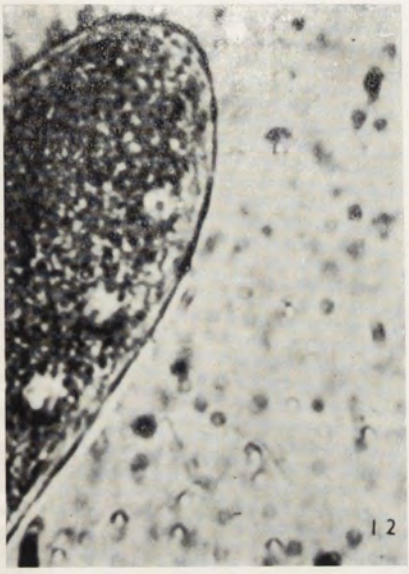

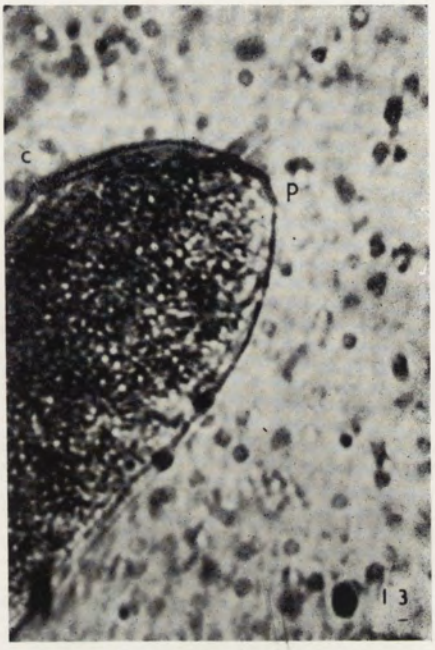

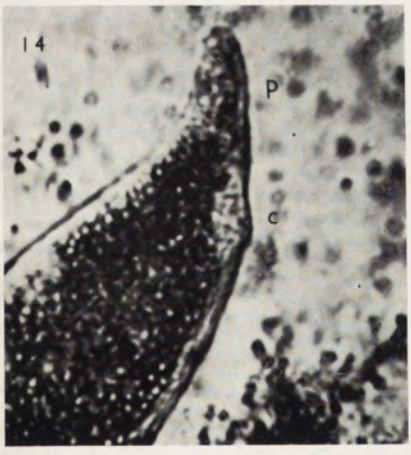

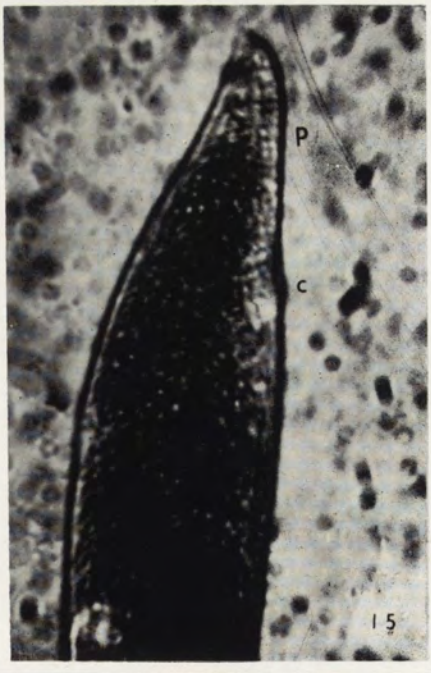

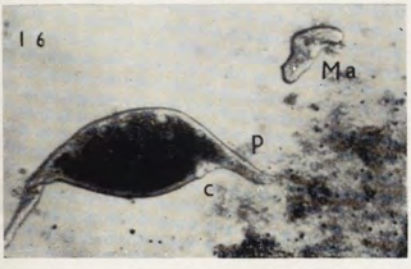

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Changes of the DNA content in macro- and micronucleus of *Paramecium putrinum* in the interdivision phase

Изменение содержания ДНК в макро- и микронуклеусе *Paramecium putrinum* в период от деления до деления

In ciliates Ma is usually many times more voluminous than Mi and — as follows from the study of Grell 1950, 1953, Fauré-Fremiet 1953 and others — is a polyploidal nucleus. Ma plays not only the main role in the vegetative life of the ciliate but is endowed with a considerable genetical activity (Sonneborn 1947). Mi are inert in the vegetative period, their activity manifests only in the sexual process — in conjugation or autogamy (Poljansky i Raikov 1960). The function and interdependence of those two nuclei is as yet not clear in many respects.

A comparative study of the DNA content in both nuclei during the whole development cycle of the ciliate is of a considerable importance in this line. Such investigations carried out by some authors proved that the DNA synthesis in Ma and Mi is not a synchronized process. In *Chilodonella uncinata*, DNA synthesis in Mi occurs earlier than in Ma (Seshachar 1950), whereas in *Euplotes eurystomus* and in *Tetrahymena pyriformis* it initiates already during division of Ma when it starts the DNA synthesis in the beginning of interphase (Prescott et al. 1962, McDonald 1960, 1962). In a mass culture of *P. caudatum* DNA synthesis in Mi occurs generally later than in Ma (Walker and Mitchison 1957). The investigations of DNA content in Ma and Mi of different species of *Paramecium* executed by Cheissin and oth. proved also that in the majority of individuals, DNA synthesis in Mi occurs later than in Ma. Nevertheless, a strict constancy of beginning of synthesis has not been found. So in 30% of ciliates studied, DNA synthesis in Mi initiates earlier than in Ma (Cheissin and Ovchinnikova 1964).

The beginning of DNA synthesis in Ma and Mi may coincide in time as found by Woodard et al. 1961 in *Paramecium aurelia*. The same has been stated by Cheissin et al. 1963 in the study of DNA synthesis in *Paramecium caudatum*.

Those evidences are surely not sufficient for establishing any regularity in the interdependence of Ma and Mi. Besides, in different laboratories, studies are carried out at different conditions of the organisms culture which complicates the comparison of results obtained by the research workers.

The present study is a microspectrophotometrical analysis of the time of DNA synthesis in Ma and in Mi of *P. putrinum*.

I wish to express my gratitude to Professor E. M. Cheissin for his permanent attention and help in this work.

Material and methods

The study was executed on a non-synchronized clone M-12 of *P. putrinum* kindly provided by A. W. Jankowski.

Ciliates were kept in Petri dishes in the medium of Losina-Losinsky 1931 at the temperature 22°C. As nutrient, yeast was applied. The mean time of interdivision amounted 15—15.5 hrs.

Material was prepared as follows: individuals in stage of division were placed in a separate microaquarium. After division, every daughter-individual was placed after a definite period of time on a quartz slide and fixed. As fixation fluid, absolute alcohol and acetic acid (9:1) was used.

RNA was eliminated by means of ribonuclease (conc. 1 mg/ml) at the temperature 37°C during 6—7 hrs.

Determination of DNA content in Ma and Mi was executed by photographic method with ultraviolet light microscope MUF-6 at the wave-length 265 mμ, followed by photometry of negatives. The quantity of DNA was calculated by the equation $Q = D \times S$, where Q — quantity of substance, D — optical density, and S — area of the nucleus $\times 10^{-5}$ cm² (Brodskij 1956, Cheissin et al. 1963, Raikov et al. 1963, Cheissin et al. 1964). In the text below, data of the content of DNA are given in arbitarary units. Coefficient 10^{-5} is omitted.

Results

Results of determination of DNA content in Ma and Mi of *P. putrinum* in interphase are presented in the Table 1 and shown in Figs. 1 and 2.

Table 1

Changes of DNA content in Ma and Mi of *Paramecium putrinum* in interphase

Hours		0	2	4	6	8	10	12	14	15	15—15,5	
											*	**
Q_{Ma}	range	0.09 0.18	0.09 0.16	0.10 0.19	0.09 0.19	0.09 0.17	0.12 0.20	0.11 0.21	0.16 0.39	0.15 0.36	0.20 0.36	0.20 0.35
	mean.	0.13	0.13	0.13	0.13	0.12	0.16	0.16	0.22	0.25	0.26	0.26
	n	33	31	30	35	31	30	29	30	33	24	28
Q_{Mi}	range	0.009 0.013	0.008 0.012	0.008 0.013	0.007 0.012	0.007 0.013	0.008 0.013	0.009 0.016	0.011 0.030	0.014 0.027	0.008 0.013	0.007 0.012
	mean.	0.011	0.010	0.010	0.011	0.010	0.011	0.014	0.020	0.019	0.010	0.010
	n	14	15	15	16	16	17	16	14	18	12	18
Q_{Ma}/Q_{Mi}		12	13	13	12	12	14	11	11	13	26	26

* Full fission of the paternal Ma into two daughter ones is not yet accomplished.

** Fission of the paternal Ma into two daughter ones is accomplished but the daughter individuals remain linked by a cytoplasmic connection.

As follows from the Table 1 and Fig. 1, quantity of DNA in Ma of the just divided ciliates amounts 0.13. This value remains at the same level up till the 10th hour when a slight rise of DNA content up to 0.16 occurs. This rise remains for some time (about 2 hrs.), and is followed by a rapid increase of DNA content till the very beginning of Ma division. Presumably the DNA synthesis in time of its division fails to occur or is very insignificant since the values of DNA content at the 15th hour in the groups A and B (Table 1) remain nearly at the same level.

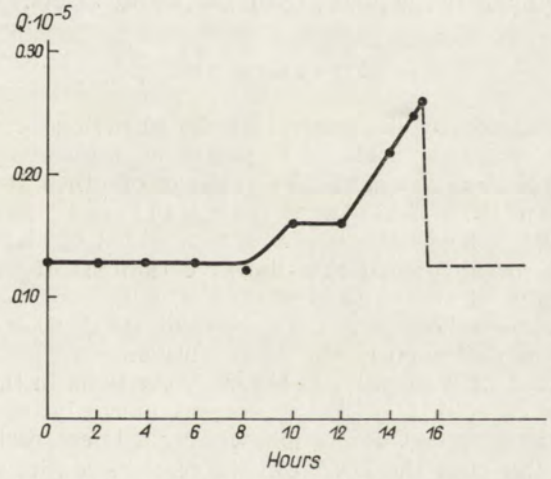

Fig. 1. DNA content in Ma of *Paramecium putrinum* in the interphase period

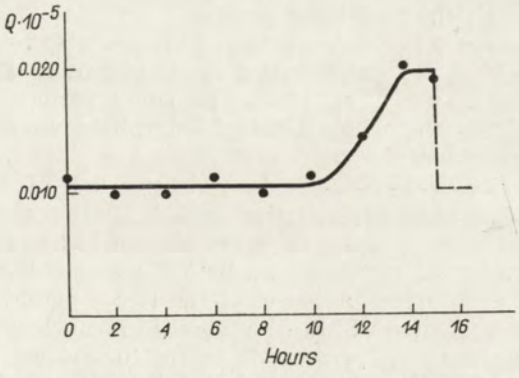

Fig. 2. DNA content in Mi of *Paramecium putrinum* in the interphase period (divison of Mi occurs immediately before division of Ma)

The mean DNA content in Mi (Table 1, Fig. 2) in ciliates immediately after division is 0.011. This remains nearly unaltered till the 12th hour. Subsequently a rapid duplication of DNA in Mi occurs which concludes approx. 1 hour prior

the division. Division of Mi occurs very quickly immediately before the division of Ma.

Measurements proved that the ratio of DNA content in two daughter Ma (Q_1/Q_2) amounts in average 1.2 but in single individuals a more irregular distribution of DNA is manifested. In this case, the ratio reaches the value up to 1.4 or even 1.5.

The ratio of DNA content in pre-synthetic Ma to pre-synthetic Mi, and of post-synthetic Ma to post-synthetic Mi are in average 12 and 13 respectively.

Although in *P. putrinum* the interdivision period lasts 15.5 hrs. at t = 22°C, in separate individuals it fluctuates from 14.5 up to 16 hrs.

Discussion

Our results obtained by the method of the ultraviolet cytophotometry indicate that DNA synthesis in Ma of *P. putrinum* initiates at the second half of interphase. This result supports the findings of other authors (Walker and Mitchison 1957, Gall 1959, Kimball and Barka 1959, Woodard et al. 1961, Cheissin et al. 1963) provided by the study of various ciliates. It should be mentioned that the investigations of those authors — as well as the results of Kimball and Perdue 1962 who revealed the unevenness of DNA synthesis in *P. aurelia* — indicate that DNA synthesis when initiated, proceedes continuously till its conclusion.

Determination of DNA quantity in Ma of *P. putrinum* in the period between two divisions indicates that synthesis proceeds unevenly: after about 10 hrs. a slight rise of DNA content occurs followed by a latent period prolonged for about 2 hrs. At this time the DNA content remains at the same level. After conclusion of this period of "rest" an intense DNA synthesis follows, which is prolonged up till the very onset of Ma division. Initially the DNA synthesis takes place possibly in a small number of genoms which is followed by a corresponding number of endomitoses (the latent period) and only then DNA synthesis occurs in all the remaining genoms.

The lack of continuity in DNA synthesis has been also revealed in the study of changes in the DNA content between two divisions in *Spirostomum ambiguum* (Ovchinnikova et al. 1964). The latent period in this ciliate lasts about 20 hrs. whereas the whole time of interphase amounts about 72 hrs. However if in *P. putrinum* the DNA synthesis takes place in very few genoms prior the latent period, so in *S. ambiguum* about a half of the DNA content is synthesized before the onset of the latent period. Synthesis of this half content of DNA proceeds at a very slow rate. This delay of DNA synthesis is followed by its intense synthesis in the remaining half of genoms. It should be however stressed that in *S. ambiguum,* the onset of the latent period is associated with a considerable reconstruction of Ma (the moniliform nucleus becomes spherical etc.) while in *P. putrinum* no perceptible reconstruction was observed.

Hanson and Twichell 1962 found by means of autoradiographic method that in Ma of *P. trichium* (=*P. putrinum*), DNA synthesis sets on already during its division and continues for some time at the beginning of interphase. Results of the present study do not support the findings of those authors. This discrepancy may possibly be accounted for by difference of strains chosen for experiment, as in the case of similar controversive results obtained by Walker and Mitchison 1957 and McDonald 1958 in

their study of time of DNA synthesis in various strains of *Tetrahymena pyriformis.*

The DNA synthesis in Mi of *P. putrinum* begins later than in Ma (approx. 2 hrs.), proceeds very intensely and is completed approx. 1 hr. earlier than in Ma. Consequently, DNA synthesis in Mi occurs within a clearly delimited period G_2 which is not observable in Ma. The above results however follow from experiments on mass cultures. Walker and Mitchison 1957 found that DNA synthesis in Mi is delayed when compared with Ma.

In *P. putrinum,* the DNA content in Mi and more so in Ma (Table 1) shows considerable fluctuations over the whole interphase period (for Ma approx. 2 times). The errror of the photographic method amounts approx. 7% (Brodskij 1956, Ovchinnikova et al. 1963). This error is somewhat higher for Mi because of its smaller size. Fluctuations of the same range have been reported in the study of other authors (Cheissin et al. 1963, Ovchinnikova et al. 1964). No doubt, such a considerable dispersion of the DNA content cannot be explained by the error in measurement.

Dispersion of the DNA content in the ciliates of the same clone may result from an uneven distribution of DNA in the daughter cells (Scherbaum et al. 1958, Kimball and Barka 1959, Ruthmann 1964), or — as mentioned by Cheissin et al. 1963 — from the fact that division of Ma may occur at different time, independently of its increased DNA content. Our date support rather the results of the first mentioned authors because in the sister individuals a considerable dispersion of DNA content is observable. The ratio of DNA content in sister individuals — as shown above — amounts for Ma 1.2 in average. In some cases however this ratio may rise considerably.

The variability of DNA content may be accounted for by the lack of synchronization of the clone as well. Therefore the duration of the interphase period and rate of DNA synthesis may be different in separate individuals.

Cheissin and Ovchinnikova 1964 reported in their recent publication that in *P. putrinum,* 3—4 endomitotic cycles should occur before the content of DNA specific for this species is formed in presynthetic Ma during its formation from the anlage. Data of the present study fully confirm this result.

The ratio of DNA content in the presynthetic Ma to that in Mi amounts in average 12. If Mi is postulated to be diploidal, so the number of genoms in Ma should be about 24. This number coincides with the results of Cheissin and Ovchinnikova 1964 on the material stained after the Feulgen's method.

It should be stressed however that the problem of diploidy of Mi remains still disputable. Already Chen 1940 and subsequently Ray 1958 and Dysart et al. 1962 revealed that in *P. bursaria* and in some species of *Tetrahymena* it may be more than diploidal. Nevertheless, some other investigations proved that in this case the differences in the DNA content may be mistaken for polyteny of chromosomes (Alfert and Balamuth 1957). Cheissin et al. 1964 found that the coefficients of DNA content in Mi of different lines may display ratio: 2:1, 4:1, 6:1 and 8:1. These authors are inclined to the view that the higher content of DNA in big Mi is not involved by polyploidy but rather by formation of polytenic chromosomes.

Consequently the problem of the real number of genoms in Ma in ciliates — and particularly so in *Paramecium putrinum* — needs further investigation.

Summary

A prolonged rest period precedes the onset of DNA synthesis in Ma and Mi of *Paramecium putrinum*. DNA synthesis in Ma begins in the second half of interphase and is not continous, showing a latent period of about 2 hrs., followed by a new synthesis. The DNA synthesis in Mi begins later than in Ma and is concluded one hour earlier. Variability of DNA content in Ma is accounted for mainly by irregular distribution of DNA to the sister individuals after division as well as by different duration of the interphase period in separate individuals. Formation of DNA content specific for presynthetic Ma is accomplished when 3—4 endomitotic cycles have been concluded. Ratio of DNA content in presythetic Ma to DNA content in presynthetic Mi is approximately 12.

РЕЗЮМЕ

Изучено содержание ДНК в макро- и микронуклеусе *Paramecium putrinum* в период от деления до деления. Работа выполнена методом ультрафиолетовой цитофотометрии.

Полученные данные показали, что синтез ДНК в микро- и микроиуклеусе *Paramecium putrinum* не синхронизирован по времени. В макронуклеусе синтез ДНК начинается во второй половине интерфазы и, с некоторым латентным периодом (около 2-х часов), продолжается вплоть до самого начала его деления. Синтез ДНК в микронуклеусе начинается позже, чем в макронуклеусе, но завершается за час до деления макронуклеуса.

Отношение количества ДНК в пресинтетическом макронуклеусе к количеству ДНК в пресинтетическом микронуклеусе равно приблизительно 12.

Вариабильность в количестве ДНК макронуклеуса объясняется главным образом неравномерностью распределения ДНК сестринскими особями, а также различной продолжительностью интерфазного периода у отдельных особей.

При сравнении содержания ДНК в макро- и микронуклеусе видно, что для образования количества ДНК, свойственного пресинтетическому макронуклеусу *Paramecium putrinum*, должно произойти 3—4 эндомитотических цикла.

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Purification and some properties of ribonucleases from *Paramecium aurelia*

Oczyszczanie i niektóre własności ribonukleaz *Paramecium aurelia*

Little is yet known of the ribonucleases from protozoa. The only ribonuclease which was biochemically investigated, considering its specificity, was that of *Euglena gracilis* (Felling and Wiley 1960). Besides, Eichel and Figueroa 1963 succeeded in the 40-fold purification of ribonuclease from *Tetrahymena pyriformis*. According to these authors *Tetrahymena pyriformis* contains two ribonucleases: one, which is endogenous and the other which is exogenous. There is nothing to choose between them except the difference in sensibility to the temperature. It is known that the sensibility of an enzyme to an elevated temperature decreases with the increase of its purity degree (Shuster 1957, Stock and Vandendriessche 1961). It is possible that the exogenous ribonuclease from *Tetrahymena pyriformis* derives from the decomposing cells of protozoa.

Experiments intending to the isolation of the nucleic acids and nucleases from *Paramecium caudatum* and *Paramecium aurelia* and the investigation of their properties, have been started in these Departments several years ago (Gross et al. 1961, Gross et al. 1963, Skoczylas et al. 1963).

Nucleic acids were studied as parallel with RN-ases. The reason for this was to find such experimental conditions, under which the enzymic degradation of RNA during the isolation procedure would be limited to a minimum.

As the role of the ribonuclease (or one of its fractions) in the RNA metabolism has been recently emphasized in many reports (Kessler and Engelberg 1962. Ledoux et Vanderhaege 1957, Leslie 1962, Roth 1962, Tal and Elson 1963), the investigation of the ribonucleases of *Paramecium* seemed to be important.

A method of the purification of the ribonuclease from *Paramecium aurelia*, its separation into two enzymically active fractions and the results of the investigations of some of their properties (including the effect of inhibitors and examination of specificity) have been reported in this paper.

Material and methods

Paramecium aurelia (strain 51, syngen 4, KK without Kappa) from Sonneborn's collection was used. Protozoa were cultivated in mass cultures according

to Sonneborn 1950. Conditions necessary for a mass culture, the method of condensation and killing of protozoa and the way of preparation of a wet cell mass were described in previous paper (Skoczylas et al. 1963).

Usually, the yeast RNA (BDH), purified according to Kunitz 1940 with the modification of Woodward 1944, was used as a substrate for the RN-ase. Besides, three RNA fractions obtained from *Paramecium aurelia* by means of fractionation with streptomycin and NaCl, according to Harshaw 1962, were used for the examination of specificity. These fractions differ in the solubility in 1 M NaCl and in the precipitation by means of streptomycin sulphate. The h-RNA fraction yields a precipitate with streptomycin sulphate and is insoluble in 1 M NaCl. The s-RNA fraction does not produce a precipitate with streptomycin sulphate and dissolves in 1 M NaCl. The third — intermediate — p-RNA fraction is precipitated, like h-RNA fraction, by streptomycin sulphate but is soluble in 1 M NaCl. Polyuridylic (Poly-U) and polyadenylic (Poly-A) acids (Miles, sodium salts) were used as substrates, as well. The effect of ribonuclease on the "core" was also studied. The "core" was obtained from yeast RNA, purified by exhausted digestion with pancreatic RN-ase (Schuchard). A method of the preparation of "core" (with the application of continuous microdialysis) was elaborated in the Department of Biochemistry, Medical Academy, Łódź (Gross and Walter 1963).

Protamine sulphate (Mann) and ammonium sulphate (Malinckrodt Chemical Works) were used for the purification of RN-ase. A carboxymethylcellulose column (Bio-Rad Lab.) was used for the separation of RN-ases. The other reagents were of anal. grade.

The RN-ase activity was determined according to Anfinsen 1954. This method consists in the determination of an increment of acid soluble compounds liberated from RNA by ribonuclease. The extinction increment at 260 mμ by 0.001, under the given experimental conditions, was taken as a unit of an enzymic activity. The DN-ase activity was determined according to Schneider and Hogeboom 1952. DNA prepared in these Departments (Skoczylas et al. 1959) by the detergent method was used as a substrate for the DN-ase. Calcium p-nitrophenylphosphate, synthetised by Dr. Gołaszewski, was used as a substrate in the determination of a non-specific phosphodiesterase activity. The extinction value at 440 mμ, which is characteristic of p-nitrophenol liberated during the reaction, was taken as a measure of enzyme activity.

Adenosine 3′- and 5′-monophosphates (K & K) were used as substrates for the qualitative determination of activity of 3′-AMP and 5′-AMP nucleotidases. Adenosine, liberated in the course of the reaction, was separated chromatographically from the non-decomposed substrates and indentified against the standard (K & K) adenosine. 96% ethanol and 1 M sodium acetate (7.5:3) were used as a solvent.

The protein was estimated according to Lowry et al. 1951. The protein in the crude extract from protozoa was determined by biuret method (Gornall et al. 1949).

Results and discussion

Preparation of a crude extract

10—20 g of a wet mass of protozoa were ground with a glass powder in a porcelain mortar, 10—20 ml of a 0.1 M citrate-phosphate buffer, pH 6, were

added and the mixture was left in a cold room for 4—5 hours. After centrifugation (10′, 10000 r.p.m.) the obtained precipitate was re-extracted as above. As the second extract did not show any enzymic activity, the repeated extraction of the precipitate was given up.

Purification of a crude extract

75 mg of protamine sulphate were added for every 100 mg of protein in the crude extract. The protamine was added slowly at careful stirring. The mixture was left in a cold room for 1 hour and the precipitate of the residual protein was removed by centrifugation.

Salting out with $(NH_4)_2SO_4$

Specific activity of the roughly purified crude extract was determined and then the extract was fractionated with ammonium sulphate. Fractions: 0.00—0.35, 0.35—0.55 and 0.55—0.85 sat. were collected. The fraction 0.55—0.85 sat. showed to be the most active one. It was dissolved in a small amount of water and dialysed against the distilled water in a cold room for 24 hours, until no sulphate ions were present in the dialyzate. It was then dialysed for further 4 hours against two portions of. 0.01 M phosphate buffer, pH 6.

Investigation of some properties of RN-ase

RN-ase from *Paramecium*, purified by salting out with $(NH_4)_2SO_4$, between 0.55—0.85 sat., was used for further study. The dependence of the enzyme activity on the pH is presented in Fig. 1.

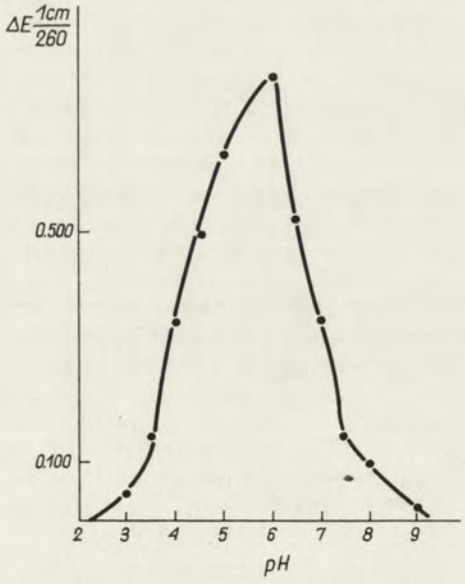

Fig. 1. Dependence of the RN-ase activity of fraction 0.55—0.85 sat. on the pH (pH 2.2—8.0 citrate-phosphate buffer, pH 8.5—10.0 glycine buffer)

It was found that the elevated temperature exerts an inhibitory influence on the RN-ase from *Paramecium aurelia*. The sensibility of the enzyme to the temperature depends on the purification degree. The crude extract is very thermolabile but the purified fraction, even after 15′ heating at 100°, retains 28% of its activity. Stock and Vandendriessche 1961 observed also the increase of thermostability of RN-ases in the course of purification.

Table 1

Effect of temperature on the RN-ase activity from *Paramecium aurelia* at various stages of purification. The activity of a non-heated preparation was taken for 1.00

Purification stage	Heating time				
	0	70°C 5 min.	70°C 15 min.	100°C 5 min.	100°C 15 min.
Crude extract	1.00	0.00	0.00	0.00	0.00
Fraction 0.55—0.85 sat. $(NH_4)_2SO_4$	1.00	0.59	0.46	0.43	0.28

The RN-ase from *Paramecium aurelia* is slightly inactivated by metal ions (Table 2, but Ca^{2+} and Fe^{2+} ions activate it to a small degree. ADTA, NaF and heparin act as inhibitors. Streptomycin exerts no influence. Phenol inactivates it entirely. This was stated in control experiments during the preparation of RNA from *Paramecium aurelia*. The investigated ribonuclease belongs to the group of enzymes which are rather resistant against the generally used inhibitors. For example, Cu^{2+} ions in 0.02 M concentration decrease its activity by only 19%, as related to the control. The much more significant activatory effect of the Fe^{2+} ions was observed by Walczak (personal communication) for the RN-ase from *Thiobacillus thioparus*.

The RNA fraction from *Paramecium aurelia*, "core" prepared from yeast RNA and sodium salts of Poly-U and Poly-A were used as substrates in the examination of the specificity of the investigated ribonucleases. The same quantitative ratio substrate/enzyme was preserved in all experiments.

A partially purified RN-ase from *Paramecium aurelia* is a few specific enzyme. It decomposes "core", prepared from yeast RNA, with almost the same velocity as it does in the case of a commercial RNA prepared from yeast. It decomposes synthetic polynucleotides with certain preference of the pyrimidine internucleotide bonds. However, the nucleotide composition of a substrate does not seem to be very important. The s-RNA from *Paramecium aurelia*, soluble in 1 M NaCl (purines/pyrimidines about 1), is decomposed a little more quickly than Poly-A. The "core" (much more purines than pyrimidines) is decomposed more quickly than Poly-U.

The investigated RN-ase shows higher affinity to RNA from the same source than to yeast RNA. This is true, however, only for the highest polymerized fraction (h-RNA). It might confirm the data of Merol and Davis 1962 who found that the s-RNA fraction is more difficult to be hydrolysed than the higher polymerised RNA. It is possible that the RN-ase from *Paramecium*

aurelia exerts its influence less readily on the substrates of ordered secondary structures and such structure is ascribed to s-RNA (McCully and Cantoni 1962) and to synthetic polynucleotides.

Table 2
Effect of metal ions and other agents on the relative RN-ase activity of 0.55—0.85 sat. fraction

Agent	Molar conc.	Relative activity	Agent	Molar conc.	Relative activity
Hg^{2+}	10^{-2}	0.00	Cu^{2+}	$2 \cdot 10^{-2}$	0.81
Mg^{2+}	10^{-2}	0.92	F^-	10^{-4}	0.93
Mg^{2+}	$2 \cdot 10^{-2}$	0.91	F^-	10^{-2}	0.86
Ca^{2+}	10^{-2}	1.03	Li^+	10^{-2}	0.98
Mn^{2+}	10^{-2}	0.94	Li^+	$2 \cdot 10^{-2}$	0.97
Fe^{2+}	10^{-2}	1.14	EDTA	10^{-2}	0.93
Zn^{2+}	10^{-2}	0.63	Heparin	0.05 mg / sample	1.00
Co^{2+}	10^{-2}	0.60	Heparin	0.5 mg / sample	0.90
Cd^{2+}	10^{-2}	0.58	Streptomycin	0.1 mg / sample	1.00
Cu^{2+}	10^{-2}	0.84	Streptomycin	1.0 mg / sample	1.00

Table 3
Comparison of RN-ase activity from *Paramecium aurelia* (fraction 0.55—0.85 sat.). The activity related to the yeast RNA was taken for a unit

Substrate	Relative actiivity
Yeast RNA	1.00
RNA from *Paramecium aurelia*:	
h-RNA fraction	1.20
p-RNA fraction	0,87
s-RNA fraction	0.48
"Core" (yeast) RNA)	0.82
Poly-U (Miles, sodium salt)	0.76
Poly-A (Miles, sodium salt)	0.36

Fractionation on carboxymethylcellulose column

The column, 2×10 cm., was filled with a suspension of CM-cellulose in water and washed subsequently with 0.1 N NaOH and water (neutral reaction) and then with HCl and water (neutral reaction). This procedure was repeated and then the column was washed with 300 ml of 0.01 M phosphate buffer, pH 6. Franction 0.55—0.85, dissolved in 0.1 M phosphate buffer, pH 6, was flown through the column and followed by 200 ml of the same buffer in order to remove the non-adsorbed protein.

Separation was achieved by passing through the column 150 ml of a 0.1 M phosphate buffer, pH 6, and then 150 ml of a 0.1 M phosphate buffer, pH 7.5. Finally, the column was washed with 0.5 N NaOH to remove the rest of protein.

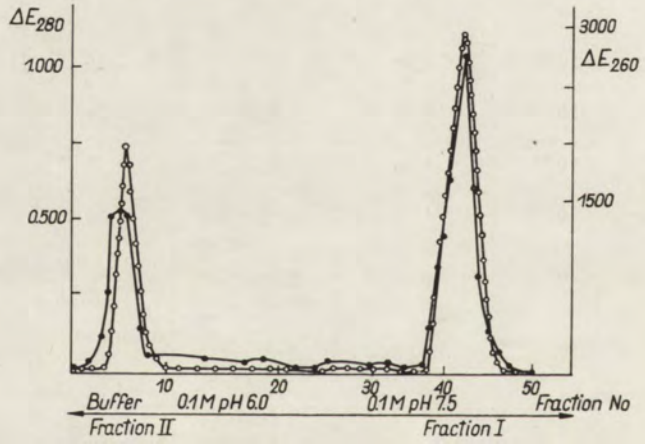

Fig. 2. Seperation of 0.55—0.85 RN-ase fraction on CM-cellulose column. Flow rate — 0.75 ml/min. Volume of fraction — 5 ml. Dotted line — RN-ase activity. Continuous line — amount of protein (E_{280})

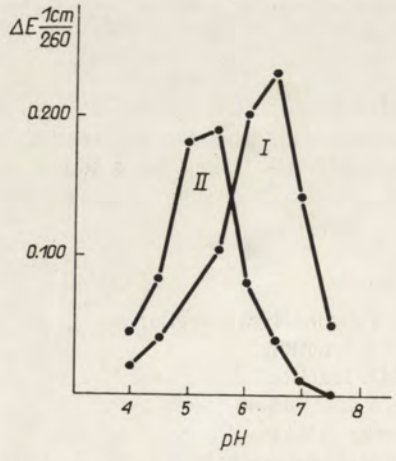

Fig. 3. Dependence of RN-ase II and RN-ase I activity on the pH

The eluate was collected in 5 ml portions in which the content of protein and enzymic activity were examined (Fig. 2).

Two distinctly separated fractions were obtained: RN-ase II and RN-ase I. The optimum pH of RN-ase II activity was about 5.5 and of RN-ase I — about 6.5 (Fig. 3).

Both fractions were free from activity of the accompanying enzymes, like: DN-ase, non-specific phosphodiesterase and 5′-AMP and 3′-AMP nucleotidases and were purified 33-fold (RN-ase II) and 20-fold (RN-ase I), as compared with the crude extract (Table 4).

Table 4
Purification of RN-ases from *Paramecium aurelia*

Purification stages	5′-AMP nucleotidase	3′-AMP nucleotidase
Crude extract	+	+
Crude extract after preliminary purification with protamine sulphate	+	+
Fraction 0.55—0.85 sat with $(NH_4)_2SO_4$	−	+
Most active fraction (pic II)	−	+−
Most active fraction (pic I)	−	−

Summary

A method of isolation and purification of RN-ases from *Paramecium aurelia* mass cultures has been described. The enzyme was separated on CM-cellulose column into two active fractions differing in optimum pH (fraction II about 5.5, fraction I about 6.5). The enzyme (before the seperation on CM-cellulose) showed a slight sensibility on the action of inhibitors (except Hg^{2+} ions), low specificity and higher affinity to higher polymerised RNA fraction from the homologous material.

STRESZCZENIE

Opisano metodę izolowania i oczyszczania ribonukleazy z masowych kultur *Paramecium aurelia*. Enzym ten rozdzielono na kolumnie z karboksymetylocelulozy na dwie czynne frakcje różniące się optymalnym pH (II około 5.5; I około 6.5). Otrzymany enzym (przed rozdzieleniem na kolumnie) wykazuje nieznaczną wrażliwość na działanie inhibitorów z wyjątkiem jonów Hg^{2+}, a także małą specyficzność i większe powinowactwo w stosunku do wyżej spolimeryzowanej frakcji RNA z homogennego materiału.

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Cultivation of some *Peritrichida*

Culture de quelques *Peritrichida*

This paper is concerned with the cultivation of that very special group of ciliates called peritrichs. The peritrichs are special in many ways. For example, the type genus of the peritrichs was described by the talented, perceptive, pioneer microbe-hunter, Antony van Leeuwenhoek, who in 1676 communicated a description of *Vorticella* to the editor of the Philosophical Transactions, London; Leeuwenhoek's communication on *Vorticella* can be found in the archives of the Royal Society of London; this justifies the use of the adjective special when mentioning peritrichs. The number of species of peritrichs is overwhelming, of the order of 1000; this is special. The peritrichs have survived the uncommon experience of having been demoted in rank and promoted back again in the past decade; this too is special.

Indeed the peritrichs are special in other ways. Hutnerisms can be paraphrased to fit them, for example: you can lead a peritrich to a particle but can you make it eat? Or, the introduction to a recipe for axenic peritrich pie: catch and axenize a peritrich.

Some peritrichs have been caught and axenized in our laboratories. Ways in which knowledge of the collection and cultivation of peritrichs has been applied to their isolation and growth in non-axenic and axenic nutrient media will be mentioned. References to housekeeping details of non-axenic maintenance, comments on the early faltering excursions into axenic territory, summarization of published findings will be given. Finally, declarations and speculations derived from unpublished findings acquired recently in our laboratory by my colleague Dr. David McLaughlin and our assistants will be presented.

Housekeeping details

Forty years ago Noland 1925 asserted that food was perhaps the most critical factor influencing the distribution of fresh-water ciliates. His assertion has been confirmed many times (Bamforth 1962; Dragesco 1960; Fauré-Fremiet 1943, 1951; Jirovec 1961; Provasoli 1958; Sandon 1932). Food is an important housekeeping detail pertaining to the cultivation of peritrichs. In early studies on peritrichs (Finley 1936, 1939) food was provided by means of a crude bacterized broth medium; the recipe for it was derived from Woodruff's (1912) suggestions. Woodruff surely inherited the idea from distinguished European biologists. When it became commercially profitable to pulverize and package hay then Cerophyl replaced alfalfa hay; but, the main food organisms were bacteria. In time *Bacillus cereus* was substituted for unknown airborne

microorganisms (Finley, McLaughlin and Harrison 1959). Consequently another important housekeeping detail was added because it was necessary to use exceptionally clean glassware and to observe other rules long practiced by microbiologists in order to avoid unwanted contamination. Numerous concoctions preceded our basal broth medium, for example, concoctions of pea seeds, egg yolk, dehydrated lettuce and dried cabbage. But none of these were as satisfactory or as convenient as our basal broth medium.

Early axenic experiments

The housekeeping details just now mentioned were derived by rule of thumb rather than by experimentation. In the late 1950s we were convinced by the peritrichs, that many of them, especially *V. microstoma*, demanded punctiliousness. The word is appropriate because axenic cultivation of *V. microstoma* demands preciseness especially in the observance of exact details pertaining to controlled and repeatable laboratory conditions. Extensive experimentation led to the adoption of maintenance methods, glassware, equipment, and buffers (Finley, McLaughlin and Harrison 1959). Levine 1959 independently proposed an axenizing medium at the same time; his basal component was egg yolk.

The widespread use of proteoses and peptones as substitutes for hay infusions, and the intense interest stimulated by the bacteria-free studies of the Lwoffs in France in the decade 1923—1933 (Lwoff 1951), George W. Kidder's restatement of obstacles to be surmounted in bacteria-free research (Kidder and Dewey 1951), and brilliant experimental designs that came out of Seymour Hutner's laboratory in 1953 all prompted the supplementation of our basal broth medium with that ubiquitous substance called proteose peptone. Thus a potent substitute for basal broth (Table 1) was concocted (Table 2, Peri Broth). It is an excellent nutrient medium for *B. cereus* and other bacteria, and so it is almost ideal for monoxenic cultivation of common peritrichs. When used monoxenically it can be diluted and buffered for the purpose of holding in check the prolific growth of bacteria which occurs in the undiluated medium; this gives the peritrich population a better chance to survive in laboratory environments. When used axenically it is inadequate for peritrichs, as expected.

Table 1

Basal broth for polyxenic growth of peritrichs

Alfalfa hay*	2 grams
Wheat kernels	3 „
Distilled water	100 ml

* Obsolete. Substitute Cerophyl.

Soon it became evident that the preferences and tolerances of peritrichs were not similar to the preferences and tolerances of either *Tetrahymena* or *Paramecium*. The path from monoxenic to axenic cultivation of *Vorticella* and *Telotrochidium* was tortuous and difficult. Failures of singly-added components to shore up axenic basal media indicated the need for other metabolites time and again. Trials and failures led to a moderately effective basal medium for axenic maintenance (Table 3).

Table 2
Basal medium for monoxenic growth of common peritrichs (Peri Broth)

Components	Amounts (mg/100 ml)
Proteose-Peptone (Difco)	20
Cerophyl-Wheat broth	
Cerophyl	300
Wheat kernel broth	200

Table 3
Basal medium for axenic maintenance of common peritrichs

Components	Amounts/ml in 5 ml final volume (μg)
Acid hydrolyzed gelatin (Eastman)	4.30
Aqueous liver extract, 1:20 (N.B.C.)	0.56
Hydrolyzed yeast nucleic acid (C.F.)	0.56
Glucose (Fisher)	2.82
*Serine	40.0
*EDTA	5.0
*$CaCl_2$	0.80
*$FeCl_3$	5.0
*KCl	20.0
*Dl β-Hydroxybutyric acid	20.0
*$MgSO_4 \cdot 7H_2O$	40.0
*Riboflavin (approx limits)	0.04—4.0
Penicillin G	2 500 USP units

* Additions subject to variation for experimental purposes.

Later axenic experiments

Additions and substitutions led to the elimination of one of the three crude components in our basal axenic medium (liver extract, yeast nucleic acid, gelatin). With the cooperation of *Telotrochidium* we (Finley and McLaughlin 1965) designed a medium containing four basic components (liver extract, hydrolyzed yeast nucleic acid, glucose, and dl-β-hydroxybutyric acid), 18 amino acids, 7 vitamins, 10 salts supplying trace metals, uridylic, cytidylic, guanylic, and adenylic acids; thymidine-5-diphosphate, nicotinamide mononucleotide, and choline; phosphate buffer, EDTA, and penicillin (Table 4, T-3 medium, and Table 5). It did not contain gelatin, thus one of the crude components was eliminated successfully.

The complexity of T-3 medium reflects the need for balance, balance in the sense that when an axenic medium provides acceptable and useful metabolites sufficient to meet the nutritional needs of a protozoan the deletion or addition of a single component may unbalance it and make it unsatisfactory. All experienced nutritionists know this. I mention it merely as a precaution

to those who would tackle cultivation problems. It should be understood that all components in our T-3 medium may not be essential metabolites. It is impossible to prove that each of 18 amino acids is essential for the nutrition of *Telotrochidium*. But the 18 amino acids together with the other components make a balanced medium suitable for prolonged serial transfers and capable of producing large populations of healthy organisms.

Table 4

Medium T-3 for axenic growth of *Telotrochidium henneguyi*

Component	ml in 10-ml final vol.	Component	ml in 10 ml final vol.	Component	ml in 10 ml final vol.
Liver hydrolysate	0.0056	Glycine	4.1	Biotin	1×10^{-4}
Yeast nucleic acid	0.0056	DL-Alanine	5.6	Thiamine-HCl	0.04
EDTA	0.05	L-Proline	9.0	Pyridoxine·HCl	0.1
L-Arginine	5.4	L-Tryptophan	1.0	Pyridoxamine.	
L-Histidine	8.9	$FeCl_2\cdot6H_2O$	0.05	2HCl	0.01
L-Lysine·HCl	5.9	$CaCl_2$	0.01	Riboflavin	0.02
DL-Tyrosine	0.5	KCl	0.2	GDP	3.0
L-Cystine	0.1	$MgSO_4\cdot7H_2O$	0.4	DL-β-hydroxybu-	
DL-Phenylalanine	3.4	KH_2PO_4	3.4	tyric acid	0.2
L-Methionine	2.5	K_2HPO_4 (anhyd.)	5.4	Glucose	0.03
L-Threonine	6.7	$Fe_2(SO_4)_3(NH_4)_2\cdot$		Uridylic acid	0.04
L-Leucine	3.5	$\cdot24H_2O$	0.4	Cytidylic acid	5×10^{-4}
DL-Isoleucine	4.9	ZnCl	0.02	Guanylic acid	5×10^{-5}
DL-Valine	4.9	$MnCl_2\cdot4H_2O$	0.04	Adenylic acid	0.1
DL-Serine	0.4	$CuCl_2\cdot2H_2O$	0.02	TDP	2.5×10^{-6}
L-Glutamic acid	20.8	Folic acid	0.01	NMN	2.5×10^{-8}
L-Aspartic acid	5.9	Cyanocobalamin	1×10^{-4}	Choline Chloride	5×10^{-4}

Table 5

Rages and optima in T-3 medium*

Factors investigated	Limits acceptable	Optimum
Temperature	18—25°C	23°C
pH	6.0—8.0	6.8
Buffer (phosphate)	2×10^{-1}—1×10^{-5}M	2×10^{-1}
Penicillin G (axenic)	700—7 000 USP units/ml	5 000 USP units/ml

* Probably applicable for all common peritrichs.

Dr. David McLaughlin, my colleague, removed the two remaining crude components from T-3 medium. i. e., he removed liver hydrolysate and yeast nucleic acid. This was accomplished by increasing the concentration of the T-3 supplements and also by adding either uracil and sodium acetate or citric acid, sodium acetate and uracil. Thus again deletion of crudes lead to imbalances corrected by additions and adjustments in concentration of supplements.

With axenic media, as with puddings, the proof is in the eating. *Telotrochidium* "ate" the modified medium and thrived rather well. The results show progress toward the development of chemically defined axenic media for peritrichs (Figs. 1, 2). The results also suggest that some peritrichs utilize solutes. Now there can be no doubt that it is possible to overcome the obstacles inherent in bacterized and polyxenic cultivation of peritrichs.

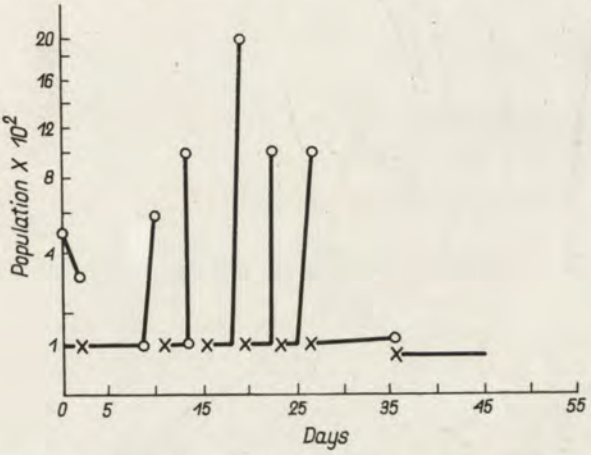

Fig. 1. Results from serial transfers in axenic T-3. Initial inoculum 500 motile telas. Inoculum 2 consist of 100 motile telas taken from day 2 population. Subsequent transfer inocula consisted of 100 motile telas from exponentially growing cultures on days 10, 14, 17, 25. On day 36 only 70 motiles were transferred from a stationary population

Cultivation of other peritrichs

Colonial and loricated peritrichs are not so easily tamed as *Vorticella* and *Telotrochidium*. We have had flickering success with Peri Broth. Extensive analyses (Finley and McLaughlin 1961), of natural habitats gave useful clues to environmental preferences. Total alkalinity as $CaCO_3$, nitrogen ammonia, nitrite, nitrate, sodium, potassium, chloride, light, and temperature in natural habitats should be measured, recorded, and duplicated in laboratory habitats, if possible; this procedure is moderately successful, non-axenically, with *Carchesium, Epistylis,* and *Zoothamnium. Pyxicola* is the only loricate that we have cultivated axenically for prolonged periods of time. The challenge of axenic cultivation of colonial and loricated peritrichs is still outstanding.

Comparisons and predictions

Peritrichs have been noticeably absent from reviews on nutrition and metabolism of ciliates and also not mentioned in annual reviews on comparative physiology and nutrition of ciliates. This is understandable because, in the main, review articles on ciliates have been restricted to work done with organisms in defined or nearly defined axenic culture media; and, as Holtz

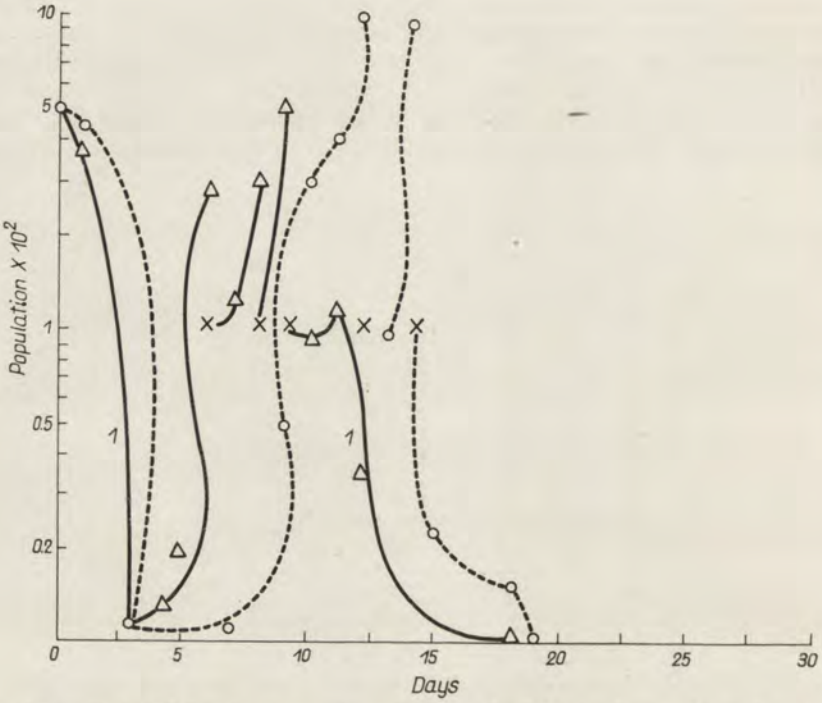

Fig. 2. Growth in T-3 minus yeast and liver. I. Effect of uracil, sodium acetate and citric acid. Inoculum = 500 motile telas; X = serial transfers (100 motiles from exponentially growing cultures). o - - - o = T-3 plus 10γ/ml uracil and sodium acetate. Δ——Δ = T-3 plus 10γ/ml uracil and sodium acetate and citric acid. 1 = decline leading to encystment. (T-3 conc. = 10 X)

wrote recently (1964), "strains of only 6 species of approximately 6 000 species of ciliates were cultivable axenically in completely defined media".

Perhaps now is the time to mention comparisons between axenic cultivation of peritrichs and the most highly domesticated ciliates, namely, tetrahymenids and paramecia. It is not feasible to make component-by-component comparisons but categories of nutrients can be considered. Peritrichs, like tetrahymenids and paramecia grow well in autoclaved peptone media supplemented with glucose. Peritrichs, tetrahymenids and paramecia grow axenically when provided with common amino acids of proteins, purines and pyrimidines, sources of carbon and energy, B-vitamins, and trace metals. Like most animals, peritrichs and the other ciliates grow axenically with little or no dietary fat. It remains to be seen whether peritrichs are more or less fussy about their diet than the tetrahymenids, paramecia, and other ciliates. At the moment we can say that the fundamentals of axenic peritrich cultivation have been exposed, and that they appear to be similar to the fundamentals pertaining to axenic cultivation of tetrahymenids and paramecia.

The total metabolic capacities of peritrichs may yet be revealed even though the improbability of success from such a venture is more likely than the probability. In any event, the solutions to many problems previously beyond reach with peritrichs are now approachable if not attainable. The implications of the

potentialities of peritrichs as laboratory tools for working on applied biochemical problems of nutrition are only beginning to be understood. Each fruitful search for nutritional requirements of laboratory animals should contribute fundamental knowledge of energy-yielding reactions and ultimately each contribution of that kind should lead to new or better methods for the detection and correction of man's nutritional deficiences.

Summary

Two generalizations are supported by this paper: 1. peritrichs grow well in monoxenic media; 2. they grow axenically when provided with common amino acids of proteins, purines, pyrimidines, sources of carbon and energy, B-vitamins, and trace metals. A bacterized broth containing proteose peptone, Cerophyl, and wheat seeds (*Triticum aestivum*) is recommended for monoxenic methods. The broth is inoculated with *Bacillus cereus* and any one of the non-parasitic peritrichs in order to obtain monoxenic populations. Four components (liver extract, hydrolyzed yeast nucleic acid, dl-beta-hydroxybutyric acid, and glucose are recommended as the foundation of a basal axenic medium. The foundation components must be supplemented and balanced according to details supplied in the text.

RESUME

Cette étude soutient deux généralisations, à savoir: 1. les peritricha se cultivent bien dans les milieux monoxéniques; 2. ils se cultivent anexiquement quand on les nourit avec des amino-acides ordinaires des protéines, des purines, des pyrimidines, des sources de carbone et d'énergie, des vitamines B, et des traces de métaux. Un bouillon bactérisé contenant du proteose peptone, du Cérophyle, et des semences de blé (*Triticum aestivum*) est recommandé pour les méthodes monoxéniques. Le bouillon est inoculé de *Bacillus cereus* et de n'importe lequel des peritricha non-parasitiques en vue d'obtenir des populations monoxéniques. Quatre composants (extrait de foie, acide nucléique de levure hydrolisée, acide dl-beta-hydroxybutyrique, et de la glucose) sont reccommandés comme base d'un milieu axenique fondamental. Les composants de base peuvent être complétés et balancés selon les détails fournis dans le texte.

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A cytophotometrical investigation of the cytoplasmic RNA content in the course of oocyst formation in the intestinal rabbit coccidia *Eimeria intestinalis* Cheissin, 1948

Цитофотометрическое исследования количества цитоплазматической РНА в процессе образования ооцист у кишечной кокцидии кролика *Eimeria intestinalis* Cheissin, 1948

In the previous paper (Beyer and Ovchinnikova 1964) the authors reported the quantitative measurements of the cytoplasmic RNA during macrogametogenesis in two rabbit coccidia — *Eimeria magna* and *E. intestinalis*. A steady rise of RNA was followed up to the formation of the zygote. However no further studies were made of the RNA content in the zygote (oocyst) during its intracellular existence and after leaving the host's cell.

However, the observations like those would contribute much into our knowledge of the metabolic dependence of the parasite on the host.

The present study is concerned with photometrical measurements of the cytoplasmic RNA in zygotes (oocysts) of *E. intestinalis*.

Material and methods

A detailed description of the techniques is given in the previous paper (Beyer and Ovchinnikova 1964).

The measurements were performed on the Soviet microspectrophotometer MUV-4 by the scanning method in λ 579 mμ. Rabbits infected by sporulated oocysts of *Eimeria intestinalis* were sacrificed on the 11th day after infection. At this time the following stages of the life cycle can be observed: 1. mature macrogametes ready to fertilization; 2. zygotes and oocysts localized inside the epithelial cells; 3. oocysts discharged into the lumen of the intestine. The quantity of RNA was compared between the three above stages which are referred to as size classes V_1, VI and VII, respectively (see Fig. 1). The data obtained were treated statistically.

It is to be noted that just after fertilization of the macrogamete, the wall surrounding the zygote is very thin. The condensation of the wall occurs later. The walls of oocysts excharged from the host cells are much denser if compared to those of younger, still intracellular oocysts.

The total quantity of the cytoplasmic RNA (Q) was calculated according to the formula:

$$Q = \frac{DV_{cyt}}{h}$$

where Q is the total quantity of cytoplasmic RNA, D — optical density, V_{cyt} — volume of the cytoplasm, h — thickness of the section.

$$V_{cyt} = V_{cell} - V_{nucleus}$$

The oocyst has an ellipsoid-like form the volume of which can be calculated according to the formula:

$$V_{cell} = \frac{3}{4}\tilde{\eta}Rr^2$$

where R — a greater radius of the oocyst, and r — a lesser radius of the oocyst.

The nucleus of the oocyst is ball-like and its volume can be calculated according to the formula:

$$V_{nucleus} = \frac{4}{3}\tilde{\eta}r_1^3$$

where r_1 is the radius of the nucleus.

Finally:

$$Q = \frac{D}{h} \cdot \frac{4}{3}\tilde{\eta}(Rr^2 - r_1^3)$$

Results.

The results obtained are given in the Table 1.

Table 1

The results obtained from the photometric estimation of RNA in mature macrogametes and oocysts of Eimeria intestinalis

Size classes of developmental stages	Developmental stages of the parasite	The number of cells measured	RNA quantity (Q) $(M \pm m).10^{-6}$	Volume of the developmental stages (V_{cyt}) $(M \pm m).10^{-6}$
V_1	Mature macrogametes	14	56.02±2.00	95.00±3.50
VI	Zygotes and oocysts localized inside the epithelial cells	33	45.00±2.00	96.50±3 20
VII	Oocysts discharged into the lumen of intestine	16	45.00±3.00	114.00±4.00

As is seen from the table the quantity of the cytoplasmic RNA in the zygote (oocyst) is less than in the mature macrogamete. The differences are statistically significant with probability equal to 99.9 per cent ($t > 3.00$). At the same time the oocysts inside and outside the host cell (VI and VII, see the table) have the equal quantity of RNA.

The cytoplasmic volume of a just formed zygote (oocyst) was found not to be changed in comparison with that of the mature macrogamete (the differences are statistically significant, $t = 0.32$). Unlike this, the cytoplasmic volume of the oocyst ready to be discharged, or already discharged from the host's cell into the lumen of intestine, is greater than that of the intracellular oocysts ($t > 3.00$). Compare mma, z and 00 with 00_1 and 00_2 in the Pl. I.

The data obtained are represented graphically in Fig. 1 as a segment V_1—VII. In Fig. 1 there are two curves representing the data of the present communication (V_1—VII) and of the earlier data of the authors — Beyer and Ovchinnikova 1964 (I—V). A continuous line shows changes in the quantity of RNA during the growth of the macrogamete and oocyst. A spotted line reflects changes in the cytoplasmic volume of the respective stages. Roman numerals show the size classes, V and V_1 representing one and the same stage of the mature macrogamete according to the earlier and present data of the authors, resp.

As is seen from Fig. 1 (continuous line), the quantity of the cytoplasmic RNA of the macrogamete increases as the latter grows. By the time of zygote formation the content of RNA in the macrogamete reaches its maximal value (V_1). Afterwards this content falls a bit to a certain level which remains unchanged up to the time of the oocyst elimination. An extremely dense wall impedes further measurement of the RNA content in the discharged oocysts.

As the macrogamete grows, its cytoplasmic volume is seen to increase constantly (Fig. 1). The volumes of the macrogamete and of the zygote (oocyst) still localized within the host's cell do not differ much. Unlike this the volume of the oocyst which is discharging or has been already discharged from the hosts cell is much greater if compared with that of the zygote.

Discussion

The previous investigation of the cytoplasmic RNA content in macrogametes of *Eimeria magna* and *E. intestinalis* enabled the authors to follow a direct relation between the volume of the parasite and the RNA quantity. The increase in the RNA content of the macrogamete as its growth seems rather natural. It is just at this stage of the parasite's development that the reserve materials are accumulated to which are to be consumed later on, i.e. in the non-living media.

A question arises: up to which moment in the parasite's development a constant rise of RNA and consequently its synthesis proceeds? In other words, up to which moment of its development the parasite keeps its active relationship with the host. There is no doubt that all the food demands of the parasite are provided by the host, i.e. by the epithelial cell of the intestine which constitutes the environment of the parasite. The breach of the relationships between the parasite and the host seems very likely to result in stopping the increase in the RNA content.

The results obtained show the stoppage of the RNA increase to occur as early as at the stage of zygote. Moreover, in the zygote the RNA content

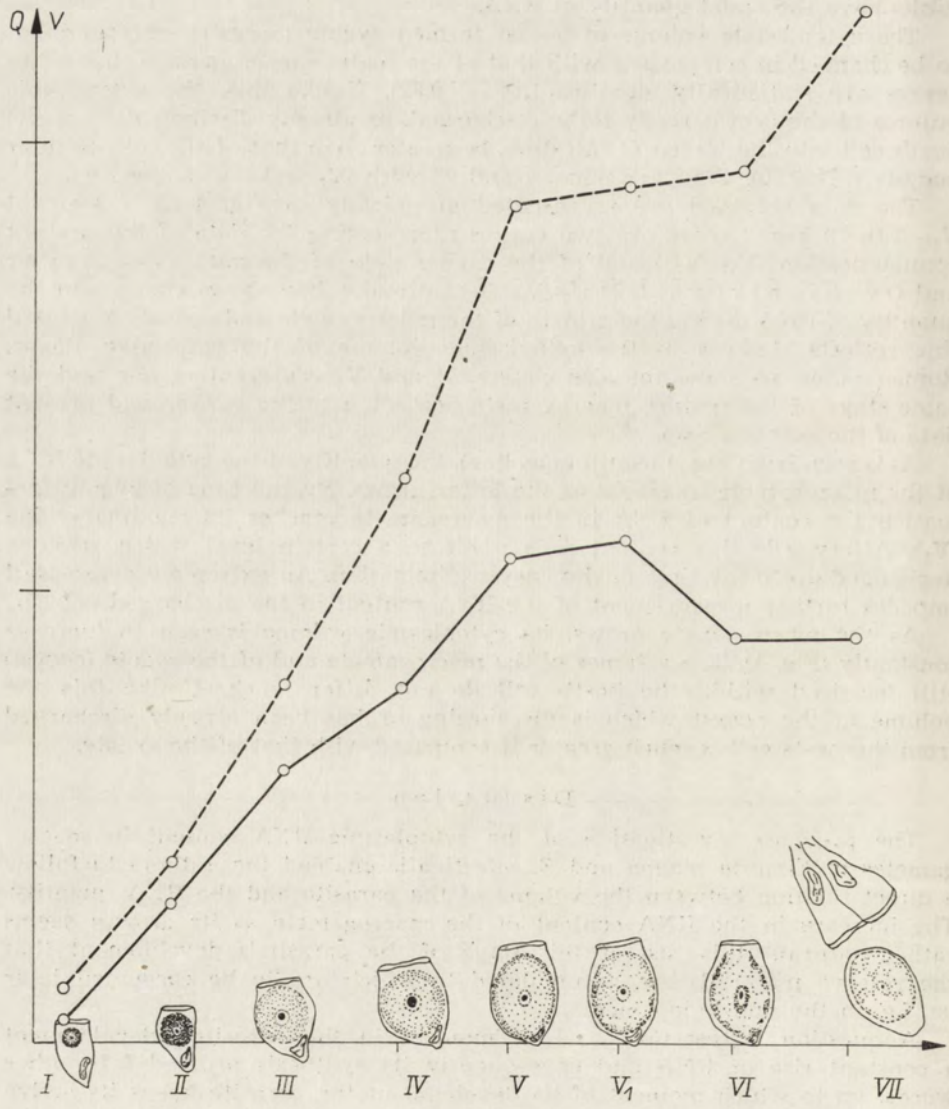

Fig. 1. Changes in the cytoplasmic RNA content in the growing macrogametes and in the oocysts of *Eimeria intestinalis*. V — volume of the cell; Q — RNA quantity per cell. Continuous line represents changes in Q value, dotted line — changes in V value. Roman numerals represent size classes of the development stages examined

is even lower than that in the macrogamete. It looks like a certain portion of RNA being spent during the formation of oocyst wall. The protein and mucoprotein granules constituting the oocyst wall are known to appear first as early as in the macrogamete (Cheissin 1958).

In oocysts discharged from the intestinal cell into the lumen of intestine, the quantity of RNA remained unchanged in comparison with that in the intracellular oocysts. At the same time the cytoplasmic volume of the former appears to be greater. Th adsorption of water from the environment by the oocyst cytoplasm might account for this increase. The latter fact is seconded as well by a decrease in the optical density of the cytoplasm in discharged oocysts as compared with intracellular ones.

Not long before being discharged from the host's organism, the oocyst wall becomes dense which impedes the penetration of oocyst by fixatives and dyes. To avoid technical errors, neither unstained not shrunk oocysts underwent measurings.

The results of the present study allow to suggest that the breach in the host-parasite relationship should occur at the stage of zygote localized within the host epithelial cell. The parasite being localized intracellularly, its metabolism is determined by the further conditions it will face in the non-living environment.

After being eliminated from the host's cell, the oocyst finds itself in the lumen of intestine where the parasite should withstand a digestive action. A very dense oocyst wall contributes much in the protection of the parasite. While in the lumen, the oocyst wall gets more and more dense, so that after being disharged in the non-living environment it is found easily to withstand the external mechanical influences.

The oocyst can exist in the non-living medium only if water and oxygen are available. No entrance of nutrition substances from the environment occurs. The process of sporulation is realized at the expense of the energy released at the utilization of the reserve materials of the oocyst — glycogen, lipids, proteins. The existence of the oocyst in the non-living medium is provided for the same source of energy.

After fertilization, the character of the parasite metabolism turns from an anaerobic into an aerobic one (Beyer 1962, 1963). The importance of this phenomenon is evident: the aerobic oxidation of the substrate is process more economically profitable, taking into account that the amount of the reserve material, of the oocyst is limited, while the period of its existence in the non-living environment before invading a new host may be very long.

Thus, the stage of zygote (oocyst) is a connection between endogenous and exogenous stages in the life cycle of coccidia. The total metabolic reorganization providing for the life of the parasite in the non-living environment has been found to occur still at the intracellular localization of the oocyst.

Summary

The cytoplasmic RNA during the formation of zygote (oocyst) was investigated by the scanning method at the wave length 579 mμ, on the microspectrophotometer MUV-4. The greatest RNA content was recorded in the mature macrogametes, before fertilization and formation of the zygote. During the building of the oocyst walls a slight decrease in the RNA

content is observed. In the oocyst the RNA content is constant as well during their intracellular localization as after excharging into the lumen of intestine. Stopping of the RNA increase in the zygote is considered as result of breaching of the active relation between parasite and its host.

РЕЗЮМЕ

Методом сканирования в длине волны 579 mμ на микроспектрофотометре МУФ-4 проведено исследование цитоплазматической РНК в процессе формирования зиготы — ооцисты. Максимальное количество РНК зарегистрировано в зрелых макрогаметах, перед оплодотворением и образованием зиготы. Во время образования оболочек ооцисты наблюдается некоторое уменьшение количества РНК. В ооцистах, локализованных внутри клеток и выведенных в просвет кишечника, количество цитоплазматической РНК одинаково. Прекращение увеличения количества РНК в зиготе рассматривается как результат нарушения активной связи между паразитом и хозяином.

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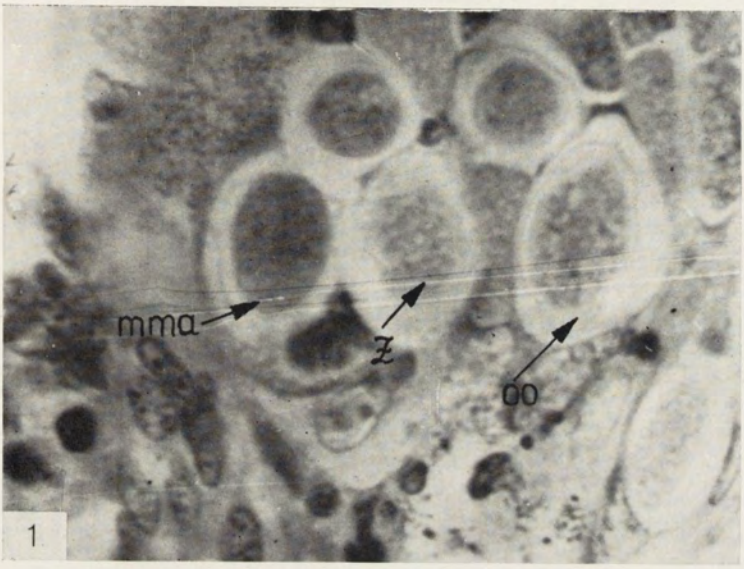
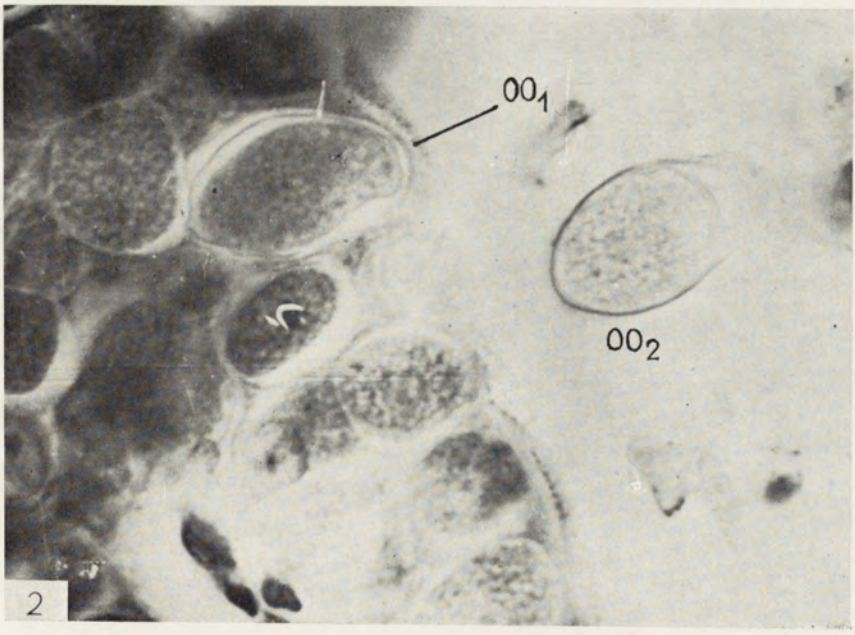
EXPLANATION OF THE PLATE I

1—2: Oocysts of *Eimeria intestinalis* (mma — mature macrogamete, z — zygote, OO — oocyst localized within a host cell, OO_1 — oocyst not long before its discharge into the lumen of intestine, OO_2 — oocyst in the lumen of intestine). All stained with gallocyanin-chromalum (× 3135)





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О способе питания вегетативных стадий *Myxidium lieberkuhni* (Bütschli)

On the mode of nutrition of vegetative stages of *Myxidium lieberkuhni* (Bütschli)

Слизистый споровик *Myxidium lieberkuhni* паразитирует в мочевом пузыре щук. Среди конечных продуктов азотистого обмена, выводимых с мочей пресноводных рыб, главное место занимает аммиак — 56% (в процентах ко всему азоту выводимому мочей), затем мочевина — 5.7%, затем мочевая кислота — 0.2% (Гинецинский 1964). Аммиак очень токсичен, и обитание в такой среде, как моча рыб, требует, видимо, каких-то приспособлений у паразитических организмов. В связи с этим интересно было установить как и за счет чего питаются миксоспоридии из мочевого пузыря.

Вегетативные стадии *M. lieberkuhni* представляют собой крупных плазмодиев до 300 μ длины. Зрелые плазмодии имеют вытянутую по одной оси червеобразную форму. От основного ствола часто отходят боковые ветви — неотделившиеся почки (Табл. I 2—4, II 5—6). Часть плазмодиев *M. lieberkuhni* плавает в полости мочевого пузыря, часть же их прикрепляется к стенке пузыря с помощью особых „ножек", представляющих собой плазматические выросты различной формы (Табл. I 1, II 5—6, III 9, IV 10). Эти выросты могут возникать и на узких концах плазмодиев, и на его широкой боковой поверхности, в зависимости от того, какой частью тела он примкнул к стенке мочевого пузыря (Табл. I 1, IV 10). С помощью „ножки" плазмодий часто довольно глубоко проникают в эпителий, тело же их направлено в полость пузыря (Табл. I 1).

Относительно того, каким образом происходит внедрение плазмодия в стенку мочевого пузыря, за счет чего он питается и каково его влияние на ткани пузыря не существует единого мнения. Так, Тибигер (Tibiger 1906, цит. по Bauer 1922) считает, что паразит этот не вызывает каких-либо заметных патологических изменений в тканях хозяина, потому, что плазмодии находятся в слизи, которая пластом покрывает стенки мочевого пузыря. Бючли (Bütschli 1882), Бальбиани (Balbiani 1888) и Пфеффер (Pfeiffer 1890) придерживаются мнения, что *M. lieberkuhni* погружает особые отростки или конец тела в вещество клетки, высасывая её. Однако Лаверан (Laveran 1902) отрицает такую возможность и утверждает, что плазмодии сидят на клетках эпителия, никогда не внедряясь ни в клетки, ни в межклеточные пространства.

Коон (Cohn 1896) различает два способа прикрепления *M. lieberkuhni* к эпителию: погружение одного конца тела в гипертрофированную клетку и неподвижное прикрепление к эпителию с помощью подошвообразного расширения.

Наиболее подробно изменения стенок мочевого пузыря под влиянием *M. lieberkuhni* изучал Бауэр (Bauer 1922). В вопросе о способе прикрепления паразита он присоединяется к мнению Коона, однако, на приведенном им в статье рисунке, плазматические выросты *M. lieberkuhni* изображены скорее проходящими по межклеточникам. Бауэр отмечает, что ему удавалось наблюдать гипертрофию эпителиальных клеток, а также все стадии дегенерации клеток в результате внедрения паразита. Разрушение клеток, по его мнению, начинается с ядра. По данным Бауэра, клетки среднего слоя эпителия тоже изменяются в связи с непрямым раздражением и токсическим воздействием. Реакция других слоев ткани незначительна, хотя изредка наблюдается эдематозное набухание membrana propria и подслизистой, гиперемия и слабая лейкоцитарная инфильтрация.

Материал и метод

Для того, чтобы разобраться в этих разноречивых данных, нами гистологически и гистохимически было обработано значительное количество мочевых пузырей щук (более 100 щук), зараженных *M. lieberkuhni*. Микроскопические срезы через пузырь окрашивались железным гематоксилином Гейденгайна, сулемовым раствором бромфенолового синего по Мезиа, Бреверу и Алферту, для выявления суммарного белка, метиловым зеленым-пиронином по Унна при pH 4.7, галоцианином и по методу Фельгена для выявления нуклеиновых кислот, кислым фуксином по Кулю и Альтману, позволяющему выявить митохондрии, суданом III для определения локализации нейтрального жира, а также подвергались реакции PAS на полисахариды и реакции на кислую фосфатазу по Гомори. Для выявления гиалуронидазы использовалась вискозиметрическая методика (Наточин 1959 а). Кроме того, приготовлялись срезы на ультрамикротоме и изучались под электронным микроскопом.

Результаты исследования и их обсуждение

Изучение обработанного перечисленными методами материала убедило нас в том, что с помощью „ножек" *M. lieberkuhni* проникает в межклеточные пространства эпителия мочевого пузыря, а не в клетки (Табл. I 1, III 9, IV 10). Лежащие в непосредственной близости к „ножке" эпителиальные клетки имеют нормальные ядра и часто остаются совершенно нетронутыми, хотя временами, благодаря механическому сжатию, раздвиганию слоев эпителия и отделению клеток от остальной ткани, действительно наблюдается гипертрофия клеток и отслаивание части эпителия. Электронномикроскопические исследования подтверждают факт внедрения плазмодия по межклеточникам и на приведенных микрофотографиях видно, что клетки непосредственно прилегающие к „ножке" паразита имеют нормальные ядра и цитоплазмы (Табл. III 9). На фотографии (Табл. IV 10) видно, что вырост цитоплазмы, обозначенный цифрой 1, раздвинул две смежные клетки, а там, где он кончается легко разглядеть примыкающие друг к другу оболочки клеток эпителия (стрелки). Вырост цитоплазмы, обозначенный цифрой 2, тоже внедряется в межклеточное пространство. На границе между цитоплазмой паразита и цитоплазмой эпителиальной клетки четко видна оболочка последней (стрелка).

Многими работами установлено, что ферментом способствующим проникновению ряда бактерий, паразитических простейших, личинок паразитических червей в ткани хозяина является гиалуронидаза (Davis 1936, Duran-Reynals 1942, Гинецинская 1950, Levert and Lee 1951, 1954, Stierwalt and Evans 1952, Evans 1953, Oshio and Furata 1955, Tempelis and Lysenko 1957, Успенская 1963 и др.).

Представлялось вероятным наличие гиалуронидазы и у *M. lieberkuhni*. Поэтому мы попытались выявить этот фермент, используя вискозиметрическую методику (Наточин 1959 а). При постановке опыта необходимо было иметь в виду, что моча щук сама по себе содержит большое количество гиалуронидазы (Наточин 1959 б) и нужна предварительная тщательнейшая отмывка от нее плазмодиев. Все попытки выявить гиалуронидазу у *M. lieberkuhni* не увенчались успехом и, при условии достаточно тщательной отмывки плазмодиев от мочи, она не была обнаружена. Этот, казалось бы, неожиданный результат легко объясним в свете данных, приведенных в работе Гинецинского (Гинецинский 1962) а том, что почечные кислые мукополисахариды у рыб не являются ни гиалуроновой кислотой, ни хондроитинсульфатом „С", а так как мочевой пузырь щук — простое расширение мезонефридиальных протоков, то, следовательно, и его мукополисахариды не подвергаются деполимеризующему воздействию гиалуронидазы. Таким образом, можно думать, что фермент, выделяемый „ножкой" должен отличаться от гиалуронидазы, хотя может быть близок к ней по характеру действия.

Факт внедрения плазмодия в межклеточники опровергает представление о том, что *M. lieberkuhni* питается клетками эпителия мочевого пузыря. Если „ножка" служит только для прикрепления, то ферментативная её деятельность должна сводиться к выделению гиалуронидазоподобного фермента, растворяющего мукополисахариды стенок пузыря. Если же мукополисахариды межклеточников утилизируются плазмодием, тогда всасывание их может происходить либо непосредственно через „ножку", либо, растворенные ферментами, выделяемыми „ножкой", вещества могут поступать в мочу, обогащая ее, а из мочи уже всасываются поверхностью тела плазмодия.

С помощью гистохимической методики было выяснено, что „ножка" PAS-отрицательна и, следовательно, не содержит полисахаридов. В ней так же не наблюдается скоплений жира, тогда как в теле плазмодия содержится большое количество обоих из указанных веществ. Реакция же на суммарный белок с сулемовым раствором бромфенолового синего наиболее интенсивна в „ножке", цитоплазма которой более компактна чем в теле. В „ножке" по методу Куля обнаруживается большое скопление митохондрий. Этот факт подтверждается и электронномикроскопическими данными. Поскольку митохондрии играют главную роль в продуцировании и накоплении энергии, то, видимо, в этой части плазмодия возникает повышенная потребность в энергии. Скопление митохондрий может быть связано с секреторной деятельностью „ножки".

Для того, чтобы понять значение этих фактов, важно было выяснить, какие участки плазмодия играют большую роль в усвоении питательных веществ. С этой целью мы считали целесообразным установить локализацию в плазмодии кислой фосфатазы — фермента, который обычно связывают с процессами клеточного питания: фаго- и пиноцитозом (Birns 1960, Seaman 1961, Novikoff 1961, Холтер 1962, де Дюв 1962, Müller, Töth and Törö 1962, Barka 1962, Сергеева 1963, 1964).

Мы подвергали плазмодиев обработке по методу Гомори в модификации, описанной в работе Сергеевой 1963. Оказалось, что фосфатазной активностью обладает лишь тело плазмодия, а „ножка" совершенно не дает реакции (Табл. II 5—6). Часто можно видеть особей, у которых основная часть тела забита спорами и неокрашена, тогда как неотделившиеся от основного ствола почки дают интенсивную реакцию на кислую фосфатазу (Табл. I 4). Таким образом, в разные периоды жизненного цикла разные части плазмодия принимают неодинаковое участие в питании. Тот факт, что „ножка" не обладает фосфатазной активностью, говорит о том, что всосывание питательных веществ осуществляется через тело плазмодия, направленного в сторону полости пузыря.

Выяснилась еще одна интересная особенность в распределении кислой фосфатазы. На поперечных и продольных срезах через плазмодиев, обработанных по методу Гомори, видно, что фосфатаза сосредоточена только по периферии клетки. Во внутренних же слоях цитоплазмы она совершенно отсутствует. Иногда слабокоричневую окраску дают некоторые ядра плазмодия, но вопрос о фосфатазной активности ядер требует специального изучения, и мы не будем на нем останавливаться. Такая локализация фосфатазы наводит на мысль, что гидролитическое расщепление некоторых веществ, поступающих из мочи, осуществляется вблизи поверхности клетки.

Поверхность плазмодиев *M. lieberkuhni*, подобно поверхности других миксоспоридий (Grassé 1960), сильно развита благодаря наличию микроворсинок. Как было выяснено с помощью светового и электронного микроскопов, ворсинки имеют 1—2.5 μ длины и от 0.1 до 0.5 μ в диаметре. У вегетативных плавающих особей они располагаются в шахматном порядке поодиночке или кустиками по 3—4, иногда между отдельными ворсинками группы видны анастомозы (Табл. IV 10, V 11, VI 13). У прикрепленных особей ворсинки обычно сосредоточены на конце, направленном к центру полости пузыря. Должно быть на разных этапах жизненного цикла и в разных участках плазмодия ворсинки могут исчезать и возникать вновь. Так, например, в местах возникновения почек ворсинки не обнаруживаются (Табл. V 12).

Наличие поверхности, покрытой микроворсинками, невольно наталкивает на сравнение её с поверхностью кишечника млекопитающих и наводит на мысль о возможности у миксоспоридий пищеварения по типу пристеночного. В самом деле, ворсинки *M. lieberkuhni* по размерам близки к размерам, известным для ворсинок кишечников млекопитающих (Уголев 1962). Внутри ворсинок проходит каналец (Табл. V 12), что согласуется с описанием микроворсинок кишечника, данным Гольди и Шестопаловой (цит. по Уголеву 1962). На срезах через плазмодиев, обработанных по методике Гомори, видно, что кислая фосфатаза как раз сосредоточена в зоне ворсинок, что вполне соответствует её распределению в кишечнике млекопитающих. Более того, в литературе есть данные, что такой фермент как кислая фосфатаза, может локализоваться и на внешней поверхности мембраны ворсинок (Ogawa et al. 1962).

Однако расположение микроворсинок у *M. lieberkuhni* отличается от расположения их в щеточной кайме кишечника млекопитающих. В последней расстояние между ворсинками равно нескольким сотням Å, на основании чего Уголев рассматривает щеточную кайму как пористый катализатор (Уголев 1962). У *M. lieberkuhni*, как уже говорилось, ворсинки располагаются в шахматном порядке, по одной или группами, и, как это можно видеть под электронным микроскопом, в большинстве случаев расстояние между ними в 2 и более раз больше чем высота ворсинок. И хотя ворсинки подвижны и могут сбли-

жаться и раздвигаться, вряд ли такую поверхность можно рассматривать как пористый катализатор. Возможно, что в случае *M. lieberkuhni* именно такое, сравнительно редкое, расположение ворсинок оказывается более выгодным. Если считать, что в конечном счете, *M. lieberkuhni* получает пищу из мочи, которая менее богата питательными веществами и более жидкая среда чем химус, здесь может быть важна непрерывная смена порций мочи, омывающей поверхность плазмодия. В таком случае слишком тесное расположение ворсинок препятствовало бы свободному току жидкости и способствовало бы образованию застойной зоны. Для миксоспоридий, обитающих в мочевом пузыре, принцип пористых катализаторов может и не иметь значения. Однако для того, чтобы рассуждения не были спекулятивны, нужно точно знать, какие вещества, находящиеся в моче, используются *M. lieberkuhni* для питания.

Уголев 1962 отмечает, что щеточная кайма является универсальной структурой широко распространенной в животном мире. Основываясь на данных о локализации ферментов на поверхности клеточной мембраны дрежжей и бактерий он приходит к выводу, что пищеварение за счет ферментов, сорбированных на внешней поверхности клеточных мембран, возникло в эволюции значительно раньше, чем появились микроворсинки. Возникновение же ворсинок — важный, но более поздний этап в эволюции пристеночного пищеварения.

Таким образом, можно себе представить, что развитие поверхности клетки шло различными путями, вырабатывая в каждом отдельном случае свои приспособления. Так, у миксоспоридии *Sphaeromyxa sabrasasi*, тоже живущей в мочевом пузыре рыбы, согласно исследованиям Грассе (Grassé 1960), микроворсинки образуют между собой анастомозы, благодаря чему возникает ячеистая или сетчатая структура. Иначе выглядит поверхность опалин (Noirot-Timothée 1959) и грегарин (Kummel 1958), где образуется продольная микроскладчатость или гребенчатость.

Все эти изменения поверхности клетки, видимо, являются различными путями эволюции, ведущими к одной цели — к увеличению клеточной поверхности в связи с осмотрофным питанием.

Полученные нами предварительные данные требуют дальнейшей разработки и уточения, как имеющие значение для понимания механизма питания у миксоспоридий. И хотя приведенные факты достаточно убедительно показывают, что в конечном счете, питательные вещества поступают в тело плазмодия из мочи, тем не менее остается неизвестным из каких веществ, находящихся в моче, синтезирует плазмодий гликоген, жиры, белки и нуклоиновые кислоты, присутствующие в значительном количестве в цитоплазме и ядрах паразита. Не ясно также каково произхождение кристаллов, постоянно встречающихся в цитоплазме, начиная с самых первых дней существования вегетативных особей. Со времен Бючли считается, что это кристаллы гематоидина (Bütschli 1881). Возможно, что в питании *M. lieberkuhni* какую-то роль играет слизь, выделяемая железами пузыря и окружающая плазмодиев. Все эти вопросы подлежат еще выяснению.

Резюме

Плазмодии *M. lieberkuhni* внедряются в эпителий мочевого пузыря по межклеточникам с помощью особых плазматических выростов — „ножек". Однако попытки обнаружить у плазмодиев гиалуронидазы не увенчались успехом, что объясняется отсутствием в межклеточниках мочевого пузыря щук гиалуроновой кислоты или хондроитинсульфата „С", которые здесь заменены какими-то дру-

гими мукополисахаридами. Кислая фосфатаза локализуется лишь в теле плазмодия, находящемся в полости пузыря, тогда как ножка совершенно не обладает фосфатазной активностью. В связи с этим делается вывод, что всасывание пищи осуществляется не через ножку из тканей хозяина, а через поверхность тела плазмодия из мочи. Поверхность тела плазмодия покрыта микроворсинками. Кислая фосфатаза сосредоточена по периферии тела плазмодия в зоне микроворсинок. Наличие поверхности, покрытой микроворсинками и локализация в их зоне кислой фосфатозы, дают возможность предложить, что питание миксоспоридий осуществляется по типу пристеночного. Однако, расположение микроворсинок у *M. lieberkuhni* существенно отличается от расположения их в щеточной кайме кишечника млекопитающих. Строение поверхности *M. lieberkuhni* также отличается от строения поверхности других миксоспоридий, опалин и грегарин, что говорит о различных путях эволюции, ведущих к одной цели, к увеличению поверхности в связи с осмотрофным питанием.

SUMMARY

Data concerning the mode of nutrition of *Myxidium lieberkuhni* (Bütschli) are reported. Plasmodia of *M. lieberkühni* were found to penetrate the epithelium of the urinary bladder of the host (white-spotted pickerel *Esox lucius*) through the intercellular space by means of special cytoplasmic outgrowths or "roots". However, all the attempts to find hyaluronidase in plasmodium gave no results. The lack of both hyaluronic acid and chondroitin sulphate "C" in the intercellular space of the urinary bladder of fish is supposed to account for the latter fact (both substances are known to be substituted in fish by some other mucopolysaccharides). Acid phosphatase, the enzyme closely connected with cell nutrition (pinocytosis and phagocytosis) was demonstrated only in the body of plasmodium. The cytoplasmic "roots" revealed no phosphatase activity. This fact allows to drow the conclusion that the absorption of nutrients by plasmodium is realized not through its "roots" from the host's tissue, but through the body surface from the host's urine. The plasmodium has a number of microvilli spread throughout the body surface. Acid phosphatase is localized on the perifery of the body in the zone of microvilli. The presence of the surface covered with microvilli and the localization of acid phosphatase in region of microvilli seem to substantiate the supposition that the nutrition of *Myxosporidia* may be realized like the contact digestion of higher animals. However, the exact arrangement of microvilli in *M. lieberkuhni* essentially differs from that in the brush border of the mammalian intestine. At the same time, the structure of the body surface in *M. lieberkuhni* is not alike that of *Opalina*, *Gregarina* and of other *Myxosporidia* which allows to speculate on the possibility of different ways of evolution leading to one and the same result — enlargement of the absorbing surface related to the osmothrophic nutrition.

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ПОДПИСИ К ТАБЛИЦАМ I — VI

1: Поперечный разрез стенки мочевого пузыря щуки с внедрившимся в нее плазмодием *M. lieberkuhni;* железный гематоксилин (× 800)

2—4: Локализация кислой фосфатозы плавающих особей *M. lieberkuhni* (2 — × 400, 3 — × 200, 4 — × 250)

5—6: Локализация кислой фосфатазы у прикрепленных особей *M. lieberkuhni* (5 — × 400, 6 — × 200)

7—8: Локализация кислой фосфатазы в зоне микроворсинок (×1800); 7 — продольный разрез, 8 — поперечный разрез

9: Эпителий мочевого пузыря щуки с внедрившимся в него плазмодием *M. lieberkuhni* (× 14500)

10: Эпителей мочевого пузыря щуки с внедрившимся в него плазмодием *M. lieberkuhni* (× 10250)

11: Расположение микроворсинок на поверхности плавающей особи *M. lieberkuhni* (× 22400)

12: Поверхность *M. lieberkuhni* в месте образования почки (× 22400)

13: Микроворсинки прикрепленной особи *M. lieberkuhni* на конце направленном в полость мочевого пузыря щуки (× 21500)

EXPLANATION OF PLATES I—IV

1: Cross section of urinary bladder of *Esox lucius* with penetrated *M. lieberkuhni* (× 800)

2—4: Activity of acid phosphatase in attached specimen of *M. lieberkuhni* (2 — × 400, 3 — × 200, 4 — × 250)

5—6: Activity of acid phosphatase in unattached specimen of *M. lieberkuhni* (5 — × 400, 6 — × 300)

7—8: The localization of acid phasphatase in the zone of microvilli in *M. lieberkuhni* (× 1800); 7 — longitudinal section, 8 — cross section

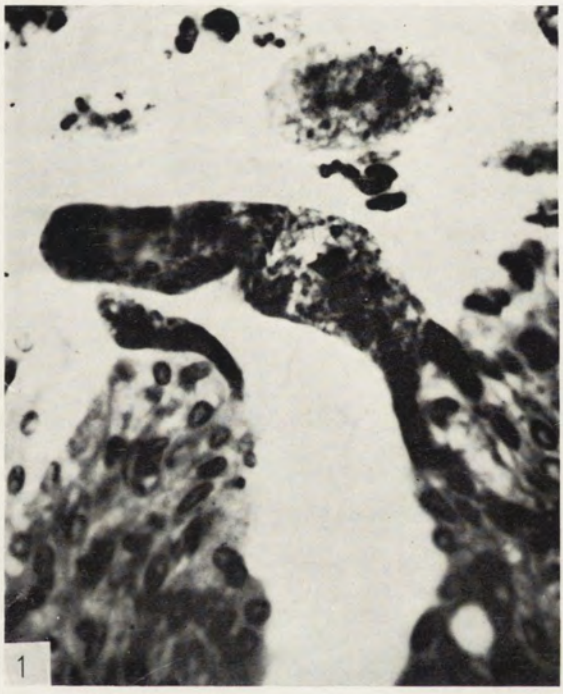
9: Epithelium of urinary bladder of *Esox lucius* with penetrated *M. lieberkuhni* (× 14500)

10: Epithelium of urinary bladder of *Esox lucius* with penetrated *M. lieberkuhni* (× 10250)

11: Distribution of microvilli on the surface of unattached specimen of *M. lieberkuhni* (× 22400)

12: The surface of *M. lieberkuhni* at the place of bud formation (× 22400)

13: Microvilli on distal end of attached specimen of *M. lieberkuhni* (× 21500)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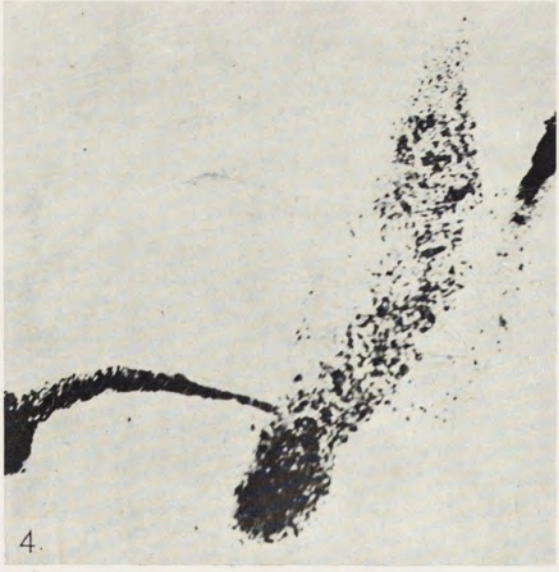
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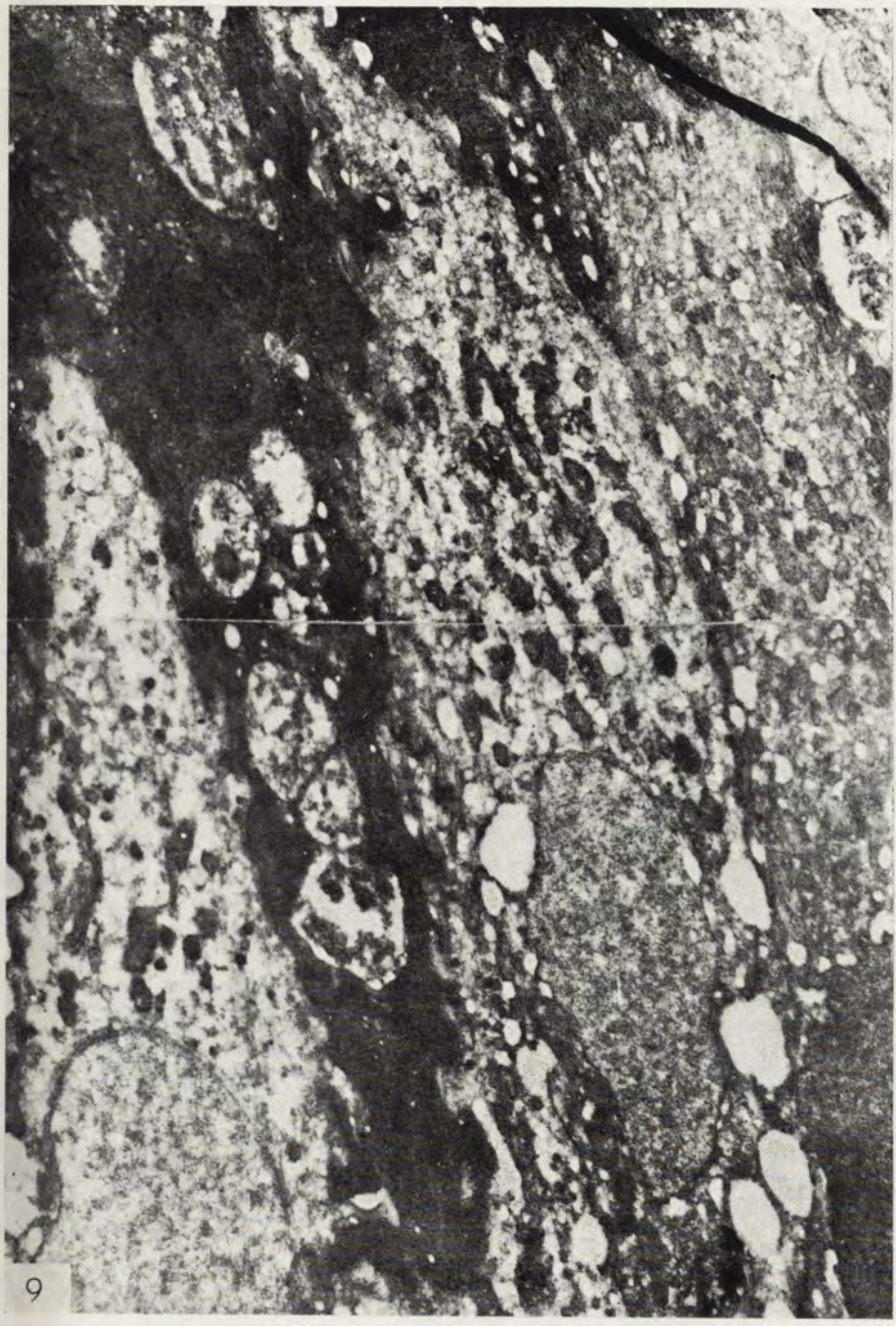

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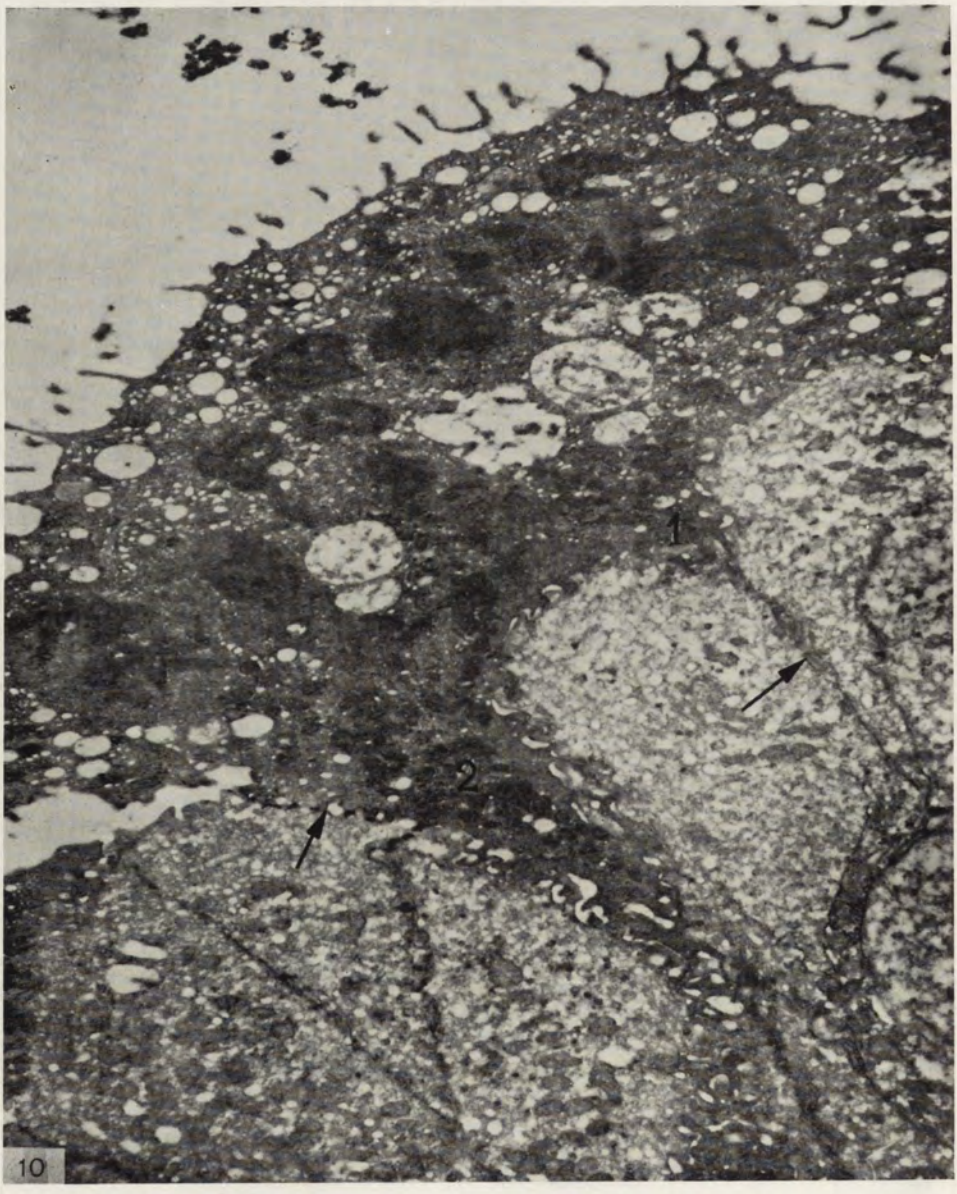

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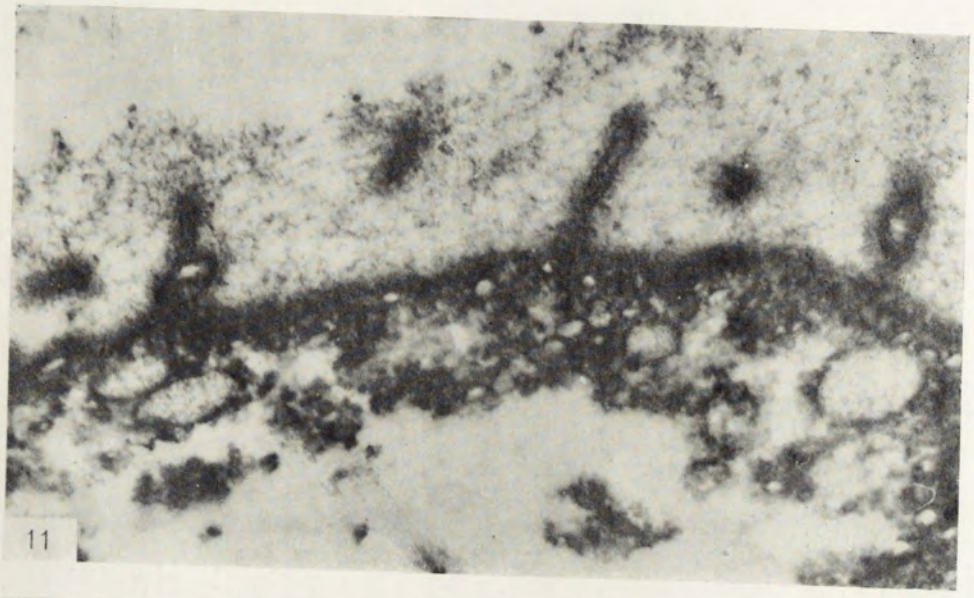

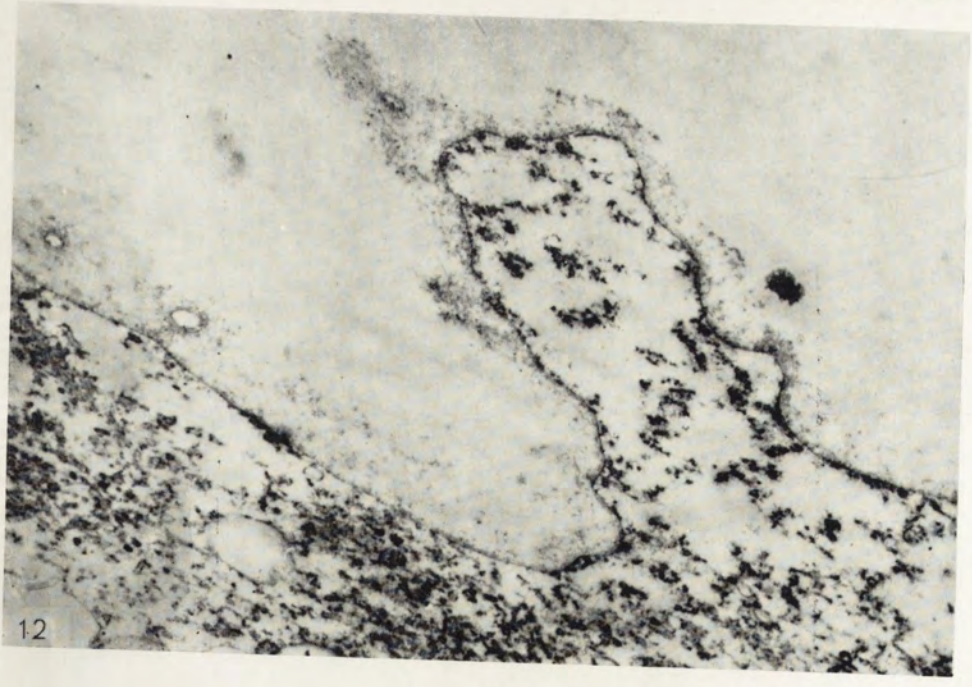

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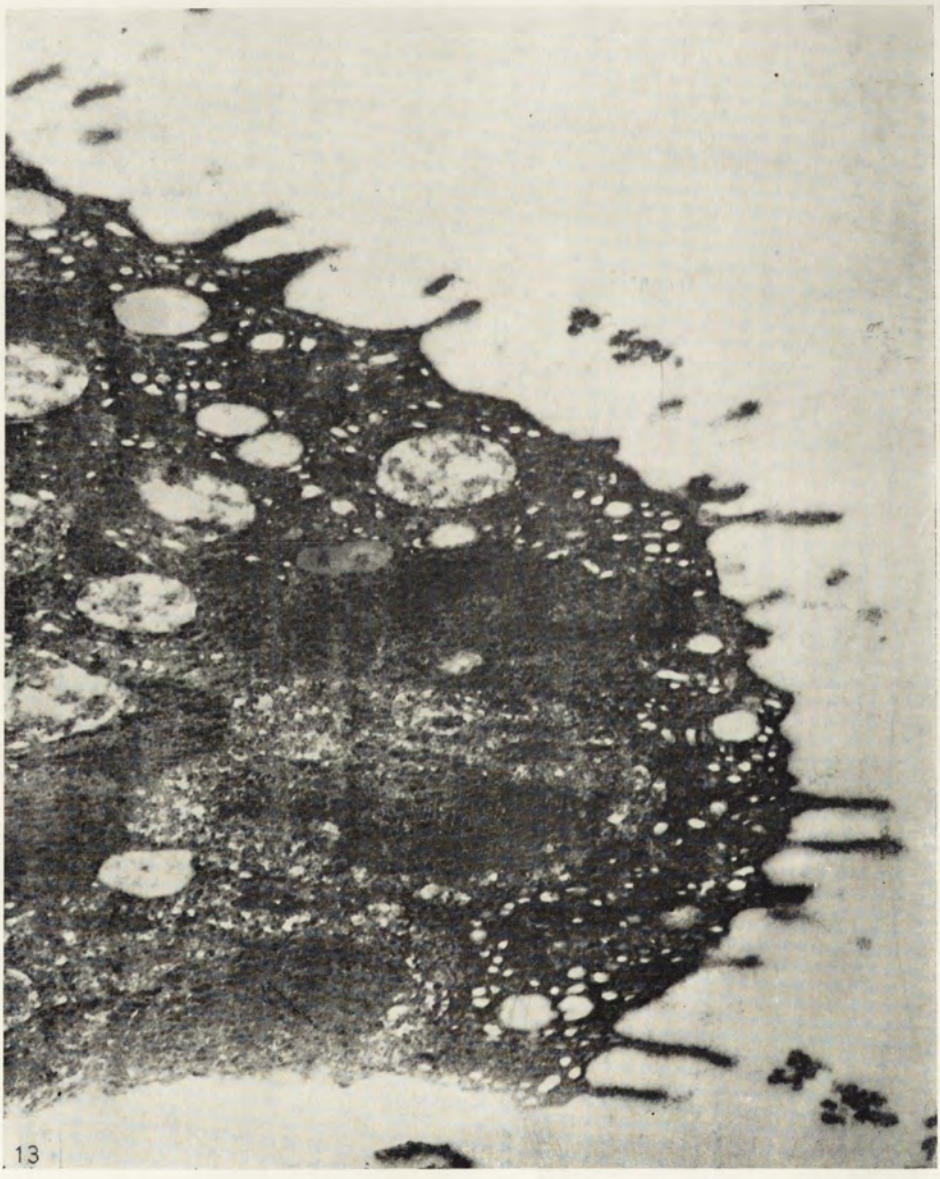

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