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THE NENCKI INSTITUTE OF EXPERIMENTAL BIOLOGY

The Nencki Institute of Experimental Biology was founded in 1918/1919 shortly after Poland regained its independence. It was based on three pre-existing laboratories affiliated with the Scientific Society of Warsaw (Towarzystwo Naukowe Warszawskie): Laboratory of Neurobiology (functioning since 1911), Laboratory of Physiology (since 1913) and Laboratory of General Biology (formed in 1918). The formation of the Institute and the extension of its laboratories was considerably promoted by a donation in 1909 from Nadine Sieber-Shumova, a close co-worker of Marceli Nencki (1847-1901) from Berne and St. Petersburg.

The Institute, named after Marceli Nencki, soon became the leading research establishment in the country. Between 1920 - 1937 it was supplemented with the Departments of Experimental Morphology and Biometry and field stations on the Hel Peninsula (Baltic Sea), at Lake Wigry and on the River Pripet. Although the Institute continues to be associated with the Scientific Society of Warsaw and was thus formally independent of Warsaw University, it was in fact closely related to this school, its scientific staff being mostly recruited from university professors and assistants, and its laboratories and field stations being available to university students.

Immediately after the war, the surviving members of the Nencki Institute re-established the Institute and chose Łódź for its temporary seat. The post-war Institute was initially composed of three Departments, namely that of Biochemistry (headed by Włodzimierz Niemierko), Neurophysiology (Jerzy Konorski) and Biology (Jan Dembowski, who was also appointed the first Director of the whole Institute). In 1952 the Institute was in the newly founded Polish Academy of Sciences, and Professor Dembowski became the first President of the Academy. During 1953 - 1955 the Institute's Departments were gradually transferred to a new building in Warsaw.

The Institute is the largest non-university establishment for basic biological research in Poland. The Institute awards annually about ten doctor's degrees in biological sciences. Broad contacts of the Institute with scientific institutions and organizations all over the world enable its young scientists to spend their research tenure, usually of one or two years, in leading laboratories abroad. Numerous foreign scientists visit the Institute to deliver lectures and seminars, discuss problem of mutual interests and, sometimes, perform research work in the Institute's laboratories.

In 1990 the Nencki Institute was invited to become a member institution of a newly founded organization - Global Network for Molecular and Cell Biology (MCBN) within UNESCO. Together with a second Polish member - institution, the Institute of Bioorganic Chemistry in Poznań, the Nencki Institute recently initiated the formation of the Polish Network of MCBN, to which over 50 other Polish scientific centers declared their participation.

In its present shape the Nencki Institute (directed by Maciej Nałęcz) consists of four Departments: Cellular Biochemistry (headed by Barbara Grzelakowska-Sztabert), Cell Biology (Stanisław Fabczak), Muscle Biochemistry (Renata Dąbrowska), Neurophysiology (Bogusław Żernicki), and three separate Laboratories: Cell and Tissue Culture, Computer Programming and Electron Microscopy.

The Nencki Institute was the host and organizer of the VIth International Congress of Protozoology in 1981.

Editor

ACTA PROTOZOOLOGICA

The Contributions of Women to the Science of Protozoology

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Summary. Although women scientists have played a role in the development and advance of the discipline of protozoology, their impact and their visibility have been relatively minor, at least until the middle of the present (20th) century. Reasons for this are explored here, and historical data are presented that indicate the continuing dominance still today of male biologists as first authors in the published protistological literature. Nevertheless, protozoology, and the biological sciences in general, seem to offer more rewarding research opportunities for women than do such fields as astronomy, chemistry, physics, and mathematics.

Key words. Women, protozoology, history of protozoology.

INTRODUCTION

One year ago, the weekly journal Science published a thought- provoking 26-page section on "Women in Science" (Benditt 1992), presumably the first in an annual series on this important subject. It is clear that some scientific fields or areas remain far more discriminatory with respect to the involvement of non-males in research leadership positions - whether intentionally so or not - than biology (e.g., abundant data exist showing this to be true for astronomy, mathematics, physics, engineering, chemistry, and medicine). Nevertheless, "subtle obstacles and unconscious assumptions are taking the place of explicit sexism" (Benditt 1992), and even in areas of the biological sciences the ratio of successful men to women seems to continue to be tipped unfairly in favor of male researchers.

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The objective of the present paper, however, is not to bewail such inequity philosophically nor to offer solutions to the complex problem. Rather, here I wish to present a brief historical review of the place of women in the growth and development of the specific biological field of protozoology/protistology, supported by data on the relative numbers of female versus male workers/researchers in that area over the past 300 years, arbitrarily using a cut-off date of ca. 1950. [In May 1992, I spoke on this topic in an invited paper given at the Third East Coast Conference on Protozoa, Mount Vernon College, Washington, D.C.: see abstract by Corliss 1993].

THE PRE-TWENTIETH CENTURY PERIOD

Virtually no woman appears to have been recognized for direct (i.e., published under her own name with or without other authors) contributions to protozoology before the present century. Surely persons of the female persuasion must have been involved in some early re-

search efforts, if only in a minor supporting role, but they received no credit for it in conventional ways. Recall that A. van Leeuwenhoek, Dutch lens-grinder today considered "father" of many fields, including bacteriology, haematology, parasitology, and protozoology (see Dobell 1932), thanks his loving daughter Maria for her help. His researches took place more than 300 years ago, and we shall never know the degree - if any - that Maria might have been involved in direct handling of any scientific material for her father.

Historians of protozoology make little reference to female workers in the field, and none for any period of time before the 1900s. Cole (1926) includes no women among his 142 citations to past leaders in the area. In my own (Corliss 1992b) recent chronicle of the first 300 years of protistology, covering the span of time from roughly 1650 to 1950, only 20 works (< 5%) are mentioned in which women were first authors out of 442 references to "firsts" in the field and to major monographs of extended significance. And these involve only a scant dozen different persons, the majority of whose papers cited actually have post-1950 dates, with none earlier than 1916. In a preceding survey of "great microscopists of the past" (Corliss 1978-1979), I had called attention to half a dozen early protozoological contributions by women, papers bearing dates between 1901 and 1913.

Although there was an explosive growth in both phycology and protozoology during the second half of the 19th century and although there were undoubtedly female assistants working in the laboratories of the great male leaders of the time, the names of such "junior colleagues" seldom, if ever, appeared on any published papers of note. This fact is confirmed by perusal of the entries in the "Protozoa" section of pre-1900 volumes of the Zoological Record.

THE TURN OF THE CENTURY

In the very late 1800s and early 1900s, women were finally enabled (allowed?) to enjoy a limited degree of independence, probably long overdue, in participation in zoological and protozoological research: the Age of Enlightenment had arrived?

Yet Goldschmidt (1956), in his perceptive book on the development and growth of zoology in Germany during the period roughly 1865 to 1915 (based mostly on firsthand observations during his own training there), very briefly mentions the names of only four women among some 200 males (this equals 2%). Actually, the great German institutions of research deliberately used to exclude females, even as students: two of Goldschmidt's tales that illustrate this point are relevant here:

In one instance, a determined woman entered a classroom and joined the male students in awaiting arrival of the distinguished professor. When the master strode into the room moments later, he immediately placed himself in front of the young lady and bellowed out, "What! A woman attending my lecture?!?" While the lads laughed insensitively, the mortified girl lifted her skirts and fled, never to be seen there again.

Another story of his has a more favorable ending. Shortly before the turn of the century, the renowned but socially shy Theodor Boveri was embarrassed to find a woman, an American student, in his class one day; but, reluctantly, he allowed her to stay. A year later they were married! Frau Marcella O'Grady Boveri was an excellent cytologist, however, in her own right; and after husband Theodor's untimely death, she returned to the United States to accept a professorship of zoology at Albertus Magnus College, next door to Yale University in New Haven, Connecticut, actively continuing her researches there.

But across Europe and in America as well, women were commencing to take graduate degrees in biology in considerable numbers by the early 1900s, and protozoology apparently appeared to be an attractive field in which to work. With rare exception, however, research results were published with the (male) professor-in-charge as senior author; and subsequently seldom did the woman become - or seem to have an opportunity to become - an independent leader in her area of expertise.

Examples of women whose contributions to protozoology during the first 40-50 years of the present century have gained them a lasting place in the literature will be considered in later sections of this paper, following presentation of some statistical data immediately below.

DATA FROM SURVEYS OF THE LITERATURE

While it is difficult to determine the percentage of women who may have started, or had the potential to start, professional careers in protistology in times preceding the Age of Computers and Polls in which we now find ourselves, it has not been too arduous a task to undertake a comparative survey of the literature (e.g., via authorships and photographs) in such a relatively restricted field as protozoology.

Turning to Copeland's (1956) authoritative monograph on the classification of "the lower organisms" (essentially the protists of today: algae, protozoa, and "lower" fungi: see Corliss 1984), we find that in its 820 bibliographic references, women appear as senior authors of only 34 (ca. 4%). Even fewer female names, however, occur as junior authors: most of Copeland's citations, curiously, are to single-authored works. As far as protozoology sensu stricto is concerned, however, I should point out that the great bulk of the 34 papers just mentioned are those of women who carried out research in the fields of phycology and, especially, mycology (perhaps showing a bias on Copeland's part).

R. R. Kudo's textbooks have long served America, and indeed much of the rest of the world, as the most convenient source of basic information on the protozoa. This is true even today, nearly 30 years after the appearance of his fifth and last edition (Kudo 1966). He has some 1575 entries in the Author Index of his last treatise, a section occupying 22 full pages of print! Helpfully (from the point of view of the present survey), he provided the first names (prenames) of (only the) female authors listed. Kudo was occasionally unaware whether an author was male or female, so I have depended on my own knowledge of the identity of protozoologists, before making the counts reported here, to "correct" some of the supposed "male" names to the females actually represented. Including second and third, as well as first, positions among the authorships, some 120 women's names are cited by Kudo throughout the literature references that appear at the ends of his various chapters. On the other hand, 1455 names of male workers are included: thus, only ca. 7.5% of the protozoologists mentioned are women. His literature citations, admittedly, cover the period running roughly from 1850 to 1950; but, even if one eliminates all of his pre-20th century authorships, the ratio of women to men would not be much higher, because most of his references are to post-1900 books, monographs, and specific research papers.

Consulting a considerably more recent publication, my own "people-illustrated" book on ciliates (Corliss 1979), one discovers that, of the 185 photographs included, only 18 were of individuals of the female gender (9.7%). If one excludes the 68 oldest persons portrayed, which includes three women, the percentage rises to 12.5%; if one excludes only the 18 very oldest (pre-20th century) men, the percentage drops again, to 10.8%. Thus, among distinguished ciliatologists, it appears that only ca. 10% have been women, up until the year 1975,

at least. In my pictorial footnote to the history of all protozoology (Corliss 1978-1979, published at about the same time, and already alluded to in the first section of this paper), of the included 69 photographs of greatest microscopists of the past only two (thus a scant 3%) were of women. Thus, either ciliatology is a more attractive subfield of protozoology/protistology for women or some subtle biases on the part of the writer are being revealed! But also it should be pointed out that a "requirement" for appearance in the historical review was for the person to have died; in the ciliate book, photographs of living persons (some still today, 15 years later), men and women, were included as well. Availability of portraits is another factor to be appreciated, of course, in both of the above publications. Thus the percentages cited must be considered as rather subjective estimates of the "true" numbers involved.

In my most recent, and lengthiest, consideration of the history of protozoology sensu lato (Corliss 1992b), as mentioned on a preceding page, women leaders/innovators were conspicuous by their absence: very few senior-authored papers by females were cited among the 442 references to the covered literature up to about the year 1950. Male domination of the field, rightly or wrongly, seems to have been a fact and may yet be largely the case (see subsequent sections, below).

For still another approach to assessing the (potential) impact or role of women in protozoology, let us look at their participation in professional societies, the most logical one to consider being the international Society of Protozoologists, founded in 1947. With respect solely to membership, female workers have long been quite well represented. As of June 1953, exactly 40 years ago, there were 58 women out of a total of 412 active members (14%). The percentage has gradually risen, so that in the Membership Directory of 1990-91, we find some 200 women out of a total of 1030 - therefore, close to 20%. Thus, the number in, or contemplating, careers that include at least some interest in the protozoa would appear to have grown significantly. Perhaps more importantly, in recent years we have witnessed a large-scale entrance of women into the Society's officerships, editorships, and committeeships: in the so-called "good old days" (e.g., 1953 and preceding years, and for a number of the succeeding years), the number of women in such positions stood at zero.

As a final survey, I have carefully checked the contents of the Journal of Protozoology for the years 1991 and 1992, noting the sex of sole and first authorships of the 213 included papers. A solid 24% of them have

shown women in those "senior" positions. Although numerous contributors (of both sexes) to the Journal are not members of the Society, this figure happens to match quite closely the percentage of women given above with respect to active membership: in both cases, from a fifth to a quarter of the protozoologists involved are female.

WOMEN OF NOTE, PERIOD 1900-1925

To return to our chronological approach, during the first quarter of this century, as indicated above, female students were becoming more abundant in colleges and universities, and quite a number were entering graduate schools for advanced degrees in the biological sciences. In Europe, especially, great centers of protozoology had been developed in the late 1800s, and women were finally being accepted into these, recognized as possessors of intellects and talents easily matching those of the men of the times. A few of these early stalwart souls, from various parts of the world, were even publishing single-authored papers close to the turn of the century. A selected score of such female workers are mentioned by name in following paragraphs. Their research productions represented - and still represent - significant contributions to the protistological literature, even if most of them are not monographic in scope or may not be important historically as "firsts" in their fields. These same points, obviously, can be made concerning the great bulk of scientific papers published by men, too, in the past and still today.

Examples of unusually outstanding women in protozoology in Europe during the first 25 years (and often beyond) of the 1900s should include Clara Hamburger (highly praised in Goldschmidt's recollections of 1956: see especially her monographs of 1911 and 1913, published with von Buddenbrock on planktonic protozoa), Rhoda Erdmann, and Frieda Feulgen-Brauns of Germany; Muriel Robertson (the first woman to do extensive studies on the trypanosomes), Helen Goodrich, Annie Porter (wife of H. B. Fantham, but his superior in protozoological abilities, in my opinion), Margaret Jepps (co-author with the irascible Clifford Dobell on occasion), Marie Lebour (phycologist of note), and Sister Monica Taylor of Great Britain; Mme M. Chatton of France; and Anna Foà of Italy. I had the privilege of knowing several of these researchers personally, and I was always tremendously impressed by their broad knowledge and their demonstrated prowess in protozoology.

The situation in Germany warrants special - if very brief - mention here. Many nationalities of protozoologists (men and eventually a few women) received at least part of their training in the celebrated institutions of that country during the last decades of the 19th and well into the 20th century. Renowned as teachers as well as prodigious producers of original and often monographic research works, the following men of giant stature deserve having their names recorded here: Johannes Müller of Berlin (passed away in 1858; but the following men lived well into the present century), Otto Bütschli of Heidelberg, Ernst Haeckel of Jena, Richard Hertwig of Munich, Max Hartmann of Berlin-Dahlem, Franz Doflein of Munich and Freiburg, and Eduard Reichenow of Hamburg.

In America, where institutions sponsoring protozoological research were only starting up in the early part of this century (see following section), few young people of either sex had yet had an opportunity to carry out much independent research: the older persons, the leaders (all males), had often received their own training in the great laboratories of Europe, especially Germany, as mentioned above. But in the U.S.A., a scattering of women were authoring memorable papers on their own before the year 1925 (or slightly thereafter): for example, Libbie Henrietta Hyman (better known, however, for her later authoritative volumes on the invertebrates), Mary Scott MacDougall, Olive Swezy (prolific co-worker, often, with C. A. Kofoid of Berkeley), Mary J. Hogue, Nettie Maria Stevens, Edith A. Pickard, Dora P. Henry, Lucy Graves Taliaferro, and Minnie Watson Kamm (who single-handedly was the first producer of American monographs on the gregarine sporozoa: see Kamm 1922, Watson 1916).

WOMEN OF NOTE, PERIOD 1925-1950

In Europe, especially in Germany, France, and England, the number of women active in protozoological laboratories increased dramatically during the second quarter of the present century, judging from the numbers of published works bearing female authorships. However, it should be kept in mind that there was also a growing increase in numbers of papers generally, so that the ratio of female to male productivity probably seldom rose above one to 10.

By 1950, the great diversity of subfields of protistology becoming of research interest to biologists -

physiologists and biochemists as well as cytologists, microscopists, ecologists, evolutionists, and systematists - increased career opportunities for both sexes. Dozens of young women became involved, publishing alone or, usually, with their male mentors. There are too many European women of note during the second quarter of the present century for me to attempt an equitable listing of their names here. But I shall, nevertheless, single out for special mention four highly productive British women: parasitologists Ann Bishop and Doris Mackinnon and phycologists Irene Manton and Mary Parke, leaders in their fields and trainers of additional women (and men) of distinction in subsequent years. And in France, much of the increase in numbers of outstanding women in protozoology during this period of time stemmed from the exciting centers of research established by (male) leaders such as E. Chatton, E. Fauré-Fremiet, P.-P. Grassé, and R. Hovasse.

In America, within a few years after the turn of the century, three highly productive centers of protozoological teaching and research were established under the well-organized leadership of men trained at least in part in the great zoological institutions of Germany (see Corliss 1992a,b, for details). These were Columbia University, in New York City, residence of Gary Nathan Calkins; University of California at Berkeley, with Charles Atwood Kofoid; and Johns Hopkins University, Baltimore, Maryland, working place of Herbert Spencer Jennings, Robert Hegner, and S. O. Mast. Not long after, centers sprang up at the University of Pennsylvania (David H. Wenrich), Yale University (Lorande Loss Woodruff), Chicago (William F. Taliaferro), Illinois (Richard Roksabro Kudo), Harvard (L. R. Cleveland, J. A. Cushman), New York University (R. P. Hall), and still others across the whole country. Graduate training became the emphasis, and numerous women were among the successful masters and doctoral degree candidates, especially in the fruitful periods of the 1920s, 1930s, and 1940s (and beyond).

Nevertheless, with a few exceptions, the great majority of women carrying out researches under male professors in these locations published little after obtaining their degrees; and, on most of their papers that did appear, they were junior authors. Interestingly, Calkins, who published with a number of his female graduate students, invited no women to be among the 20 contributors to the celebrated and highly influential 1148-page "blue bible" of research in protozoology (see Corliss 1992a) of which he was organizer and principal editor (Calkins and Summers 1941).

THE PAST 45 YEARS AND THE FUTURE OUTLOOK

Although the decades of the second half of the present century are arbitrarily beyond coverage in this historical survey (and, indeed, space would also prohibit their inclusion in any depth), there are clear indications that the ratio of one to 10, or even less favorable, for women versus men active in protozoological research which obtained during the first half of the 1900s has improved dramatically in the years since ca. 1950.

In America, especially, several specific factors have undoubtedly contributed to such a change. More young people, in general, have been going to college and on to graduate school; more women have been entering the sciences (the biological ones, at least); and research on protozoa has become more fashionable, more attractive, extending well beyond the traditional morphological-taxonomic areas with the development/application of such modern techniques as electron microscopy and of biochemical/molecular and genetic approaches to studies of all kinds of cells. Certainly the heuristic volume by Calkins and Summers (1941), despite its contributors' being exclusively male, significantly brought about an increased entrance of young people of both sexes into protozoological research, not only in America but probably also abroad (Corliss 1992a, b). Then, too, in the post-World War II boom in the U.S.A., monies were becoming available to buy research equipment, even to outfit entire experimental laboratories. Universities were offering increased numbers of teaching and research assistantships and fellowships, and many leadership roles in protozoology were passing from the traditional hands of Europeans to equally qualified researchers in the New World.

In figures given in earlier sections, we have seen that some 20% of the membership in the Society of Protozoologists is occupied by women and that a quarter of the senior authorships in papers appearing in recent numbers of the Journal of Protozoology (which is now, starting in 1993, named the Journal of Eukaryotic Microbiology) are female. A number of women run their own research laboratories in American (and other) universities, training both male and female graduate students and postdoctoral fellows. Women hold important positions in both national and international organizations. It is significant that they will be playing leadership roles in the IX International Congress of Protozoology to be convened in Berlin, Germany, in July-August 1993.

Still, one may question whether or not women protozoologists are always given the same opportunities or the same powerful/influential positions (with comparable salaries, equipment, space, etc.) as their male counterparts. Are factors of a "subtle sexism" still quietly in operation? Female membership in the Society of Protozoologists, considering the large numbers of young women who seem to be enrolled in courses of protozoology and cell biology around the world today, appears to me to be low. But why should professional careers in such areas be less attractive to women? While there are outstanding exceptions, in general, research laboratories continue to be headed by males. Are women mentors and "role models" thus of insufficient numbers to help increase female participation in protistological research projects? This is a challenge to men and women alike who are in positions to cooperate on solutions to such persisting problems, finding ways and means to enlarge the opportunities for women everywhere to play a still more significant part in the continued development and the future advances of protozoology/protistology.

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ACTA PROTOZOOLOGICA

Protistan Evolution and Phylogeny: Current Controversies

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Summary. The date for the origin of unicellular eukaryotes (protists) is difficult to establish. In order to reconcile some diagnostic fossils and molecular biological data we suggest that the major monophyletic groups of protists appeared no later than the Late Archean time. A second crucial question is focused on controversies from classical vs. molecular data on phylogenetic relationships between species and a third controversy centers on the patterns of protistan speciation and adaptive radiation.

Key words. Age of protists, molecular evolution, paleontological data, extinction, diversity.

INTRODUCTION

As early as 1866 in his "Generelle Morphologie der Organismen", Ernest Haeckel elevated the protists to the rank of kingdom, a proposal supported more recently by Corliss (1984) and others. There is little doubt that the Kingdom Protista with its 45 phyla (Corliss 1984) represents a polyphyletic taxon. Since these microscopic organisms also represent the oldest eukaryotic forms known thus far in the course of evolution, a better understanding of the phylogenetic trends among protists may lead us to a better understanding of evolutionary processes in all living systems.

Although some researchers believe that fossils tell us nothing about the evolutionary process, the study of evolution is as much about pattern as about process, and

pattern may have an important bearing on process. In the last twenty years, both molecular biology and paleontology have markedly changed our understanding of protistan evolution and phylogeny. Paleontologists have divided into two major opposing groups, one group supporting the punctuated equilibrium model of Eldredge and Gould (1972), the other supporting phyletic gradualism. Also, the molecular diversity among unicellular eukaryotes raises questions about when proliferation of major protistan groups may have occurred. In the ciliates for example, the extent of divergence is greater than the separation between plants and fungi (Fig. 1). Yet, this large-scale genetic diversity apparently has had little or no effect on the morphology and physiology of the developing species.

HOW OLD ARE THE PROTISTS?

In order to reconcile the data from the fossil records and from the molecular clock hypothesis, we must con-

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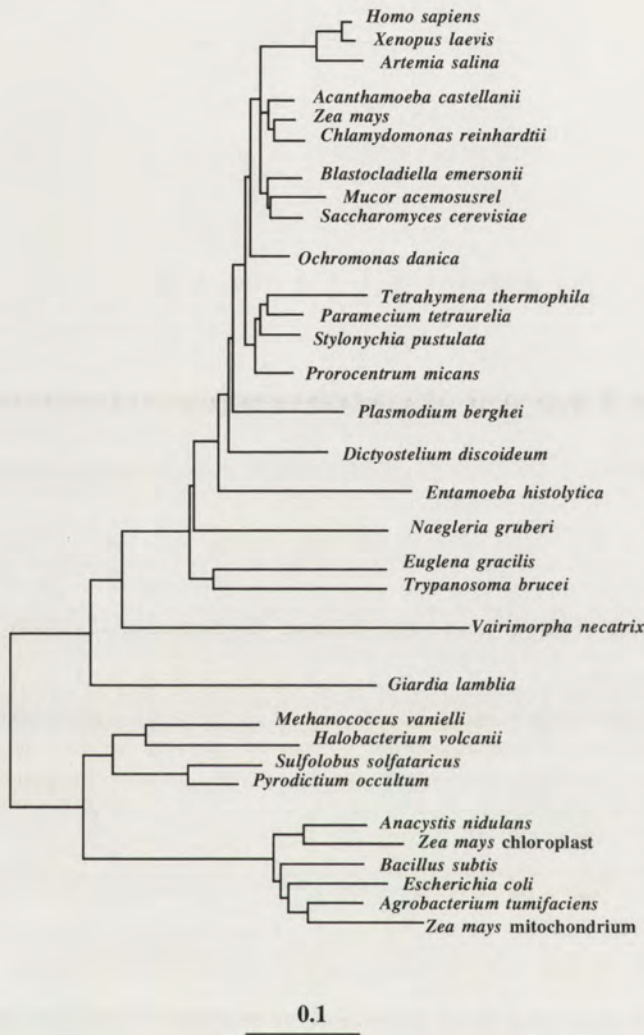
Fig. 1. Distance matrix tree inferred from approximately 900 unambiguously aligned nucleotides of small subunit rRNA gene sequences (Elwood H.J. et al. 1985), including representatives of the archaebacteria, eubacteria and eukaryota. The scale bar equals 0.1 substitutions per site (redrawn from Sogin et al. 1989 by Schlegel 1991)

sider the possibility that the major monophyletic groups of protists appeared very early in evolution, perhaps no later than the Late Archean time, i.e., more than 2.5 billion years ago (2.5 Ga). In attempting to answer questions raised by the new paleontological and molecular data then, a first crucial problem must be addressed, namely: How "old" are protists and is it possible to determine their absolute age? This problem lies largely, but not exclusively, in the realm of geology and paleontology.

The earliest generally accepted evidence of life has been found in cherts of the 3.5 Ga Warrawoona Group (Australia). According to the data of Robbins et al. (1985), metazoan life and a complex food chain were

present at 1.9 Ga ago. Based on the diagnosis of microfossils, some other authors suggest that protists and yeast might have been in existence at Middle or Late Archean time (3.5-2.6 Ga) (Kaźmierczak 1979, Pflug 1982, Pflug and Reitz 1985). Paleontological documentation has still left open the question of a time eukaryogenesis.

MOLECULAR BIOLOGICAL DATA

Molecular evolution based on the molecular clock hypothesis has provided insightful data on the origin of eukaryotic cells.

Molecular biological methods, initially applied to evolutionary studies some thirty years ago (Zuckerkandl and Pauling 1962), have also raised some important questions about phylogenetic relationships among major protistan lineages, as well as their relationships to prokaryotes, plants and animals.

The early origin of the eukaryotic cell and the problem of primary protists have thus far been considered largely with respect to (1) molecular phylogenetic analysis of the universal small subunit rRNA (ss-rRNA) (Lake 1986, 1988; Wolters and Erdmans 1986) and (2) the appearance and role of the cytoskeleton and Ca^{2+} regulated motile systems (Kuźnicki and Kuźnicki 1979, Kuźnicki 1989).

On the basis of ss-rRNA sequence data, Lake (1988) divides archaebacteria into halobacteria, metangenes and eocytes. The halobacteria are considered to be a sister group of the eubacteria, and eukaryotes may have
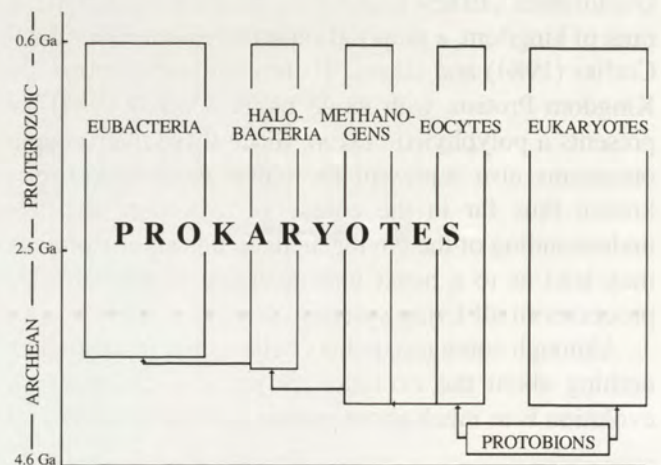

Fig. 2. The phylogenetic relationships between prokaryotes and eukaryotes and with prokaryotes at Archean and Proterozoic age (redrawn from Kuźnicki 1989)

evolved from the eocytes, a group of extremely thermophilic sulphur-metabolizing anucleate cells. As revealed by sequence variation, diversity within the eukaryotes exceeds that of both archaebacteria and eubacteria, a fact very well recognized early by cell and organismic biologists. According to Kuźnicki (1989), the separation between prokaryotes and eukaryotes occurred early (Fig. 2).

In his review on comparisons of the small subunit ribosomal RNA (ss-rRNA) sequences, Schlegel (1991) suggested that the parasitic diplomonad *Giardia lamblia* diverged first on the eukaryotic tree, followed by the microsporidian *Vairimorpha necatrix* (Fig. 1). However, their positions as the earliest diverging lineages of protists are controversial and doubtful, since both organisms are parasites. The order of branching of the major protistan groups is different, depending on the authors and analytical methods used. Certainly, the molecular data have not been applied to evolutionary questions without controversy, even when two of the most useful methods, a distance matrix and parsimony, are employed.
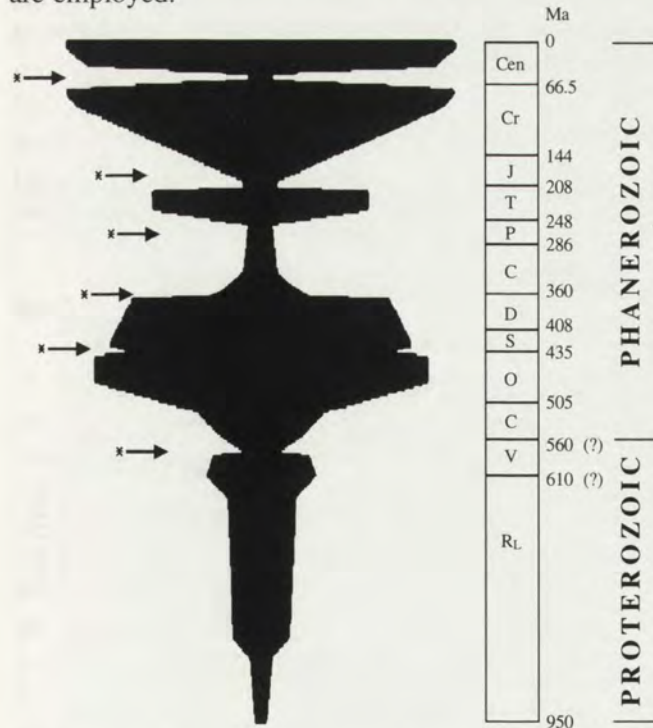

Fig. 3. Diagram showing major patterns of change in phytoplankton-cyst diversity during evolution from the late Proterozoic to present. Asterisks with arrows indicate times of major extinction. Stratigraphic intervals are indicated by initials as follows: R$_L$ - Late Rophean; V - Vendian; C - Cambrian; O - Ordovician; S - Silurian; D - Devonian; C - Carbonian; P - Permian; T - Triassic; J - Jurassic; Cr - Cretaceous; Cen - Cenozoic (Paleocene, Eocene, Oligocene, Miocene, Pliocene, Pleistocene). Partially adapted from Knoll (1989). Data from several authors
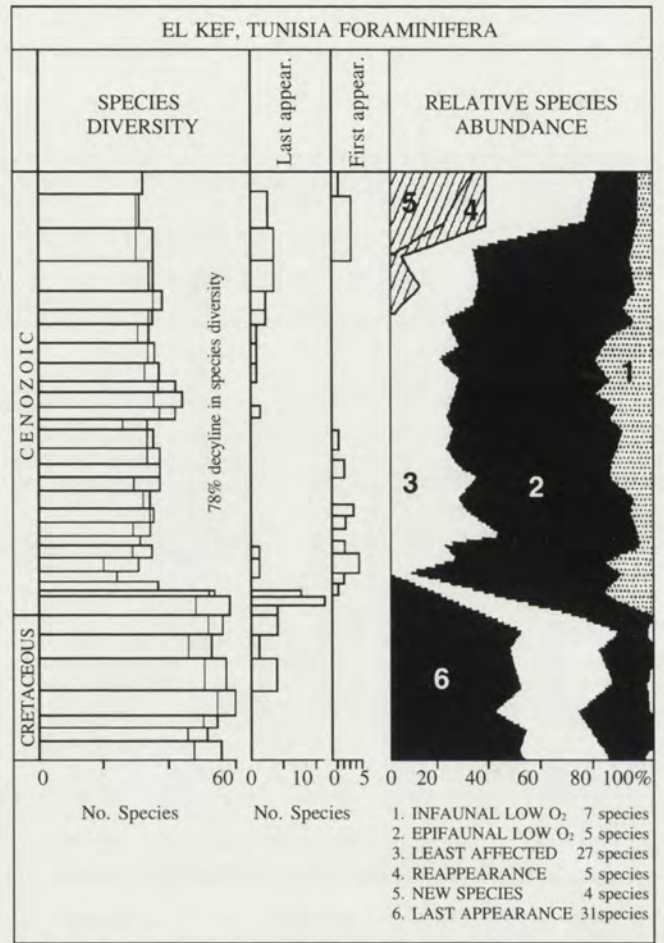

Fig. 4. El Kef, Tunisia. Species diversity, first and last appearances of species and relative abundances of species grouped into: low oxygen infaunal or epifaunal, fauna disappearing at K/T boundary, fauna first appearing or reappearing after the boundary and fauna relatively unaffected by the K/T boundary event. Adapted from Keller (1988)

Although the analysis of small subunit rRNA is a very promising tool for phylogenetic investigations, both within the protista and among other groups of organisms, the information derived from this molecule alone is not sufficient to reveal phylogenetic relationships between free-living and parasitic species, for example. The analysis of ss-rRNA, as shown by Schlegel (1991), has led to controversy between a number of authors and the interpretations of Woese et al. (1990) and Sogin et al. (1989). For more precise conclusions, other molecular studies are required, e.g., on the large subunit of RNA (ls-RNA), DNA and proteins. Obviously, the development of molecular phylogenies is only just beginning, and additional studies of fossils continue to be essential to elucidating the problem of how old the protists are. Thus, the time and mode of early protistan evolution remain an enigma.
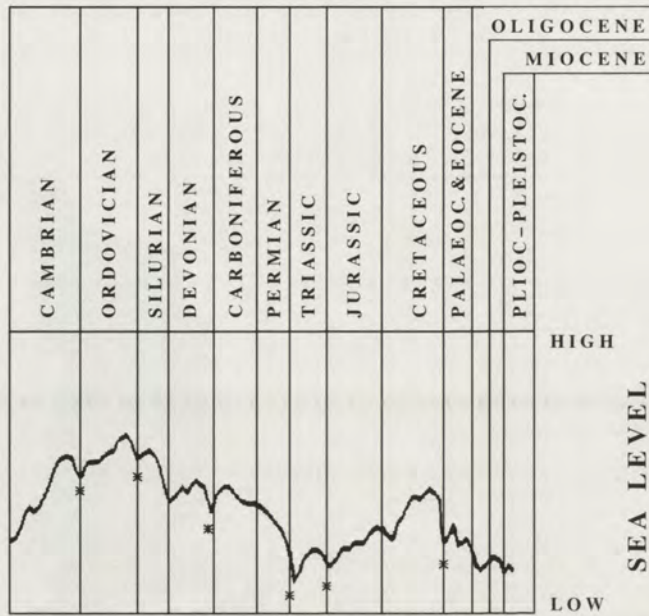
Fig. 5. The Phanerozoic sea-level of Hallam (1984, 1989) with asterisks signifying the six marine mass extinction episodes recognized by Newell (1967). Adapted from Hallam (1984, 1989)

PALEONTOLOGICAL DATA

The fossil data concern mainly marine unicellular protists and invertebrates. Over half of the preserved paleontological record consists of the organic or mineralized remains of marine planktonic algae (Knoll 1989). Undoubtedly eukaryotic microplankton play an important role in Middle Proterozoic ecosystems. The Acritarch group of completely fossil forms, for example, probably represent a polyphyletic taxon with several protistan phyla. In all likelihood, they are not the oldest among unicellular eukaryotes, since they appeared from 1.4-1.5 Ga and reached their evolutionary zenith in the Vendian (Fig. 3), followed by a major extinction ca. 580 Ma. From that time several major intervals of radiation and extinction of protists are noted. In comparison to plants and animals,than, protists may not only have a longer evolutionary history, but their fossils are much more abundant and complete.

The best preserved fossil records of protistan origin are from the late Cretaceous and Cenozoic. The history of some foraminifera, radiolaria, dinoflagellata, and diatomea is documented in detail. Late Mesozoic and Cenozoic sediments not only contain a much expanded and improved record of marine protists but also provide the best documentation of habitat in which the evolutionary events occurred. (It is generally accepted that the Cretaceous/Tertiary (K/T) boundary section near El Kef

in northwestern Tunisia is one of the most complete sections known to date, e.g., Fig. 4).

In that regard, fossils of autotrophic and heterotrophic protists have preserved isotopes, thus permitting the retrieval of information on the relative depth, temperature and productivity of the water masses in which they formed (Douglas and Savin 1978, Boersma et al. 1987). If the parameters of the physical environments can be reproduced, then the evolutionary and ecological events before, during and after mass extinction and the origin of new forms can be explained less hypothetically than in the case of higher animals and plants.

PROTISTAN SPECIATION AND ADAPTIVE RADIATION

A second crucial question, then, focuses on the patterns of protistan speciation and adaptive radiation. The best-known data from the K/T boundary show no dominance of one mode over the other. In benthic foraminifera, punctuated patterns of speciation prevail, whereas in dinoflagellates and diatoms, speciation is characterized by more gradual patterns.
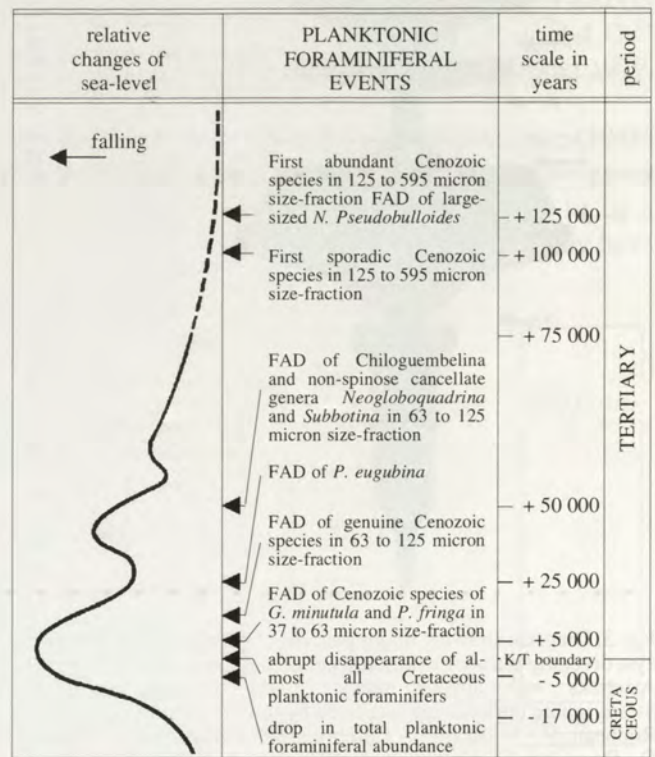

Fig. 6. Pattern-of relative sea-level change and major planktonic foraminiferal events in time-scale. Partially adopted from Brinkhuis and Zachariasse (1988)

The K/T boundary is marked by a dramatic change from oxygen-rich protists to low-oxygen tolerant protists. In effect both planktonic and benthic foraminifera dropped dramatically at the K/T boundary. About 50% of the benthic foraminifera species disappeared; however, a number of species were unaffected by the K/T boundary event (Keller 1988).

EXTINCTION EPISODES

In the Phanerozoic Era, Newell (1967) recognized six marine mass extinction episodes in which a large proportion of the clades of different organisms, protists and animals, disappeared in a relatively short period (Fig. 5). Knoll (1989) distinguished the same intervals of radiation and extinction for phytoplankton (Fig. 3), except the late Cambrian extinction. It is generally accepted that during the Phanerozoic Era the major evolutionary episodes seem to show a less dramatic reduction or no reduction at all. The final extinction of the majority of planktonic foraminifera was foreshadowed by a reduction in their total abundance some 5,000 years earlier. Crucial in understanding the K/T events is the fact of successful survival of the planktonic foraminifer *Buembelitria cretacea*. The final return of relatively stable ecosystems with an abundance of foraminifera and well-balanced dinoflagellate species occurred about 125,000 years after the K/T boundary.

DIVERSITY OF SPECIES

Thus, high rates of diversity among calcareous nanoplankton and foraminifera followed the K/T boundary mass extinction. The correlation does not include other groups of protists, especially diatoms. Comparison of the majority of the fossil records of late Cretaceous with those of the Paleocene and Eocene does not provide any evidence that mass extinction among protists was an evolutionary novelty. Species that proliferated after mass extinction were morphologically similar to those that disappeared and they occupied the same niches as their ancestral species. The truly high rates of extinction through a very short interval, followed by expansion of the species that survived and thereafter high rates of radiation concern only the calcareous planktonic forms (Fig. 6).

Clades of other marine protists at that time do not show much evidence of such relationships. Knoll (1989) questions whether the major evolutionary features of protists on the Cretaceous/Tertiary (K/T) boundary might prove equally valuable in contraining hypotheses about earlier episodes of mass extinction and high rates of speciation. The dearth of earlier fossil records limits such direct comparisons used for late Mesozoic and Cenozoic events. However, a similar mode of evolution as characterized previously for the late Cretaceous and Cenozoic was reported by Colbath (1986) for the terminal Ordovician extinction of phytoplankton. Only one group of completely fossil forms is represented among the polyphyletic Acritarchs (Corliss 1984). The resistance of the majority of protists to extinction is probably a consequence of several factors, including (1) higher individual resistance to many physical and chemical agents than other eukaryotes; (2) autotrophy, and in the case of heterotrophy a lower position in the food chains; (3) the capacity by many species to form resting stages/spores during periods of different ecological stresses; (4) free-living species occupy a relatively stable habitat; (5) a majority of the higher taxa possess widespread species.

Thus, except for the origin of parasitism or the evolution of a group of totally parasitic species, the large-scale evolutionary trends in protistan clades do not change as a consequence of extinction, and they continue the morphological and physiological characters of the ancestral species. This is generally the important difference between the major features of evolution in protists and other eukaryotes.

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ACTA
PROTOZOOLOGICA

Effects of a Gold Salt and its Intracellular Distribution in *Tetrahymena*

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Summary. In studies on effects of heavy metals and drugs containing these, *Tetrahymena* was exposed to aurothiomalate (GSTM), a drug used in therapy of rheumatoid arthritis. The drug has been reported to affect lysosomal enzymes, to accumulate in lysosomes, and to be detectable in the body years after treatment. In *Tetrahymena*, 0.5-15 mM GSTM caused only minor prolongation of the cell doubling time and affected endocytosis negligibly. These minor effects on utilization and uptake of nutrients, indicate no inhibition of lysosomal enzymes. All drug-treated cells contained small refractive granules which with time became yellow as small entities (of a similar size) in food vacuoles. Intracellularly, GSTM, or its gold component, was seen initially in food vacuoles as small dense dots, or chains, but later as conglomerates. In the cytoplasm, dense dots were found in dilated endoplasmic reticulum, small vesicles, Golgi complexes, and accumulated in dense granules (lysosomes), especially notable at prolonged exposures. The apparent binding of GSTM to less dense material in various cell compartments, indicates an intracellular handling similar to that of other heavy metals in *Tetrahymena*, i.e. a common mechanism is involved in rendering heavy metals innocuous.

Key words. Gold salt, aurothiomalate, *Tetrahymena pyriformis*, distribution, accumulation.

INTRODUCTION

The water-soluble gold salt, sodium aurothiomalate (GSTM), is an extensively used drug (Myocrisin) in therapy of rheumatoid arthritis. The drug has been used for more than 60 years, yet its mode of action is not fully understood (Brown and Smith 1980, Petering 1976, Vernon-Roberts 1979) but gold is the active moiety (Brown and Smith 1980, Lipsky et al. 1979). The drug binds to sulfhydryl-containing ligands (Sadler 1976, 1982; Vernon-Roberts 1979), affects the release and activity of some lysosomal enzymes (Brown and Smith 1980, Graabæk and Pedersen 1988, Mallya and Van Wart

1989, Metha and Webb 1982, Persellin and Ziff 1966, Sadler 1976, Vernon-Roberts 1979), affects the immune system by inhibiting monocyte function (Brown and Smith 1980, Harth et al. 1983, Lipsky et al. 1979, Lorber et al. 1979, Sanders et al. 1987, Vernon-Roberts 1979), and enhances virus infections (Gibson et al. 1990; Metha and Webb 1982, 1987). Although GSTM cures or improves the conditions in 60% of the treated cases, 40% of the patients develop gold-induced intoxication (Sadler 1976). About 60-70% of the administered gold is retained in the body deposited in various tissues (Basco et al. 1988, Brown and Smith 1980, Carter 1988, Gottlieb 1982, Lawson et al. 1977, Sadler 1976), including macrophages (Pääkkö et al. 1984, Vernon-Roberts et al. 1976), and gold has been detected 20 years after the treatment (Carter 1988, Vernon-Roberts et al. 1976).

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The high atomic number of gold makes GSTM detectable by electron microscopy and gold has been shown intracellularly in lysosomes (Davies et al. 1977, Nakamura and Igarashi 1977, Pääkkö et al. 1984, Sadler 1976, Stuve and Galle 1970, Strunk and Ziff 1970), also called aurosomes (Ghadially 1979a, b). The majority of cytosolic gold is concentrated in lysosomes (Lawson et al. 1977) where it binds to several species of macromolecules. An inhibited activity, or release, of lysosomal enzymes may in part explain the therapeutic efficacy of the drug.

The present study was undertaken with a special interest in following the intracellular distribution of the drug, or more precisely its gold component, in *Tetrahymena*. The purpose was to study how the distribution would compare to that found previously for other heavy metal compounds in this ciliate (e.g. Nilsson 1988, 1989, 1992). Possible effects of GSTM on the rates of growth and endocytosis of the cells were also studied since GSTM has been reported to affect such parameters in other types of cells (Davis et al. 1982, Lipsky et al. 1979, Lorber et al. 1979, Sadler 1976, Vernon-Roberts 1979, Vernon-Roberts et al. 1976).

MATERIAL AND METHODS

Tetrahymena pyriformis GL was grown axenically at 28°C in 2% proteose peptone enriched with in 0.1% yeast extract and inorganic salts (Plesner et al. 1964). The 100 ml cell cultures in 500 ml Fernbach flasks were aerated and agitated. The experiments were started with cells in the exponentially multiplying growth phase, i.e. with a density of 4-5 x 10^4 cells/ml.

The cell density of the cultures was determined using an electronic particle counter (Coulter Counter, model ZB). Triplet samples of 0.5 ml cell suspension were fixed with an equal volume of 1% glutaraldehyde in a phosphate buffer (pH 7.3). Cell counts were made half-hourly for 7 h (more than two normal cell generations) and again after 24 h in the drug.

The endocytic capacity of the cells was determined by a 10 min exposure to carmine particles (Nilsson 1972), suspended in a medium identical to that of the cells. After the exposure, the cells were fixed in the above-mentioned fixative and the number of labelled vacuoles was counted in 100 cells. The results were expressed as the percentage of the control value (100%). The endocytic capacity was tested after 1, 3, 6 and 24 h in drug-treated and control cells.

Sodium aurothiomalate (GSTM) monohydrate (Aldrich Chemical Co. Ltd., F.W. 401; 48.27 w% Au) was made up as 100 mM (40.8 mg GSTM/ml; 19.69 mg Au/ml) or 200 mM stock solutions in destilled water and the colourless solutions were stored in the dark. The drug (0.5-40 mM i.e. 0.099-7.88 mg Au/ml) was added to 50 ml (final) subcultures and the same volume of destilled water was added to control cultures; addition of the drug did not change the pH, or cause precipitation, of the medium. When more than one concentration of

GSTM was tested, two simultaneously inoculated 100 ml cell cultures were pooled to ensure the same initial cell density in the 50 ml subcultures. For each GSTM concentration, 3-5 experiments were performed and the cells were followed for 30 h. Recovery from a 24 h exposure to 15 mM GSTM (high cell density culture) was studied by washing the cells three times in fresh growth medium and leaving them in the medium at a high, or low, cell density.

For electron microscopy, cell samples were fixed in an equal volume of 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) for 10 min, washed in 0.1 M cacodylate buffer, and postfixed for 1 h in 1% osmium tetroxide in the same buffer. After dehydration, the cells were embedded in epon and contrasted as described previously (Nilsson 1986). The sectioned material was examined in a Zeiss EM 109 electron microscope.

RESULTS

Light microscopy

Addition of 0.5-1.5 mM sodium aurothiomalate (GSTM; 99-296 µg Au/ml) affected the rate of growth of *Tetrahymena* only slightly and after 24 h, the cell density was half of that of the control culture. The rate of endocytosis varied less than 5% (N = 12) from that of the control cells.

Increasing the concentration of the drug (2-15 mM; 0.39-2.96 mg Au/ml) prolonged the cell generation time by a factor of 1.15-1.35 early during the exposure (N = 9). No lag period was induced and the rate of proliferation decreased immediately, but after 24 h, the cell density of the drug-treated cultures was close to half of that of the control cultures, with only slight concentration-dependent differences. The normal generation time of *Tetrahymena* is, however, only less than 3 h at cell densities below about 2 x 10^5 cells/ml and it increases during the following 2-3 cell doublings before proliferation ceases at around 10^6 cells/ml. When control cells had passed through the first two cell generations, cells in low and high GSTM concentrations had passed through 1.7 and 1.4 cell generations, respectively. The findings indicate that growth is more retarded early than late during the exposure. In concentrations up to 15 mM GSTM, the rate of endocytosis was affected negligibly and irrespective of the concentration, the mean value (98% ± 3%; N = 44) corresponded to that of control cells (100%). Hence, no real toxicity was seen. Even in a high concentration of 40 mM GSTM, no cell death occurred but endocytosis and cell proliferation were inhibited; however, after 2 days the cell density of the culture was high, so the cells recovered in the drug.

Appearance of small refractive granules in the cytoplasm was typical of all drug-treated cells. The granules increased in number in a time-dependent, and some concentration-dependent, manner, during the first hours (Figs. 2-5), and after 24 h, the cells contained numerous granules (Figs. 8, 9) which in the high GSTM concentrations had a clear yellow colour. Control cells also contained refractive granules after 24 h (Fig. 7) but
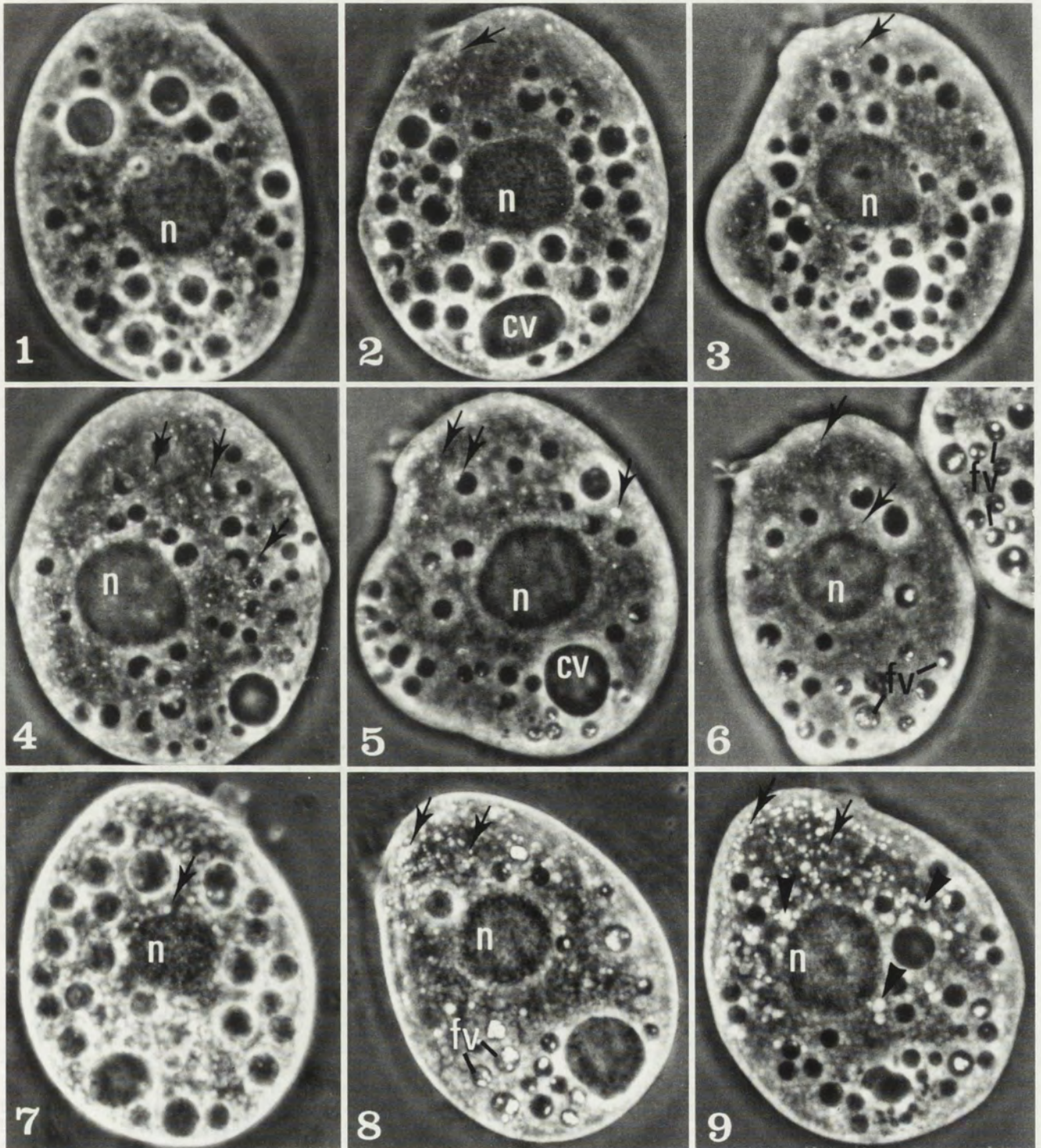
Figs. 1-9. *Tetrahymena* during a 24 h exposure to 10 or 15 mM GSTM (in vivo phase contrast microscopy). 1 - 1 h control cell; 2 - 1 h in 10 mM; 3 - 1 h in 15 mM; 4 - 3 h in 10 mM; 5 - 3 h in 15 mM; 6 - 6 h in 15 mM; 7 - 24 h control cell; 8 - 24 h in 10 mM; 9 - 24 h in 15 mM. Note, the time-dependent appearance of small refractive granules (arrows) and of similarly sized entities forming aggregates in food vacuoles (fv), note also, the less dense 2 μm granules in Fig. 9 (arrowheads). Macronucleus (n). Contractile vacuole (cv). All figures x 880
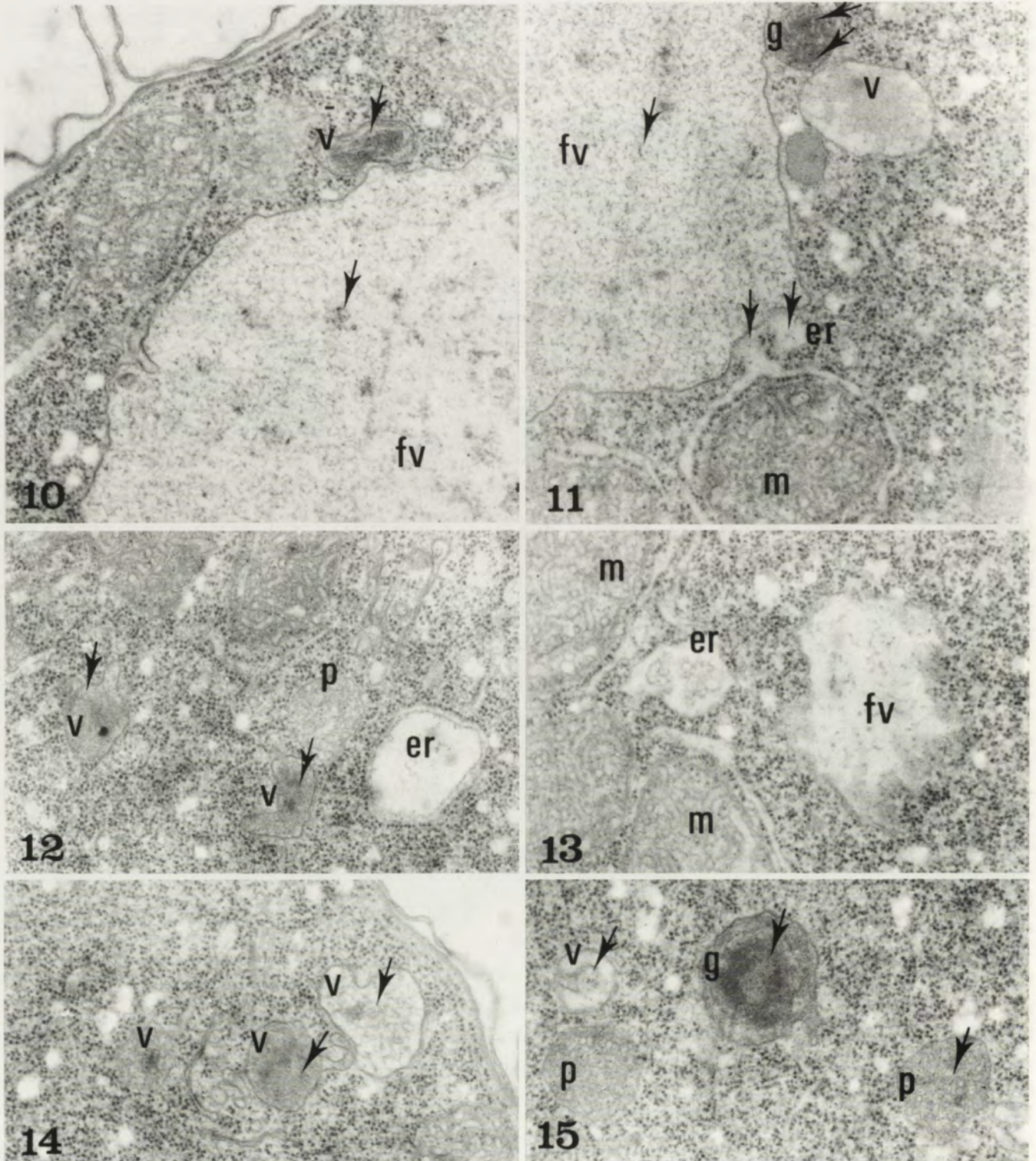
Figs. 10-15. Structural changes and intracellular distribution of gold (small dense dots) during the early exposure to 10 or 15 mM GSTM. 10 - gold (arrows) bound in food vacuole (fv) to fluffy contents and in small laminated vesicle (v). 4 h in 10 mM; 11 - gold (arrows) in food vacuole (fv), in small dense granule (g), and to a lesser extent in vesicle (v, an early mucocyst?) next to unattached mucocyst, and in dilated endoplasmic reticulum (er) adjacent to a mitochondrion (m); 12 - an early response to GSTM is the appearance of dilated endoplasmic reticulum (er) with (newly synthesized?) material. Gold (arrows) in small vesicles (v) with dense material and in dense peroxisome (p). 1.5 h in 15 mM; 13 - dilated rough endoplasmic reticulum (er) with fluffy contents adjacent to mitochondria (m) and grazing section of a food vacuole (fv). 4 h in 15 mM; 14 - vesicles (v) with contents of different density (pre-stages of granules) with scant amounts of gold (arrows), and flattened vesicles. 4h in 15 mM; 15 - dense dots of gold (arrows) accumulated in a small dense granule (g), a vesicle (v), and dense type peroxisomes (p). 4 h in 15 mM. All figures x 39,600

no coloured material. Some food vacuoles in the drug-treated cells contained after 3-6 h small entities of a size similar to that of the refractive granules (Figs. 5-6) and with time, and in a dose-dependent manner, the small entities in digestive vacuoles formed aggregates (Figs. 6, 8, 9) which after 24 h, had attained a clear yellow colour. In the high drug concentrations (5-15 mM GSTM), extrusion of compact, yellow defecation balls was frequent after 6 h and with time they accumulated at the bottom of the culture flask. Because the refractive
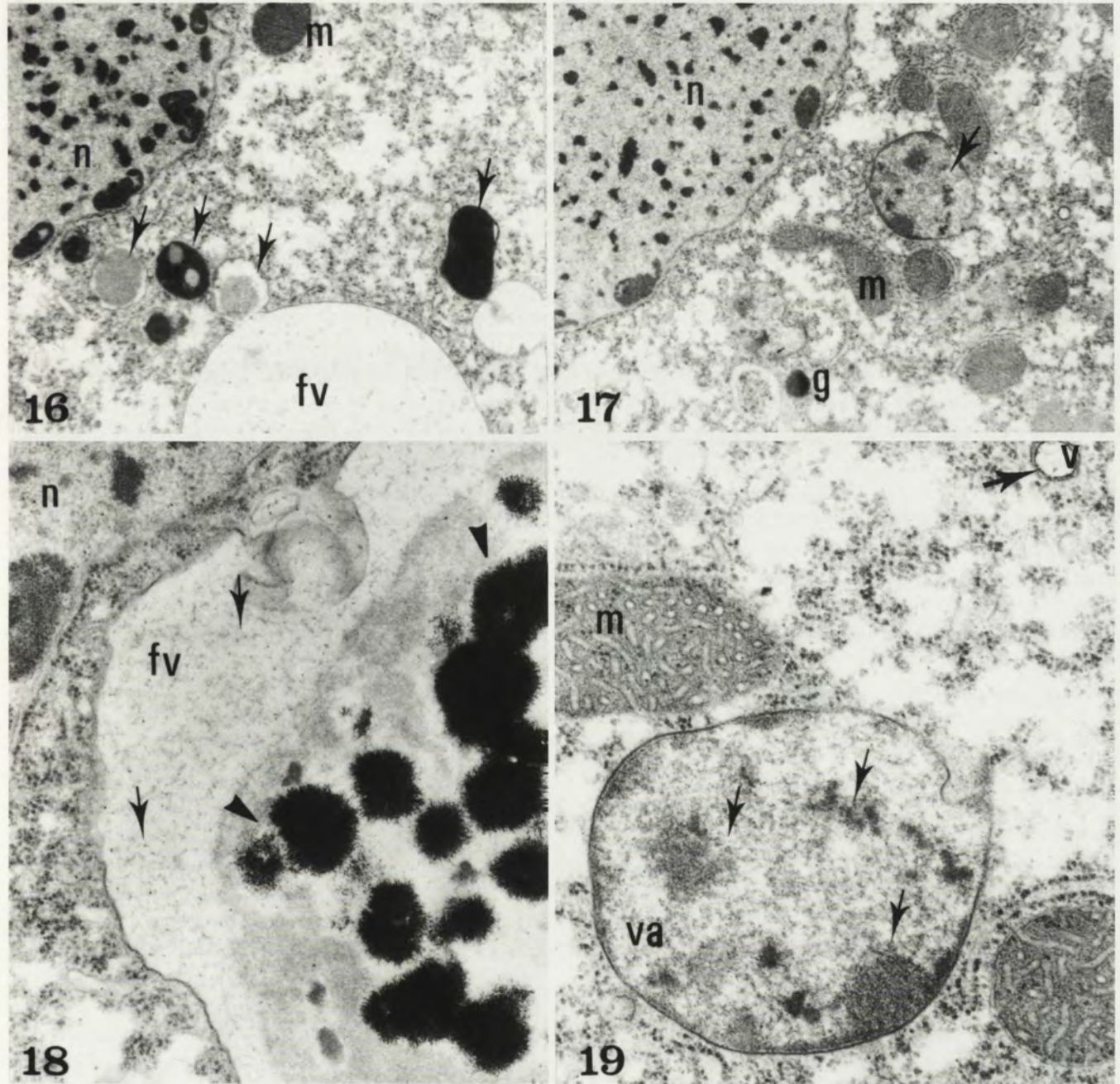
Figs. 16-19. Structural changes in control and GSTM-treated cells after a 24 h exposure. 16 - a 24 h control cell with dense mitochondrion (m), fused nucleoli (n), glycogen islands (light areas), and dense granules (arrows) with contents of varying density. One granule is fusing with a digestive vacuole (fv), believed to represent a common mode of turnover of granules in *Tetrahymena*; 17 - from a cell exposed to 10 mM, general morphology much the same as in the control cell (Fig. 16); however, apart from granules (g), small, 2 μm vacuoles with fluffy dense material are seen (arrow) as a new inclusion body, see also Fig. 19; 18 - part of a digestive vacuole (fv) in cell exposed to 15 mM GSTM. The conspicuous entities (arrowheads) seem to be conglomerates of the gold (dense dots/rods) seen in the lighter region (arrows) of the vacuole. The conglomerates are embedded in less dense debris, representing a pre-defecation ball; 19 - enlargement of the new inclusion body shown in Fig. 17. Accumulation of gold (dense dots) is seen (arrows) among the fluffy contents of the vacuole, and to a lesser extent, in the small vesicle (v). Dark type mitochondria (m). Light areas represent glycogen islands. Figs. 16, 17 x 10,200; Figs. 18, 19 x 39,600

granules, the aggregates in digestive vacuoles, and the defecation balls all had attained a clear yellow colour after 24 h in the drug, it appears likely that gold accumulates in the refractive granules which fuse with digestive vacuoles to increase their contents of gold, and that gold is eliminated from the cells through defecation with other debris. In this connection it should be pointed out that the drug did not form visible precipitate with the culture medium.

In order to study whether the drug, or gold, remained accumulated in *Tetrahymena* after end of the treatment, cells were followed after removal of the drug. After a 24 h exposure to 15 mM GSTM, the cells were transferred to fresh growth medium at a low or high cell density. In the low density cultures, a short lag period was seen before proliferation was initiated and by 5 h, the cell density had doubled. During the first couple of hours, no real change was observed, the cells still had yellow granules but also an increasing number of yellow digestive vacuoles and defecation was frequent. By 6 h of recovery, only few cells had recognizable gold-coloured material in digestive vacuoles. The washed cells left as high density cultures, did not proliferate and the disappearance of yellow material was slow, still being present in some cells 24 h after removal of the drug. Hence, retention of the drug, or its gold component, does not occur in proliferating cells but to some extent in non-dividing cells where turnover is slow.

Electron microscopy

For electron microscopy, cells exposed to 10 and 15 mM GSTM were chosen because with time their refractive granules and food vacuoles attained a clear yellow colour light microscopically. The cells were examined after a 0.5, 1.5, 4, and 24 h exposure to the drug. During this period, the cell density of control cultures increased by a factor of 25.0 (i.e. 4.6 cell doublings), whereas cells in 10 and 15 mM GSTM increased by a factor of 12.7 and 11.2, indicating that they passed through 3.6 and 3.4 cell generations, respectively. In control cultures, the fine structure of *Tetrahymena* changes when the cell generation time increases at high cell density (Nilsson 1981). Hence, these structural changes were taken into account in the interpretation of the fine structure of treated cells. In drug-treated cells, attention was focussed on possible changes in the fine structure, not seen in the corresponding control cells, and on the distribution of the gold component of GSTM, i.e. of electron-dense material not seen in control cells.

Early during the exposure (0.5 h), peroxisomes had changed into the dense type (Nilsson 1981) which is seen only later in control cultures. Moreover, the rough endoplasmic reticulum was dilated as an indication of a high rate of protein synthesis for transport. During the first 4 h, electron-dense material, as small dense dots, was abundant in food vacuoles on, or among, their fluffy contents (Figs. 10, 11). Dense dots (gold) in scant amount were seen in dilated endoplasmic reticulum cisternae (Figs. 11-13), small vesicles (Figs. 10, 11, 14), small dense granules (Fig. 11), and more abundantly in larger dense granules (Fig. 15). The dense granules have a membrane resembling that of food vacuoles and are typical of *Tetrahymena* from high cell density cultures (Nilsson 1981). No apparent difference was observed between the structure of cells in the two concentrations of the drug.

After 24 h, the fine structure of control cells had changed. Their nucleoli were fused, mitochondria and peroxisomes were of the dark type, the cytoplasm contained large glycogen islands, few ribosomes, and small dense granules (Fig. 16), all in agreement with cessation of proliferation in the high cell density cultures (Nilsson 1981). The overall fine structure of 24 h drug-treated cells (Fig. 17) resembled that of control cells, but in addition, they also contained small 1-2 μm vacuoles (Fig. 17), an atypical cell component of *Tetrahymena*. Electron-dense material (dense dots) had increased in amount in the cells, especially conspicuous in digestive vacuoles as large conglomerates embedded in less dense material (Fig. 18), presumably debris, but also in the small 2 μm vacuoles as small dense dots accumulated in less dense material (Fig. 19). The limiting membrane of the small vacuoles resembles that of food vacuoles (compare Figs. 18, 19) and often appeared with an uneven outline as though they enlarge by fusion with small vesicles containing scant amounts of gold (Fig. 20). The 1-2 μm vacuoles could represent a pre-stage of dense granules (Fig. 21) which contained varying amounts of accumulated gold (dense dots) and often appear with a composite substructure (Figs. 21, 26). Accumulation of gold (dense dots) was also seen in small laminated vesicles (Figs. 22, 23), and to a lesser extent in occasional autophagic vacuoles (Fig. 24). Scant amounts of electron-dense material were found in peroxisomes (Fig. 22), in small vesicles near parasomal sacs (Fig. 25), within alveolar sacs, a membrane system of the pellicle (Fig. 25), and within endoplasmic reticulum (Fig. 26) where the associated coated vesicles indicate a transport to or from the structure. The in-
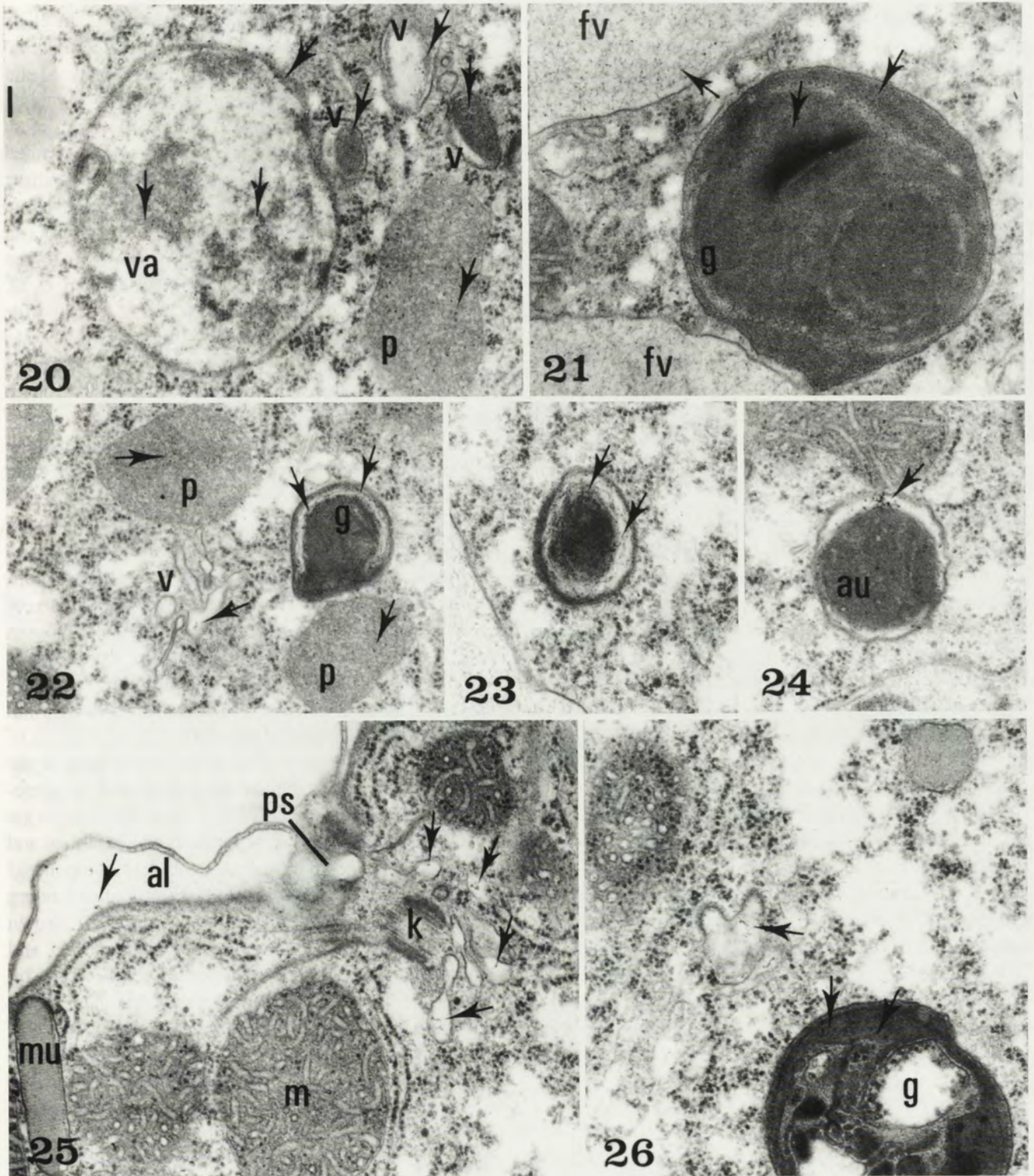
Figs. 20-26. Intracellular distribution of gold (dense dots) after a 24 h exposure to 10 or 15 mM GSTM. 20 - gold (arrows) on the fluffy contents of the special inclusion body (va) of GSTM-treated cells, in small vesicles (v), and in the dense type peroxisome (p). 15 mM; 21 - a large dense granule (g) with heavy accumulation of gold (arrows) near two digestive vacuoles (fv) containing less amount of GSTM. 10 mM; 22 - gold (arrows) accumulated in small dense granule (g), in dense type peroxisomes (p), and in scant amounts in flattened vesicles (v). 15 mM; 23 -accumulated gold (arrows) in laminated vesicle. 15 mM; 24 - gold (arrow) in an autophagosome (au). 10 mM; 25 - scant amounts of gold (arrows) in small vesicles near kinetosome (k), indicating uptake via parasomal sacs (ps), but also within the membrane-limited alveolar sac (al). Dense type mitochondrion (m) and mature (attached) mucocyst (mu); 26 - gold (arrows) in scant amounts in dilated smooth endoplasmic reticulum with coated pits, and accumulated in a composite, dense granule (g). 10 mM. All figures x 39,600

creased amount of electron-dense material in the 24 h treated cells, correlates well with the light microscopical observations of yellow-coloured material in these cells.

DISCUSSION

Sodium aurothiomalate (GSTM) in concentrations of 0.5-15 mM, had no profound effect on endocytosis in *Tetrahymena* and in agreement with this, only a slight concentration-dependent effect on cell proliferation. The fact that proliferation occurs at an almost normal rate in a wide range of GSTM concentrations, indicates a close to normal rate of uptake, turnover, and utilization of nutrients; hence, no indication was seen in *Tetrahymena* of the inhibited activity of lysosomal enzymes reported from other types of cells (Brown and Smith 1980, Graabæk and Pedersen 1988, Mallya and Van Wart 1989, Persellin and Ziff 1966, Sadler 1976, Vernon-Roberts 1979).

However, GSTM-treated *Tetrahymena* contained small refractive granules which may fuse with digestive vacuoles. With time, both structures attained a clear yellow colour light microscopically and contained dense material electron microscopically, indicative of an accumulation, and retention, of the drug or its gold component. The findings indicate that GSTM is taken up via endocytosis, in bulk via food vacuoles but also in vesicles formed at the parasomal sacs, depressions adjacent to cilia. Moreover, gold must enter the cytosol since it binds to newly synthesized material in the endoplasmic reticulum and accumulates in membrane-limited compartments, such as the dense granules, of which at least some are lysosomes (cf. Nilsson 1989). Hence, the intracellular distribution of GSTM, or its gold component, in this organism resembles that found previously for other heavy metals or heavy metal containing drugs (Nilsson 1988, 1989, 1992). However, at prolonged exposures to GSTM, *Tetrahymena* also contain small 1-2 μm vacuoles with a high amount of gold (small dense dots). These vacuoles seem to be unique to the treatment and are not seen in control cells, but they resemble the yet smaller vacuoles found in *Tetrahymena* exposed to platinic chloride (Nilsson 1992).

The reason why GSTM does not affect cell proliferation and endocytosis in *Tetrahymena*, could relate to the chemistry of GSTM and the form in which the drug (Myocrisin) is administered during chrysotherapy (Brown and Smith 1980, Sadler 1982). GSTM is polymeric and its dissolution from the yellow solid form, yealds initially a yellow solution which soon becomes

colourless (Grootveld and Sadler 1983, Kassam et al. 1987) due to a conversion of the yellow polymeric species to a colourless flexible polymer (Grootveld and Sadler 1983). The drug as prepared for chrysotherapy, is a yellow solution (Harvey et al. 1983, Kassam et al. 1987) like that of the colourless GSTM solution heated to 100°C (Kassam et al. 1987). The two solutions affect human platelets differently, they aggregate in the yellow solution (Harvey et al. 1983, Kassam et al. 1987, Kean et al. 1984) which contains longer particles than the colourless solution (Harvey et al. 1983, Kean et al. 1984). Moreover, ampoulles of the drug for chrysotherapy may vary in activity/strength (Rudkowski et al. 1991). These properties indicate that GSTM is either a mixture of gold complexes or that the polymeric structure contains gold in different chemical environments (Rudkowski et al. 1991, Sadler 1982) and that the drug used in different investigations may differ in state which could affect the action of the drug. In the present study, GSTM was used as a colourless solution but within *Tetrahymena*, the drug turns yellow with time in the digestive vacuoles. The electron-dense conglomerates seen with time in digestive vacuoles of GSTM-treated *Tetrahymena* resemble the gold deposits in lysosomes of drug-treated patients or experimental animals (Davies et al. 1977; Ghadially 1979a, b; Nakamura and Igarashi 1977; Pääkkö et al. 1984; Strunk and Ziff 1970; Stuve and Galle 1970).

In *Tetrahymena*, the accumulation of the drug, or the gold component, was clearly time-dependent in agreement with depositing of gold in mammals. A question arises on how GSTM is transported in the organism and on what determines deposition of the gold component? The drug seems to be transported in blood after binding to proteins, especially albumin (Biggs et al. 1979, Brown and Smith 1980, Lawson et al. 1977, Sadler 1976), and intracellularly, it also binds to different proteins (Lawson et al. 1977, Sadler 1976), such as metallothioneins (Butt et al. 1986, Brown and Smith 1980, Schmitz et al. 1980). Metallothioneins are small proteins having a high affinity for heavy metals (Brady 1982) and especially, cadmium is a potent inducer of the synthesis of metallothionein as a defence reaction to render the metal innocuous. Metallothioneins bind 95% of the cytosolic cadmium but only 15-50% of cytosolic gold (Schmitz et al. 1980, Sharma and McQueen 1980). Also *Tetrahymena* has metallothioneins the synthesis of which is inducable by cadmium (cf. Nilsson 1989); however, these proteins have not yet been localized to the small dense granules where heavy metals, or metal-

containing compounds, accumulates (Nilsson 1988, 1989, 1992). In a resistant Chinese hamster ovary cell line, another gold salt, auronofin, activates metallothionein gene transcription (Butt et al. 1986). Hence, a regulation of metallothionein gene transcription may play an important role in the molecular mechanism(s) of action of a gold salt and resistance to it (Butt et al. 1986) as found for heavy metals in general. The dilated rough endoplasmic reticulum seen in *Tetrahymena* early during the GSTM exposure, indicates an induced protein synthesis (metallothioneins?) for rendering the drug innocuous, such a mechanism would also explain the minor effects of the drug on the cells.

The appearance of GSTM, or the gold component, differs in the dense granules, or other intracellular sites, and in the digestive vacuoles in *Tetrahymena*. Considering the electron-dense material to represent gold, how may the difference be explained? Shortly after uptake by endocytosis, electron-dense material occurs as small dots among the normal fluffy contents (growth medium) of food vacuoles and later within less dense material at the various intracellular sites. With time in digestive vacuoles, the electron-dense material forms compact conglomerates of small rods, almost like a crystal. These conglomerates resemble, as mentioned above, the gold deposits seen in drug-treated patients or experimental animals where X-ray microanalyses of the deposits (Ghadially 1979a), reveal, in addition to gold, the presence of sulphur and phosphorus, indicative of intact GSTM molecules, a composition which remained unchanged over a period of 3 days to 18 month (Ghadially 1979a). Since the electron-dense conglomerates may relate to unaltered GSTM molecules, their compact crystalline structure in the digestive vacuoles of *Tetrahymena* could derive from removal of the binding material by digestion but also from an increased amount of drug due to transfer from dense granules after their fusion with digestive vacuoles. The appearance of a light microscopical, yellow colouring of dense granules and digestive vacuoles is time-dependent which correlates well with the increase in electron-dense material in the structures but the colour indicates a changed state of the GSTM molecule from the colourless to the yellow form, as discussed above. Since an increase in pH above 7 favours the conversion of the yellow GSTM solution to the colourless one (Grootveld and Sadler 1983), the low pH of digestive vacuoles may favour the reverse reaction to explain the yellow colouring of the refractive granules and digestive vacuoles in *Tetrahymena*, both are acidic compartments.

The turnover of gold is very slow in humans or experimental animals treated with GSTM, as mentioned in the Introduction. Yellow material was also recognizable long after removal of the drug in non-dividing *Tetrahymena*, but not after 1-2 cell doublings in proliferating cultures. Hence, retention of gold seems to correlate with a non-dividing cell state and/or a low metabolic rate.

In conclusion, no evidence was found of a profound effect of GSTM on the function of lysosomal enzymes in *Tetrahymena*. The minor effect on cell proliferation may be ascribed to the cost of activation of a common defense mechanism involved in rendering the drug, or its gold component, innocuous.

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ACTA PROTOZOOLOGICA

Cell Multiplication in *Tetrahymena setosa* and *Tetrahymena thermophila* in Synthetic Nutrient Medium. Effects of Ethanol, Cholesterol and Extracellular Medium

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Summary. Cell multiplication in cultures of *Tetrahymena setosa* and *Tetrahymena thermophila* was followed under various conditions: in single cell or mass cultures, and in variations of synthetic nutrient media. The experimental flasks were inoculated at known concentrations with cells whose extracellular medium had been replaced by an inorganic buffer. *T. thermophila* cells failed to multiply at low concentrations of cells or amino acids, but these situations were reversed by the addition of 10 µg cholesterol per ml. Also *T. setosa* failed to multiply at low initial cell concentrations, a situation reversed by the addition of a mixture of phospholipids. At high initial cell concentrations *T. setosa* was grown for months in standard, **lipid-free** synthetic medium supplemented with 0.2% ethanol. All of these results were unexpected because *T. thermophila* has been considered a non-lipid-requirer, since it has been grown in lipid-free synthetic medium for decades, and *T. setosa* has been reported to have a lipid requirement. Because there is a **critical low cell concentration** for multiplication, it is believed that these cells release multiplication promoting factors and that added lipids somehow mimic their effects. In agreement with this idea cell-free, extracellular fluid promoted cell multiplication, even across the borderline between the two species.

Key words. Standard synthetic medium, critical initial cell concentration, multiplication promoting factors, signal compounds.

INTRODUCTION

This report deals with the effect of cholesterol on growth and multiplication in two species of *Tetrahymena*. Such studies were first made long ago (Kidder and Dewey 1951, Holz et al. 1962), but there are good reasons for repeating them now: (i) we have access to a wider range of purer chemicals; (ii) we have better methods for assessing population concentrations at low values; (iii) we know that cell multiplication may depend on cell-

produced compounds (Ghiladi et al. 1992, Christensen and Rasmussen 1992); and (iv) we obtained initial results showing that previous conclusions as to a lipid requirement of *T. setosa* were incomplete. All these points argue for more detailed studies.

Most work on cell multiplication in *Tetrahymena* in the synthetic nutrient media has been done with species without lipid requirements. Kidder and Dewey (1951) published the first recipe for a **lipid-free**, synthetic nutrient medium sustaining growth and multiplication of the cell now known as *T. pyriformis* (Nanney and McCoy 1976). This medium also supports multiplication of *T. thermophila*, *T. vorax*, and *T. pigmentosa* (Schous-

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boe et al. 1992) when conventional cultivation techniques are used. Recent reports have led to the suggestion that *Tetrahymena* release chemicals rescuing moribund cells (Ghiladi et al. 1992, Christensen and Rasmussen 1992). These effects can be simulated by phospholipids and other compounds (Schousboe et al. 1992, Christensen et al. 1992, Ghiladi et al. 1992, Christensen and Rasmussen 1992). Furthermore, multiplication of *T. setosa* was reported to require addition of alcohol **and** sterols (or other lipids) (Holz et al. 1962) to Kidder and Dewey's (1951) medium.

MATERIAL AND METHODS

Cells. The following cell lines were used: *Tetrahymena thermophila*, CU 399, and *Tetrahymena setosa*, HZ-1, obtained from dr. David Beach, Upstate Medical Center, State University of New York, Syracuse, N. Y.

Cultures. Stock cultures of *T. setosa* were grown in test tubes in 2 ml of a standard synthetic medium, SSM, for *Tetrahymena* (Szablewski et al. 1991) supplemented with 40 µl 10% ethanol per ml for several months before the experiments to be reported began. For experiments *T. setosa* grew in 10-ml portions of the same type of medium for 2 days in conical flasks with screw caps, transferred to a Tris/HCl-buffer, pH 7.5, by being centrifuged for 3 min at 1500 x g and resuspended in the buffer. This procedure was repeated three times. *T. thermophila* were treated the same way, except that the medium was not supplemented with ethanol.

Nutrient medium and supplements. The standard synthetic medium, SSM, was used unless otherwise indicated. It contains 19 amino acids, 4 nucleosides, 7 vitamins, glucose, ions, and citrate (Szablewski et al. 1991). Cholesterol was dissolved in absolute ethanol and added in the required amount to empty culture flasks; ethanol was then evaporated by a stream of sterile air and sterile nutrient medium carefully added. Asolectin, a crude preparation of phospholipids obtained from the American Lecithin Company (Woodside, N.Y., USA), was dissolved at 5 mg per ml, sonicated and sterilised by filtration and used at 50 µg per ml. In some experiments SSM was diluted in the ratio of 1:1 with TRIS/HCl buffer (pH 7.5). When testing the presence of stimulatory compounds in the extracellular fluid, the buffer was replaced by extracellular fluid. Thus, the concentration of nutrients in the medium were kept constant. In all cases the concentration of ethanol was kept at 0.2%.

Preparation of cell-free extracellular fluid. Cells were starved in a 10 mM TRIS/HCl buffer (pH 7.5) in 10 ml conical flasks at a concentration of 50,000 cells per ml. After 0-1 min and 4 h samples of the culture were transferred to centrifuge tubes and precipitated for 3 min at 4000 xg. The supernatants were removed and filtered with a Millipore 0.22 µm filter.

Cloning. In 1 ml test medium: a culture sample from a late log phase of growth was diluted 100-fold with fresh medium. A sample of this culture was placed on a sterile petri dish under a stereo microscope (25x - magnification) in a flow bench. With a sterile pasteur pipette with a fine drawn out tip single cells were transferred to test tubes containing 1 ml of medium. In 0.5 µl test medium: a culture sample from a late log phase of growth was diluted to 2000

cells per ml, sucked into a Hamilton pipette and dispensed as drops of approx. 0.5 µl on top of a 2 mm layer of sterile paraffin oil in a glass petri dish in a flow bench. The drops fell through the oil and stuck to the bottom of the dish. The drops were immediately inspected under the microscope, and only drops containing a single initial cell were included in the experiment. These cultures were kept under observation every day for ten days and it was noted in which the single cells formed a culture; percentages of successful cultures were calculated.

Cell counting. Population concentrations were determined as follows: 1 ml samples were aseptically removed, 1 ml of a 4% formaldehyde solution and 8 ml of a 0.9% NaCl solution were added. The cells were then enumerated in an electronic particle counter.

RESULTS

We have studied cell multiplication of *T. setosa* and *T. thermophila* in a standard synthetic nutrient medium, SSM. We measured the effects of addition of various chemical compounds, **cell-free** extracellular fluid and various initial population concentrations. Fig. 1 shows the number of cell doublings of *T. setosa* as a function of time, number of cells transferred into SSM, and presence of either phospholipids, cholesterol or ethanol. The initial cell concentrations were either 250 or 2000 cells per ml. At 250 cells per ml the cells multiply only if asolectin is added (shortest doubling times: 8 h). At 2000 cells per ml the cells fail to multiply in the standard synthetic medium, but form cultures if either cholesterol or ethanol is added (shortest doubling times: 20 h). These results have been extended to single cell experiments.
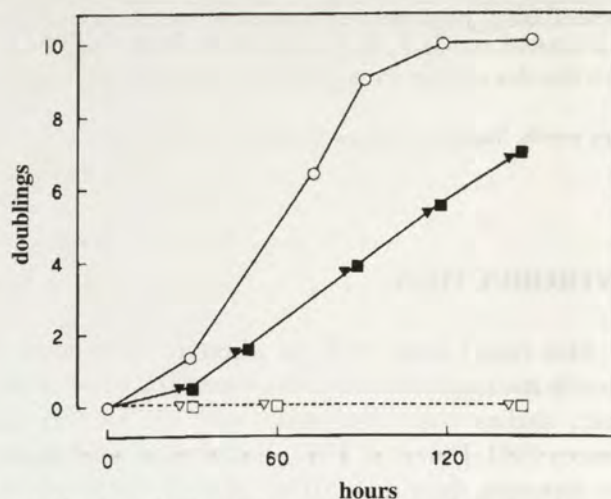

Fig. 1. Number of cell doublings as a function of time in cultures of *Tetrahymena setosa*. The cells were inoculated at either 250 cells per ml (open symbols) or 2,000 cells per ml (filled symbols) in lipid-free standard synthetic medium, SSM. **Circles:** SSM + asolectin (50 µg per ml); **triangles:** SSM + cholesterol (10 µg per ml); **squares:** SSM + ethanol (0.2%). 2,000 cells per ml in SSM did not multiply (data not shown). Before inoculation the extracellular medium was replaced by a Tris/HCl-buffer
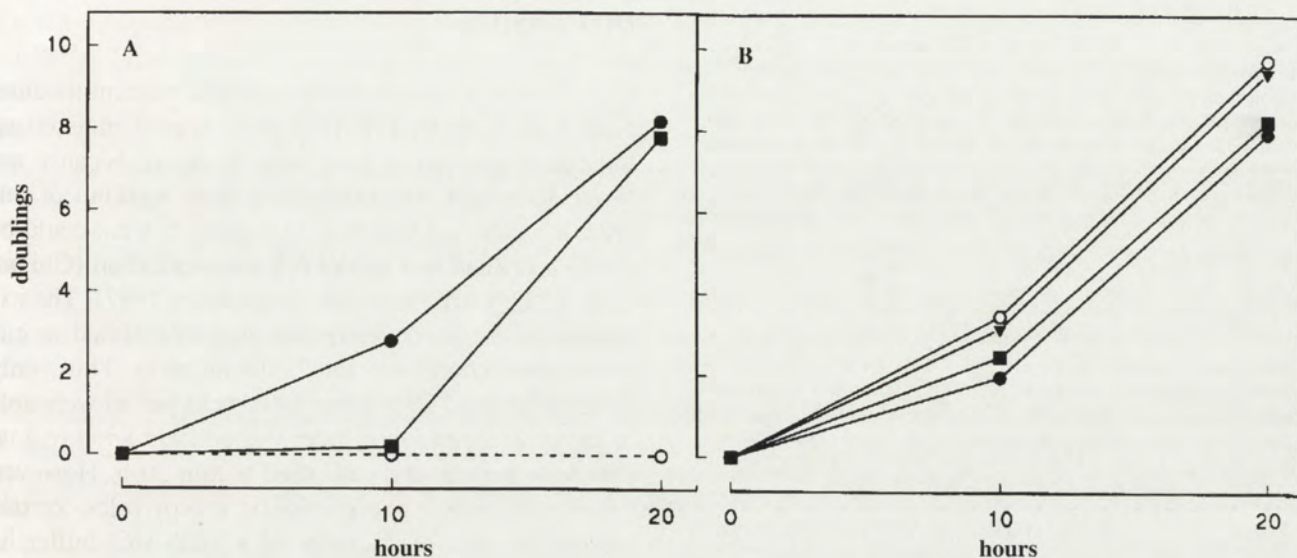
Fig. 2. Number of cell doublings as a function of time in cultures of *Tetrahymena thermophila* inoculated in SSM at various initial cell concentrations in the absence, A, or in the presence, B, of cholesterol (10 μg per ml). **Open circles:** 250 or 500 cells per ml; **squares:** 1,000 cells per ml; **filled squares:** 2,000 cells per ml. Before inoculation the extracellular medium was replaced by a Tris/HCl-buffer
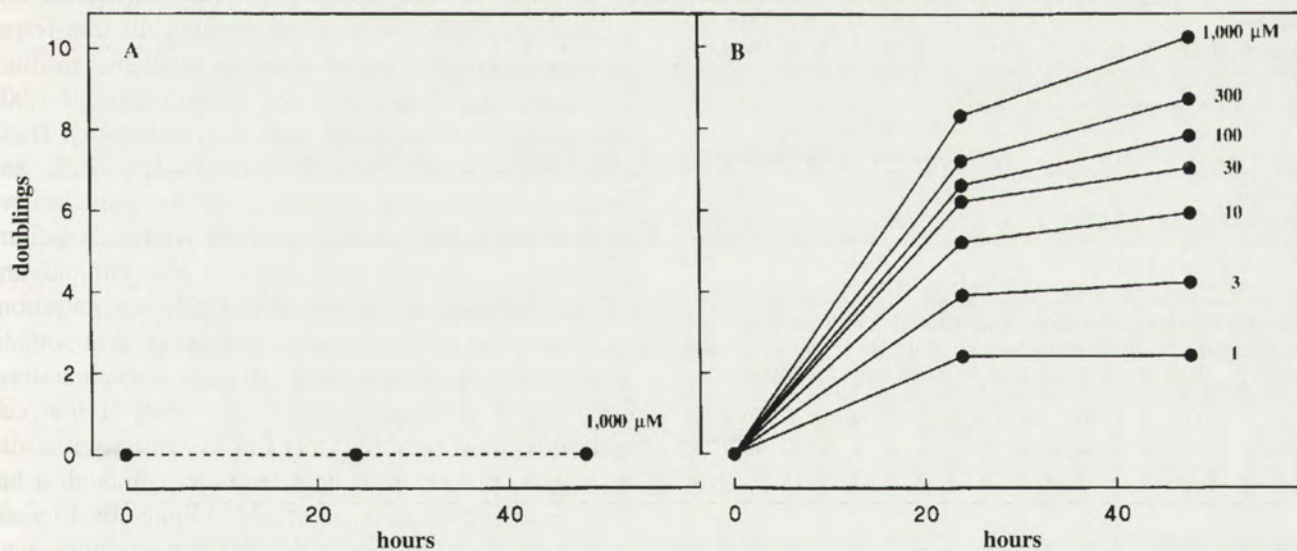

Fig. 3. Number of cell doublings as a function of time in cultures of *Tetrahymena thermophila* inoculated at 250 cells per ml at decreasing concentrations of amino acids in SSM. Before inoculation the extracellular medium was replaced by a Tris/HCl-buffer

Single cells of *T. setosa* transferred to 1 ml of culture medium do not multiply in standard synthetic medium supplemented with either nothing, paraffin oil (controls), or ethanol, but do multiply if it is supplemented with asolectin, Table 1. If the volume of the culture medium is around 0.5 μl, single cells multiply both in medium supplemented with ethanol and phospholipids, but not without these additions. We have previously shown that addition of small amounts of cell-free extracellular fluid from *T. thermophila* stimulated other cells of the same species into multiplication. We extend these observations and show that also cell-free extracellular fluid, obtained from cells starved in TRIS/HCl buffer for 4 h,

stimulate other species, Table 2. Thus, extracellular fluid obtained either from *T. setosa* or *T. thermophila* improve cell proliferation of both species. Multiplication of *T. setosa* was observed only when ethanol was added to the medium. Addition of ethanol did not not improve multiplication of *T. thermophila.* The effect of cholesterol on cell multiplication has been studied in cultures of *T. thermophila.* Fig. 2 shows cell multiplication in cultures with initial densities from 250 to 2000 cells per ml. In the absence of cholesterol, Fig. 2A, no multiplication occurs at the two lowest cell concentrations. In the presence of cholesterol the cells multiply in all cultures, Fig. 2B.

Table 1

Cloning efficiency of *Tetrahymena setosa* in 1 µl or 1 ml of SSM supplemented with either nothing, paraffin oil (1 ml layed at the top of the medium), ethanol or phospholipids. The expression "28/30" indicates that 28 out of 30 single cells formed clones

Volume	Cloning efficiency in SSM supplemented with			
	nothing	paraffin oil	ethanol	phospholipids
1 ml	0/30	0/30	0/30	28/30
0.5 µl	0/10	-	9/13	11/12

Table 2

Cloning efficiency of *Tetrahymena thermophila* and *Tetrahymena pyriformis* in 1 ml volumes of SSM with and without ethanol supplemented with either nothing, or extracellular fluid obtained from the two species. The expression "14/20" indicates that 14 out of 20 single cells formed clones

Cell	Cloning efficiency in SSM supplemented with		
- ethanol			
	nothing	extracellular fluid	
		T. setosa	*T. thermophila*
T. setosa	0/10	0/10	0/10
T. thermophila	0/10	17/20	18/20
+ ethanol			
	nothing	extracellular fluid	
		T. setosa	*T. thermophila*
T. setosa	0/10	14/20	15/20
T. thermophila	0/10	19/20	17/20

Table 3

Cloning efficiency of *Tetrahymena thermophila* in 1 ml of SSM supplemented with either nothing or cholesterol. The expression "10/10" indicates that 10 out of 10 single cells formed clones

Cell	cloning efficiency in SSM supplemented with	
	nothing	cholesterol
T. thermophila	0/10	10/10

The next series of experiments was made with medium of decreasing amino acid concentrations at an initial cell concentration not compatible with multiplication in the standard medium, Fig. 3. The amino acid complement was reduced stepwise from 1 mM to 0 mM of each single amino acid present in SSM, and cultures were inoculated with 250 cells per ml in the absence or presence of cholesterol. Cells in cultures without cholesterol did not multiply, but addition of this compound improved proliferation at amino acid concentrations from 1 mM to 0.001 mM. Only cells lacking amino acids failed to grow and multiply.

We have also investigated the cloning efficiency of *T. thermophila* in 1 ml cultures in SSM, Table 3. In the presence of cholesterol the cells "took" with high probability.

DISCUSSION

In their first review of the synthetic nutrient medium Kidder and Dewey (1951) warned against transferring too small volumes at each subcultivation, because too few cells would not survive in culture medium of this type. Recently we reported on aspects of what could be called a **critical low initial cell concentration** (Ghiladi et al. 1992, Christensen and Rasmussen 1992). The existence of these concentrations was established at circumscribed conditions for *T. thermophila*. Thus, only cultures having 750 or more initial cells per ml were able to thrive and multiply. Under this concentration (e.g. at 250 cells per ml) the cells died within 20 h. However, addition of either phospholipids, tetrapyrroles, certain proteins or cell-free samples of a TRIS-HCl buffer, in which cells have been starved for 5 h, saved the cells and supported proliferation (Schousboe et al. 1992, Ghiladi et al. 1992, Christensen et al. 1992, Christensen and Rasmussen 1992). Furthermore, single cells transferred to microdrops of 1 µl of standard synthetic medium (corresponding to an initial cell concentration of 1,000 cells per ml) formed clones with high probability. These observations indicate that *T. thermophila* produce and release multiplication promoting factors with decisive roles in cell proliferation in standard synthetic medium. Exogenous compounds such as hemin, phospholipids and certain proteins can at low initial cell concentrations substitute for cell-produced stimulatory compounds. These results have now been extended: also cholesterol can save *T. thermophila* from cell death at low cell concentrations. Therefore this cell in a strict sense also belongs to the group of lipid requirers, although it has been grown in the absence of added lipids for 40 years in cultures starting from initial cell concentrations well above the critical values. Apparently, we have uncovered the existence of a group of compounds - apart from nutrilites - essential for cell multiplication. These compounds have remained unnoticed because they have been transferred from the mother culture together with the cells at each subcultivation, unless the cells were washed with buffer prior to inoculation.

On account of these observations we have reinvestigated the separate roles of ethanol and cholesterol for the growth and multiplication of *T. setosa* in the standard synthetic nutrient medium. In contrast to previous workers (Holz et al. 1962) we have found that ethanol promotes cell multiplication in SSM. In the presence of ethanol *T. setosa* multiplied - in the presence of ethanol and without any lipid - as fast as they did in the presence

of cholesterol (without ethanol) and at the same temperature as in Koll and Erwin's (1990) experiments. At the time of our tests *T. setosa* had grown for more than four months (more than 20 subcultivations) in the absence of any exogenous lipid. The stimulatory effects of ethanol and cholesterol, however, are dependent on the initial population concentration; only at high concentrations (above 2,000 cells per ml) the cells thrive and multiply. Thus, as reported for *T. thermophila*, multiplication of *T. setosa* in SSM, supplemented with ethanol, is also related to a **critical low initial cell concentration**. Further similarities have been observed: *T. setosa* can form clones in 0.5 μl cultures of SSM supplemented with ethanol (corresponding to an initial population concentration of 2,000 cells per ml). Moreover, extracellular fluid, obtained from cells starved in TRIS/HCl buffer for 4 h, promotes cell proliferation at low initial cell concentrations. As controls, we have also made experiments with cell-free samples, where cells have been exposed to starvation conditions for 0-5 min. In these cases there was no stimulatory effect on cell multiplication. These results indicate that *T. setosa* and *T. thermophila* both release multiplication promoting compounds responsible for cell proliferation in synthetic nutrient medium. These compounds promote cell proliferation across the borderline between the two species, Table 2.

T. thermophila has been grown for many years without added lipids. Addition of cholesterol, however, was reported to make multiplication more reproducible (Everhart 1972) and addition of an impure preparation of phospholipids shortened doubling times slightly (Rasmussen and Dive 1980). Much greater effects of phospholipids were seen on the duration of the lag phases and cell survival (Ghiladi et al. 1992, Christensen and Rasmussen 1992). Cholesterol is an enigmatic compound in connection with *Tetrahymena*. It has never been identified in homogenates of these cells cultivated in the absence of dietary cholesterol (Koll and Erwin 1990). Their plasma membranes contain, apart from phospholipids, tetrahymanol, a pentacyclic triterpenoid alcohol (Holz and Conner 1973). Koll and Erwin (1990) have warned against too rash conclusions as to whether it occurs in *Tetrahymena* or not, since small amounts could be difficult to trace. They point out that cholesterol in amounts as low as 0.25 μg per ml (nanno molar concentrations) stimulates cell multiplication, a result we have confirmed (not shown).

The action of cholesterol is unknown. Exogenous compounds substituting for cell-produced multiplication promoting compounds in *T. thermophila* form an incoherent chemical group including tetrapyrroles, phospholipids, proteins (Schousboe et al. 1992, Ghiladi et al. 1992, Christensen et al. 1992, Christensen and Rasmussen 1992), and cholesterol (this report). They are active in micromolar concentrations or less. No constituent of the normal nutrient medium has this effect, although some of them, e. g. each of the 19 amino acids, are present in high (millimolar) concentrations. The exogenous stimulating compounds have no more than marginal effects on doubling times in exponential phase of growth, but they have very distinct effects on cell survival in the period before multiplication begins. The mechanism of their actions is still unknown. So far, the effects of compounds added to the standard synthetic nutrient media for *Tetrahymena* have been discussed exclusively in terms of synthetic routes and capacities. Our results as a whole indicate that they are compounds with signalling effects. The relation between cholesterol and initial cell concentration in *T. setosa* is under investigation.

We are now beginning to understand the requirements for growth and multiplication in these cells. The data presented here seem to point to a requirement of two groups of compounds for proliferation in *Tetrahymena*. Group 1 is **all the essential nutrients**, amino acids, vitamins, salts etc., taking part in processes of synthesis and degradation. Group 2 is **multiplication promoting compounds** released by the cells and taking part in processes initiating multiplication in subcultivated cells. These compounds may act as **growth factors**, and since they at least in part consist of molecules the cells can make themselves, we believe that they act on the **outside** of or **in** the plasma membrane.

The *T. setosa* cells we have been working with are from the same stock as those used by Erwin and colleagues (Holz et al. 1962, Koll and Erwin 1990), sometimes under the name of *T. setifera* (Holz et al. 1962).

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ACTA
PROTOZOOLOGICA

Motor Behaviour of Prey During First Steps of Food Capture by *Actinophrys sol*

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Summary. Samples of *Actinophrys sol*, taken 3-4 h after feeding, were supplied again with food, a flagellate *Chlorogonium elongatum* or a ciliate *Tetrahymena pyriformis*. First stages of the prey-predator interactions could then be noticed, although the heliozoa were incapable of ingesting more food. The prey moved randomly and was not attracted by heliozoa at a distance, nevertheless it accumulated around them in large numbers, by a trap mechanism. The flagellates were entrapped by adhesion to the bottom underneath *Actinophrys* cells and to the distal segments of axopodia that touch the substratum. Aggregations of the ciliates were dynamic and kept together by the changes of swimming direction, i.e. by chemokinesis.

Key words. Heliozoa, feeding behaviour, prey-predator relations.

INTRODUCTION

The procedure of food capture by *Actinophrys* and related genera of heliozoa has been subdivided by Hausmann and Patterson (1982) in two phases: prey adhesion to the predator and prey enclosure. The present study deals only with the first phase of prey-predator interaction. There was a common belief at the early period of heliozoa research that axopodia exert a toxic or paralysing effect on the food organisms which enter into contact with them (Leidy 1879, Penard 1904, Looper 1928). Later authors often reiterated this question and always raised the same arguments against that old opinion: there is no evidence of a paralysis of prey, since the ciliates

and flagellates continue swimming movements after they were fastened to axopodia, they can often escape and swim away, and eventually move inside the food vacuole until they are digested (Kitching 1960, Dragesco 1964, Rainer 1968, Suzaki et al. 1980, Patterson and Hausmann 1981). Occasionally, however, lytic agents may be released to the external medium and damage the prey organisms before their enclosure in food vacuoles (Linnenbach et al. 1983). As to the paralysing substances, only Davidson (1976) still postulated their secretion by muciferous bodies of *Ciliophrys* and *Heterophrys*. However, according to Patterson (1979), Centrohelidae to which these two marine heliozoa belong, are profoundly different from the Actinophryidae.

In the relatively modern literature, since Kitching (1960) and Dragesco (1964), the term "adhesion" is usually given to the link established between the heliozoon and its prey. The word adhesion seems, how-

ever, to be used either in its simple colloquial meaning as an undefined state of physical contact, or as the viscous attachment by a sticky layer of mucus. Prey is indeed easily attached to the glycoprotein coat which covers the surface of heliozoon and is shed out along axopodia, as observed by Kitching (1960) and by us (Grębecki and Hausmann 1992). However, the idea that as a rule, a certain category of extrusomes discharge and release this adhesive substance during feeding could not be unequivocally demonstrated by ultrastructural studies (Suzaki et al. 1980, Hausmann and Patterson 1982). The notion of adhesion is much more elaborated in the case of other motile cells, protozoan and metazoan, and involves specialized adhesive molecules with binding sites specific to the interacting surfaces. The nature of the links that bind prey to predator during food capture by heliozoa, as well as the nature of the motor phenomena which enable interaction of both cells, remain open questions.

MATERIAL AND METHODS

Cultures of *Actinophrys sol* grown in the Spa mineral water were fed on *Chlorogonium elongatum* or *Tetrahymena pyriformis*. Heliozoa were used for experiments 3-4 h after feeding. Beads of Sephadex G25 (Pharmacia, Uppsala, Sweden), ca.50 μm in diameter, were added to the slides with heliozoa as spacers supporting the coverslip and as control spheres with which the prey organisms could also mechanically interfere.

An ICM 35 inverted microscope of Zeiss (Oberkochen,Germany), equipped with Nomarski differential interference contrast and a Panasonic videosystem, was used for recording observations and experiments. The documentation was completed on videotapes in Berlin. The digital optimalization and analysis of image were done in Warsaw with an Argus 10 image processing system (Hamamatsu, Japan).

RESULTS AND DISCUSSION

Samples of *Actinophrys sol* were taken from mass cultures 3-4 h after feeding. A drop of heliozoa culture was then put on the slide and the food organisms, either *Chlorogonium* or *Tetrahymena*, were added again. At this stage of digestion, as earlier demonstrated by Patterson and Hausmann (1981) and Linnenbach et al. (1983), and confirmed by the present observations, the cell periphery becomes densely packed with empty vacuoles. Fig. 1 shows such a strongly vacuolized *Actinophrys* surrounded by freshly added *Tetrahymena*. According to Suzaki et al. (1980) it takes about 3-4 h until the capacity of food capturing is recovered. Patter-

son and Hausmann (1981) report that 20% of predator cells can catch the prey that is supplied at this stage. In the present experiments heliozoa were tested, 3-4 h after feeding, not for capturing new prey, but for its enclosure. The ingestion of food happened only in 1% of more than 200 specimens which were recorded on videotape. Nevertheless, heliozoa were still in close contact with the incessantly colliding flagellates or ciliates.

Therefore, this situation leads to dissecting early stages of the prey-predator interaction from the phase of effective feeding.

Small unsuccessful funnels, obviously due to mechanical stimulation by bumping ciliates, were occasionally found among heliozoa exposed to *Tetrahymena* 4 h after feeding (Fig. 2). Similar effects of collisions with prey and strokes of a glass micropipette were described by Kitching (1960). In our experiments, moreover, some of the vacuoles in the cortical layer of *Actinophrys* were apparently produced by intake of the external fluid, which could be provoked by the contact with ciliates. This is indicated by 1 μm latex beads, which were in many experiments added to the medium, and later could be seen inside the vacuoles (Fig. 3). Finally, the most spectacular event occurring about 15 min after mixing the predator and prey populations was the accumulation of food organisms around the heliozoans. This latter phenomenon indicates that probably Dragesco (1964) was right, when stating that a satiated heliozoon is still capable of immobilizing the prey, although it is incapable of ingesting it. In other words, according to the terms used by Hausmann and Patterson (1982), the response to new prey is blocked between the adhesion and enclosure phases of the feeding behaviour of *Actinophrys*.

Accumulation of the prey around well-fed and non-ingesting heliozoa is demonstrated in Figs. 4-7. Aggregates of *Chlorogonium* are larger and more stable than those of *Tetrahymena*. As demonstrated in Fig. 4, Chlorogonia cluster around *Actinophrys,* but are indifferent to Sephadex spheres, similar in size, which were added as a control. Pictures taken at a lesser focal depth show accumulation of *Chlorogonium* not exactly around, but rather underneath the heliozoa (Fig. 5). More accurate focusing at higher magnification reveals large number of flagellates aggregated at the bottom (Fig. 6a), a few attached to axopodia or swimming around the lower half of a heliozoon (Fig. 6b-d) and none near its upper surface (Fig. 6e, f). Accumulation of *Chlorogonium* on and beneath *Actinophrys* is due to thigmoattraction: flagellates are either fixed to axopodia or, what
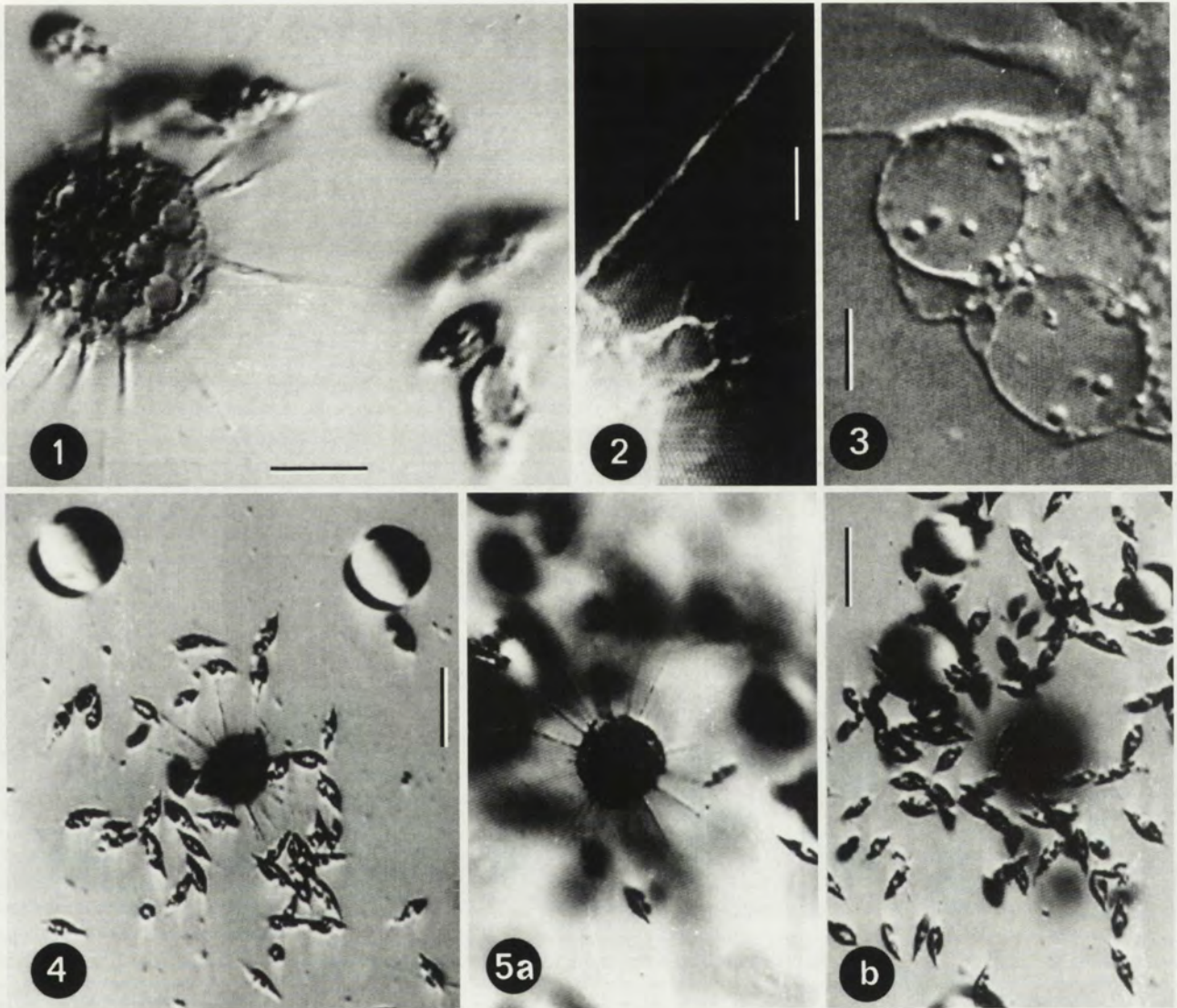
Figs. 1-5. 1- a specimen of *Actinophrys* 4 h after feeding and 10 min after addition of new prey. Note vacuolization of the heliozoon and the accumulation of *Tetrahymena* swimming around. Bar - 25 μm, 2- an example of rudimentary funnels that may be induced in well-fed and non-ingesting heliozoa by collisions with the ciliates. Bar - 5 μm, 3- latex beads internalized in the vacuoles after 30 min. of collisions with *Tetrahymena*. Bar - 5 μm, 4- aggregation of *Chlorogonium* close to *Actinophrys* and their absence around control spheres of Sephadex. Bar - 50 μm, 5- low focal depth picture of another aggregation of *Chlorogonium*. Note very few flagellates present at the *Actinophrys* cell level (a) and their massive accumulation beneath the heliozoon (b). Bar - 50 μm

seems unexpected but important, to the glass substratum between the axopodial tips (Fig. 6a). Therefore, one could suspect the diffusion of a soluble agent modifying the interaction of flagellates with the solid surface. Thus, the mechanism of prey capture seems not to be restricted to the mechanical attachment of prey by the sticky mucous layer covering *Actinophrys*.

The *Chlorogonium* cells fixed to the glass are hold fast only by their anterior ends, the flagella and cell body being free to perform oscillatory movements, and sometimes they detach and swim away. That not only validates the general use of the term adhesion (instead of paralysis) in relatively modern descriptions of the

interactions of heliozoa with prey (Kitching 1960, Dragesco 1964, Suzaki et al. 1980, Patterson and Hausmann 1981, Hausmann and Patterson 1982). Moreover, it should switch our attention to more complicated and subtle mechanisms of adhesion, which are known in other cells interacting with organic and inorganic surfaces. For example, some of the serum proteins adsorbed to solid substrata mediate the adhesion of leukocytes and fibroblasts (Curtis and Forrester 1984, Lewandowska et al. 1989). In the same manner, a protein diffusing from *Actinophrys* could magnify the adhesiveness of glass for the flagellates and thus, explain the origin of their accumulation beneath heliozoa.
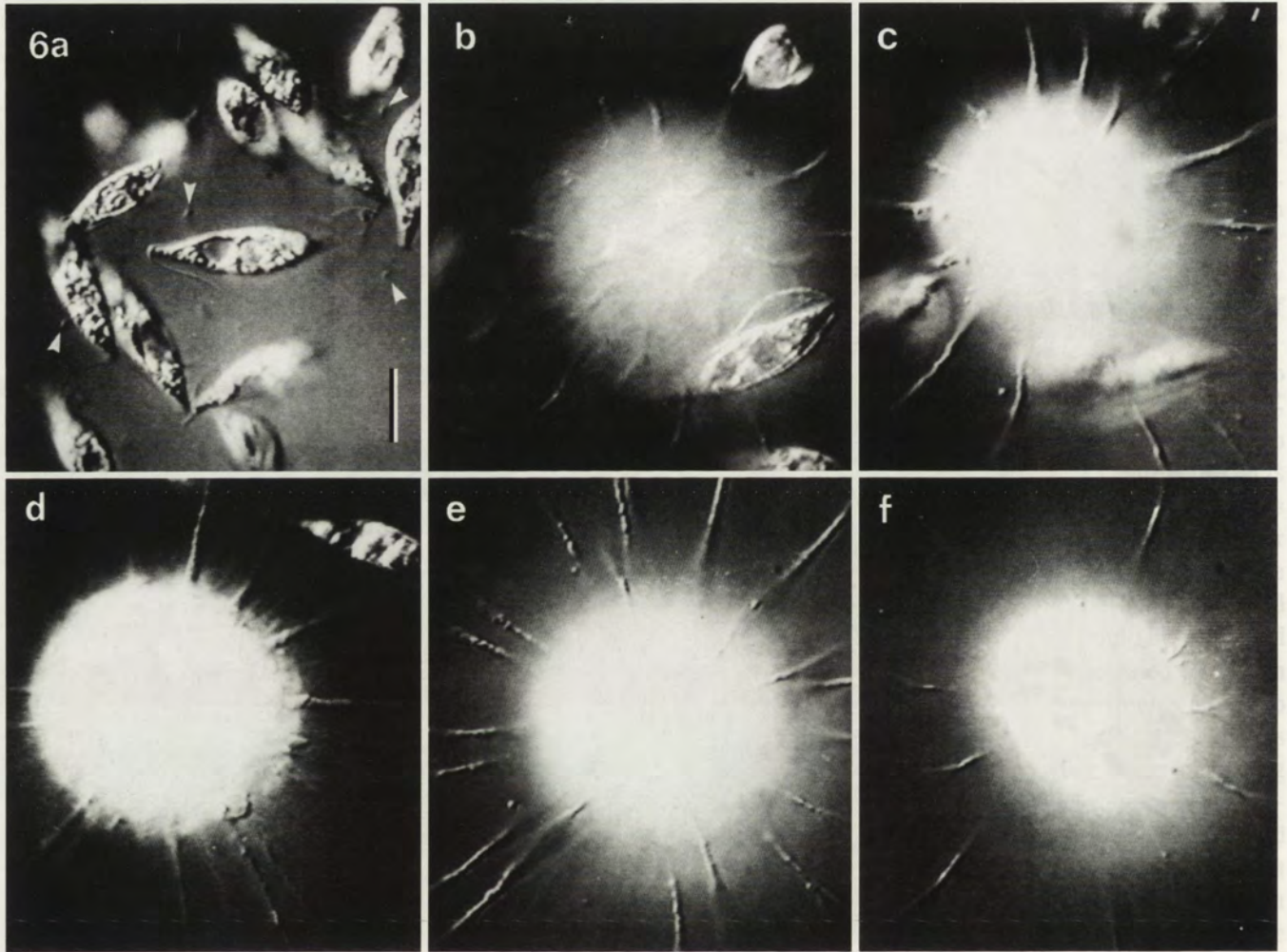
Fig. 6. Optical sections through *Actinophrys* with the accumulated aggregation of *Chlorogonium*. At the bottom level (a) a large number of flagellates adhere to the glass between axopodial tips that cling to the substratum (arrows). Only a few specimens attach to axopodia at low intermediate positions (b, c) or at the cell equator level (d). The upper levels (e, f) are empty. Bar - 10 μm
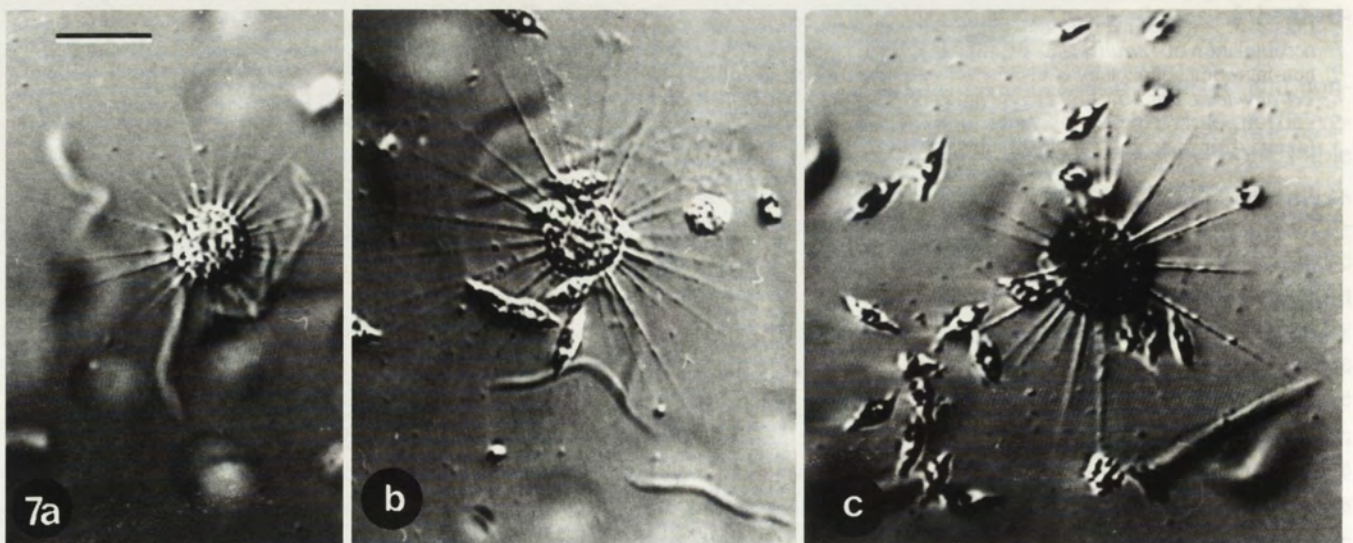

Fig. 7. Accumulation of Chlorogonia close to *Actinophrys* 2 min. (a), 5 min. (b) and 12 min. (c) after mixing both populations. Every picture was produced by digital accumulation of images from 32 frames. Immobile flagellates are shown as well as tracks of the swimming individuals. Bar - 50 μm
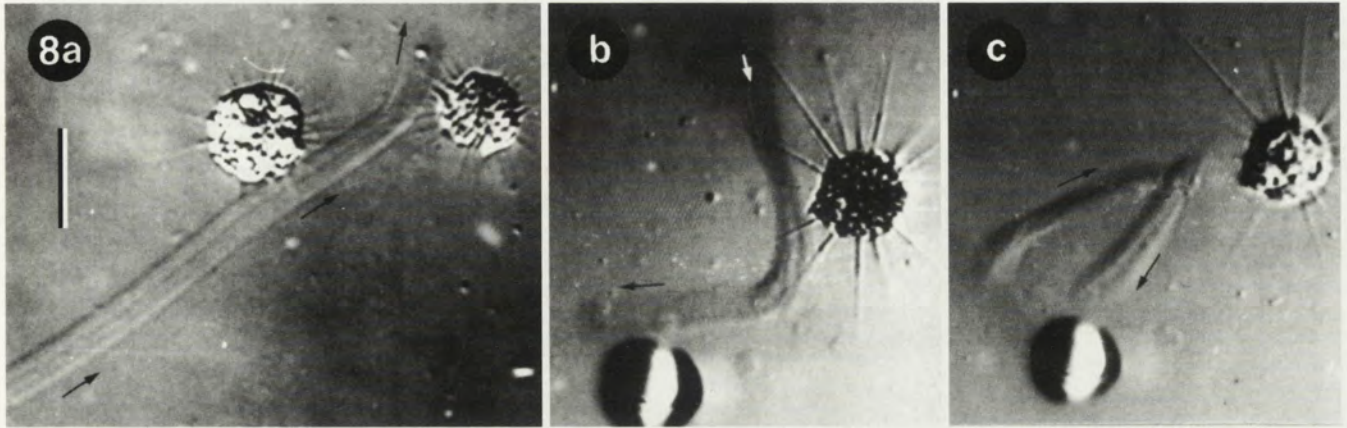
Fig. 8. Tracks of *Tetrahymena* swimming close to the heliozoa, recorded by accumulation of images from 16 videoframes (directions indicated by arrows); a - no reaction on meeting with the first *Actinophrys* cell and a smooth turn at the second one, b - another smooth turn, c - a sharp turn. Bar - 50 μm
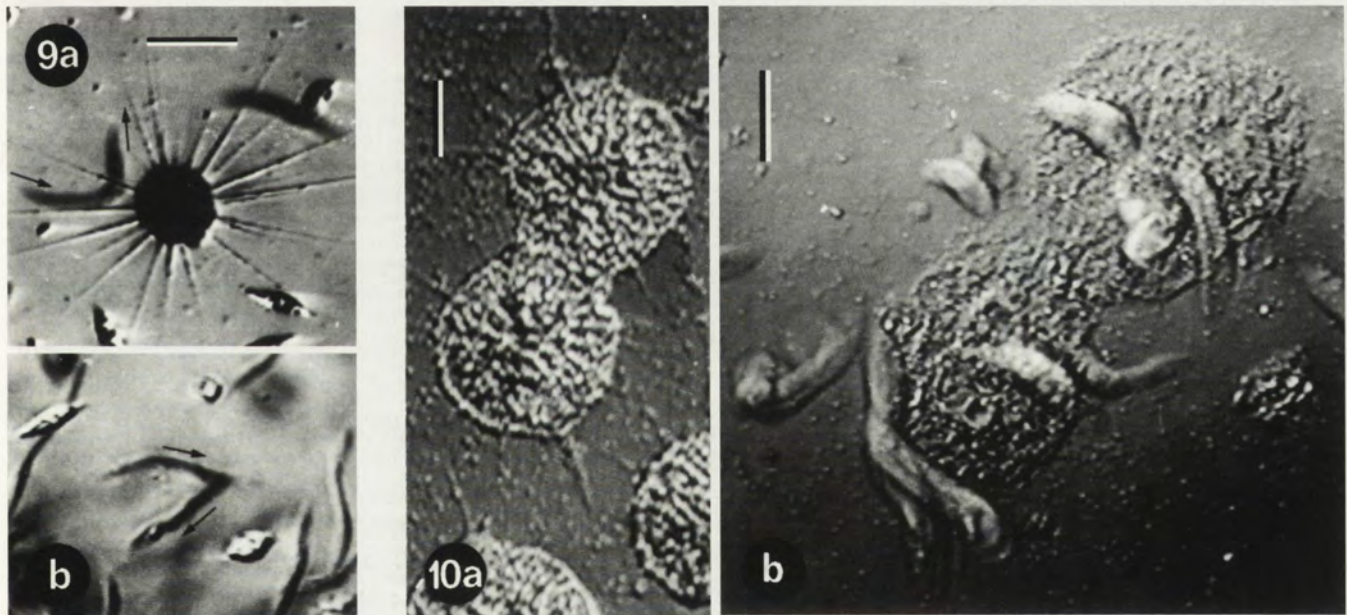

Fig. 9. Two types of changing swimming direction (arrows) by *Chlorogonium*: smooth (a) and sharp turns (b). Image accumulated from 32 videoframes. Bar - 50 μm

Fig. 10. Dry remnants of thermically killed heliozoa (a) and a group of swimming *Tetrahymena* cells aggregated on them (b). Note much shorter and in curved paths seen in the aggregation than traced by free swimming individuals, as demonstrated in Fig. 8a (in both cases 16 images were accumulated). Bars - 25 μm

The proportions of flagellates actively swimming around *Actinophrys* and those held beneath it by adhesion were recorded at regular intervals by digital accumulation of images from 32 consecutive videoframes. There was a regular increase in number of the stationary cell contours, while the tracks produced by swimming individuals gradually disappeared (Fig. 7). This clearly indicates that localized adhesion plays the role of a trap in which the *Chlorogonium* cells are accumulated, regardless of oriented or random character of their swimming.

Aggregations of *Tetrahymena* are, on the contrary, dynamic: they are composed of swimming individuals (cf. Figs. 1 and 10b), which are easily dispersed by external stimuli. Tracks of swimming *Tetrahymena* were obtained by accumulation of 16 videoframes. By this procedure three motor patterns could be recorded in the vicinity of *Actinophrys* and control spheres of Sephadex: forward swimming without any reaction (Fig. 8a), smooth deviations resulting in U-turns (Fig. 8a, b), and V-turns (Fig. 8c) that are due to immediate changes from forward to backward swimming. Similar swimming
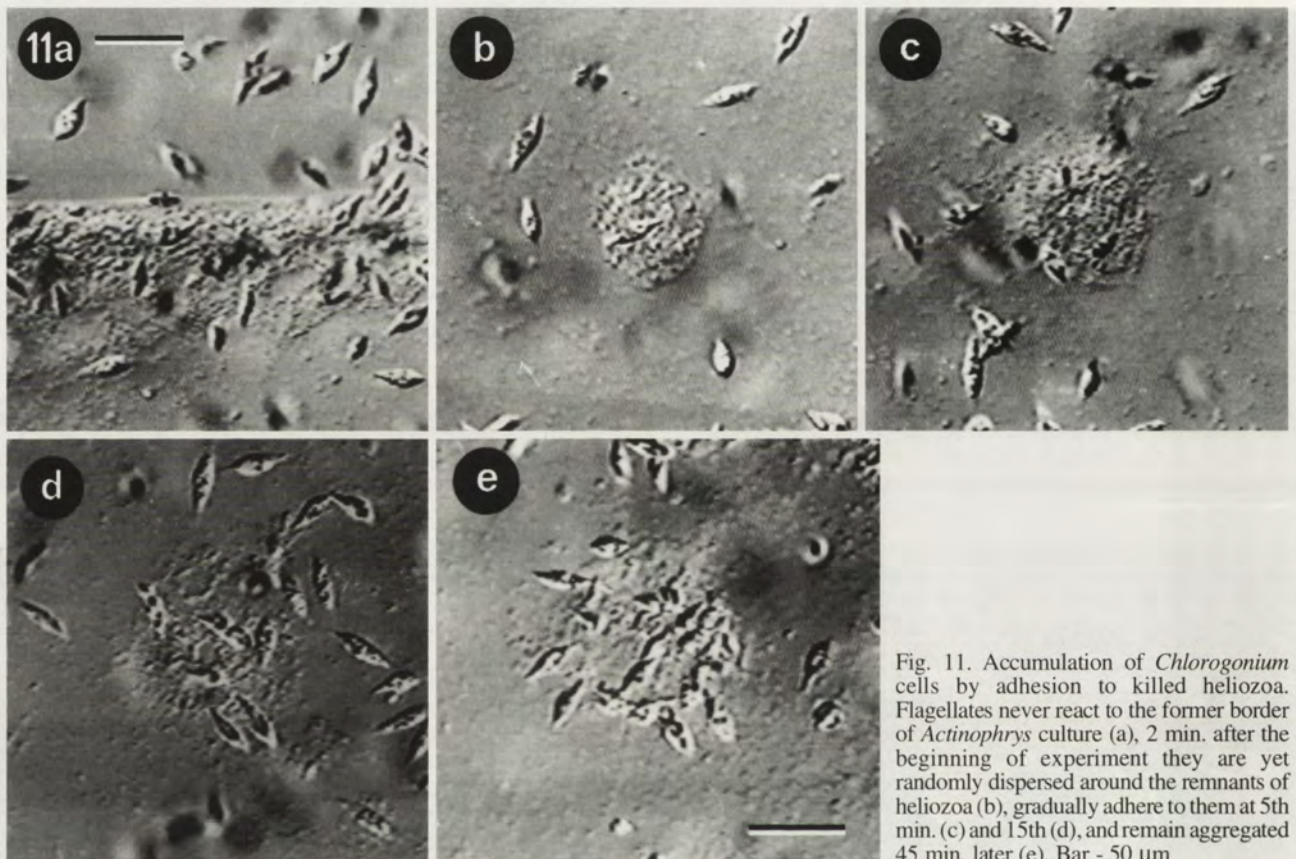
Fig. 11. Accumulation of *Chlorogonium* cells by adhesion to killed heliozoa. Flagellates never react to the former border of *Actinophrys* culture (a), 2 min. after the beginning of experiment they are yet randomly dispersed around the remnants of heliozoa (b), gradually adhere to them at 5th min. (c) and 15th (d), and remain aggregated 45 min. later (e). Bar - 50 μm

deviations were also generated by the flagellar activity of *Chlorogonium* (Fig. 9a, b).

The V-turn of *Tetrahymena* is identical with the avoiding reaction, discovered by Jennings (1906), which commonly occurs in ciliates as a result of an instantaneous but complete ciliary reversal. The U-turns are provoked by longer reversals of a part of cilia, as in *Paramecium* swimming in a Ca-deficient medium (Grębecki, 1965). Occurrence of these two kinds of turns in *Tetrahymena*, was recently mentioned by Leick and Hellung-Larsen (1992) as a quotation of unpublished observations.

Such alterations of the swimming path could constrain the movements of *Tetrahymena* to the neighborhood of an *Actinophrys*. The increased frequency of turning in the presence of a chemical agent is classified (according to Fraenkel and Gunn 1961 and Diehn et al. 1977) as chemokinesis, and is known as another trap mechanism, that produces focal aggregations of motile cells. The chemokinesis of *Tetrahymena* is most effectively induced by some peptids and proteins, including the adhesion and growth factors (Hellung-Larsen et al. 1986). Diffusion of soluble protein(s) around *Actinophrys* may be therefore suspected as a possible factor of the chemoattraction of *Tetrahymena*, as well as of the thigmoattraction of *Chlorogonium*.

Entrapping flagellates or ciliates by adhesion or chemokinesis, respectively, does not a priori necessitate any previous orientation of their swimming. Patterson and Hausmann (1981) estimated that the food capture by *Actinophrys* is preceded by random prey-to-predator contacts. This assumption was verified by analyzing (on videorecords) *Chlorogonium* and *Tetrahymena* movements in the presence of heliozoa mixed with Sephadex beads. The distribution of 1163 collisions produced by both species of prey (Table 1) disproves any orientation of their swimming before contact with an *Actinophrys*: they bumped against the control spheres of Sephadex exactly as frequently as against heliozoa. *Tetrahymena* yielded, however, nearly three times more avoiding reactions (V-turns) after interfering with *Actinophrys* than after colliding with Sephadex. This confirms that both organisms move at random before meeting a heliozoon, but become subsequently entangled either by adhesion, as the flagellates, or by frequent sharp changes of swimming direction, as the ciliates.

The accumulation of prey around *Actinophrys* is certainly propitious for food capture. It seems, however,
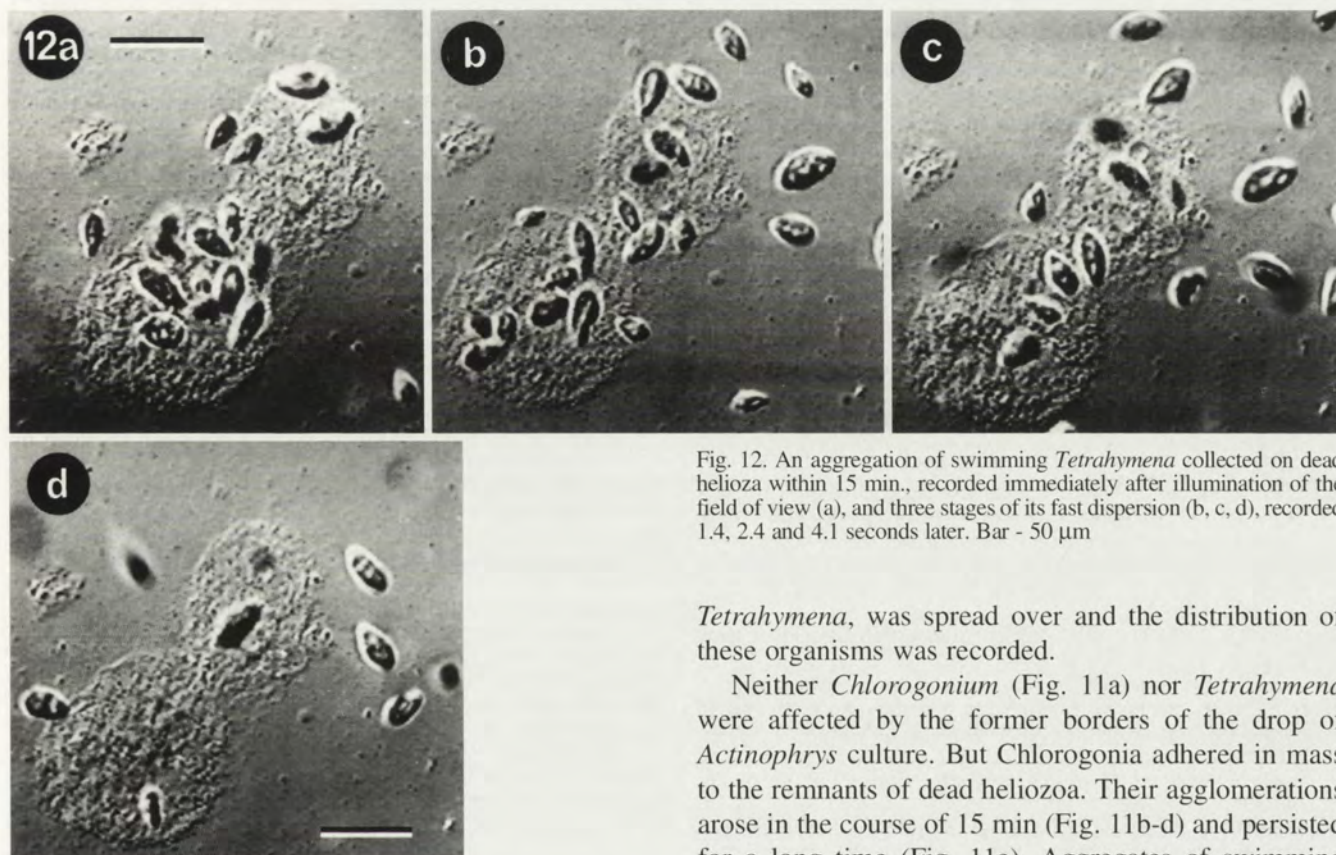
Fig. 12. An aggregation of swimming *Tetrahymena* collected on dead helioza within 15 min., recorded immediately after illumination of the field of view (a), and three stages of its fast dispersion (b, c, d), recorded 1.4, 2.4 and 4.1 seconds later. Bar - 50 μm

that the agents holding flagellates and ciliates close to *Actinophrys* are not, or not only, specifically secreted by the predator in response to the presence of prey. That is demonstrated by aggregation of *Chlorogonium* and *Tetrahymena* on killed heliozoa.

A drop of *Actinophrys* culture was heated to 50-60°C and allowed to evaporate on the slide (Fig. 10a). Then a larger drop, containing either *Chlorogonium* or *Tetrahymena*, was spread over and the distribution of these organisms was recorded.

Neither *Chlorogonium* (Fig. 11a) nor *Tetrahymena* were affected by the former borders of the drop of *Actinophrys* culture. But Chlorogonia adhered in mass to the remnants of dead heliozoa. Their agglomerations arose in the course of 15 min (Fig. 11b-d) and persisted for a long time (Fig. 11e). Aggregates of swimming *Tetrahymena* were assembled throughout a similar period of time (Fig. 12a), but could be dispersed during a few seconds by illumination of the field of view with 8000 lux of white light (Fig. 12b-d). The reaction of swimming *Chlorogonium* and *Tetrahymena* to dead heliozoa is specific: in control experiments they failed to accumulate on dried remnants of *Tetrahymena, Colpidium* or *Paramecium*.

It may be concluded that during digestion *Actinophrys* cannot absorb more food, but early stages of its interaction with prey are however manifested. Swimming flagellates and ciliates accumulate in abundance around heliozoa. Control experiments have demonstrated that these aggregations should not be attributed to chemotaxis, since the swimming of prey is unoriented. The prey is accumulated by trapping mechanisms. *Chlorogonium* is trapped beneath heliozoa by adhesion, not only to axopodia but - in the experiment - mainly to the glass substratum. Swimming of *Tetrahymena* is restricted to the vicinity of heliozoa owing to chemokinesis. Therefore, since both reactions take place in the close neighborhood of *Actinophrys*, but not exactly on its surface, their attribution only to the mucous coat becomes difficult. It may be stressed on the other hand, that adhesion of other motile cells to the

Table 1

Frequency of collisions of prey organisms (*Chlorogonium* and *Tetrahymena*) with the predator (*Actinophrys*) and an artificial obstacle (Sephadex spheres))

| | *Chlorogonium* with | | *Tetrahymena* with | |
	Actinophrys	Sephadex	*Actinophrys*	Sephadex
Number of experiments	12	14	14	10
Time total	7'05"	9'35"	14'36"	10'56"
Collisions total	303	425	245	190
Collisions per min.	43.45	44.05	17.30	18.39
S.D.	5.46	5.24	3.46	4.77
S.E.	1.58	1.40	0.92	1.51
Reaction after collision:no response	58%	62%	40%	62%
U-turn	31%	29%	32%	28%
V-turn	11%	9%	28%	10%

substrata, as well as other cases of chemoattraction in *Tetrahymena*, may be mediated by soluble proteins.

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ACTA PROTOZOOLOGICA

Genetics of Symbiotic Interactions between *Paramecium bursaria* and the Intranuclear Bacterium *Holospora acuminata*, Natural Genetic Variability by Infectivity and Susceptibility

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Summary. The creation of a symbiotic system between *Paramecium bursaria* and the intranuclear bacterium *Holospora acuminata* has been studied. The natural variability of hosts and symbionts by the feature of susceptibility and infectivity is shown. Compatible and incompatible symbiont-host genomes exist. As a rule, a particular *Paramecium* clone can only be infected by a symbiont isolated from the host of the same syngen. The stages in which the infection process was blocked in incompatible host-symbiont couples are also syngen-specific. One can define at least 5 stages in which the creation of a symbiotic system might be blocked by genetic incompatibility of host and symbiont. This provides good possibilities for genetic investigations of the infection process in this system.

Key words. Symbiosis, *Paramecium*, *Holospora*, micronucleus.

INTRODUCTION

Genetic analysis is one of the most powerful tools in the investigation of complex biological processes. This approach is based on breaking down the process of interest to the stages controlled by different genes. We have used this to study the formation of the symbiotic system between the ciliate *Paramecium bursaria* and the intranuclear symbiotic bacterium (SB) *Holospora acuminata*. *H. acuminata*, as with other members of the genus *Holospora*, has a complex life cycle, with a capacity to survive in the nucleus of the host cell and also to infect the new hosts.

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The symbiont population in the nucleus is represented by small vegetative cells, divided with transverse binding, as well as by large infectious forms which are unable to divide (Ossipov et al. 1980). Vegetative forms are not infectious and function only as reproductive cells within the host cell's nucleus (Rautian et al. 1978). They are, however, able to differentiate into infectious ones, ceasing division and undergoing significant internal reorganization. One can see dramatic changes in the cytoplasm, such as the appearance of electron-dense periplasmic material on one side of the cell (Gromov et al. 1976), and an altered polypeptide pattern (Görtz et al. 1990).

The infectious *Holospora* forms are removed from the infected cell to the medium. Then together with bacterial food they enter the ciliate's food vacuoles. Here they are not digested but enter the cytoplasm, specifically reorganizing the membranes of the host cell, and

then after interacting with the nuclear membrane they penetrate the target nucleus i.e., in the micronucleus (Mi) of *P. bursaria* in the case of *H. acuminata*. Having entered the nucleus, *Holospora* is referred to as the euinfectious form of SB (Ossipov and Podlipaev 1977), or activated form (Görtz and Wiemann 1989).

Investigation of the related bacterium *H. obtusa* showed the infectious forms to be unable to divide after their injection directly into the nucleus (Skovorodkin and Rautian 1990). This testifies to the fact that further changes to SB during transportation to the nucleus are necessary for its development in the nucleus. This result agrees with data on differences in polypeptide pattern of infectious forms before infection, and that of activated forms (Görtz et al. 1990).

Thus, the infection of *Paramecium* with intranuclear endobionts is a multistage, finely regulated process connected to the stage by stage recognition of host and symbiont. It consists of the release of SB from the digestive vacuole, its delivery to the target nucleus, the induction of protuberance(s) from the nuclear membrane, and finally penetration of the symbiont into the nucleus, followed by symbiont reproduction and differentiation into infectious forms. The genetic control of the initiation of such a symbiotic system is an important problem in general biology.

The present work is devoted to the elucidation of natural variability of symbionts and hosts, according to their ability to establish a symbiotic system. Initially we screened 57 clones of *P. bursaria* with 7 isolates of *H. acuminata* to locate incompatible partners. Investigation of stages where development of the symbiotic system was blocked constitutes the second part of the work.

MATERIAL AND METHODS

The work was carried out on *P. bursaria* clones from the collection belonging to the Laboratory of Protozoan Karyology, part of the Biological Research Institute of St. Petersburg State University. These have been collected for more than 10 years in various regions of the former Soviet Union. The clones from Murmansk, Irkutsk, Leningrad (St. Petersburg), Kaliningrad districts, from Karelia, Armenia, Georgia, Estonia, Latvia, Lithuania, Tadjikistan, Far East and Kamchatka, both with exconjugant clones appearing after cross-breeding of natural clones, are present in this collection. All clones are of three syngens which cannot be identified with the international collection of *P. bursaria* syngens (Bomford 1966), which have been lost from known international Protista collections.

Ciliates were cultivated according to conditions accepted for *P. bursaria* (Sonneborn 1970) at 18-20 °C with artificial illumination (14 h - light, 10 h - darkness).

Clones of *P. bursaria* without SB were infected by a crude homogenate of *P. bursaria* cells containing *H. acuminata*. In all infection experiments the number of infectious SB forms was not less than 100 per *P. bursaria* individual. This is sufficient to induce infection in susceptible partners. For all incompatible host-symbiont couples the experiments were repeated 3-5 times.

In the first part of the work 35 clones of syngen 1, 15 clones of syngen 2, and 7 of syngen 3 were used. Each clone was tested for it's ability to be infected by each of the 7 *H. acuminata* isolates. The presence of *H. acuminata* was confirmed in either Azur II stained preparations (Ossipov et al. 1980), or during the observation of detergent-lysed cells under an inverted "Opton" microscope, 2-3 weeks after infection. This method can reveal symbionts in very weak infections.

In the second part of the work we studied at which stage infection was blocked in incompatible partners, these being evident from the first part of the work. Clone AS62-14 of syngen 1, being the universal recipient (i.e., the clone that can be infected with every *H. acuminata* isolate), was used as a control in all experiments.

The entire process of infection was divided into 4 successive stages which were easily identified cytologically: the penetration of SB into the Mi, the beginning of SB reproduction in the nucleus - the chain formation, the division of vegetative cells, and the differentiation of infectious forms. After infection live cells of *P. bursaria* were studied daily by Nomarsky interference contrast with a "Polyvar" microscope. The presence of SB at each of the stages outlined above was recorded.

RESULTS AND DISCUSSION

The testing of infection ability of *H. acuminata* from 7 independently separated *P. bursaria* clones (isolates) was carried out as a first step. Four clones with SB were from syngen 1, and three from syngen 2. Unfortunately, we do not have *H. acuminata* from syngen 3.

Fifty seven *P. bursaria* clones were used as recipient clones: 35 from syngen 1, and 15 and 7 from syngens 2 and 3, respectively. While choosing the recipient clones we tried to embrace the maximal natural host variability. In this respect, we used unrelated clones from different natural water bodies situated in various geographic and climatic regions throughout the large territory of the former USSR, both with some exconjugant clones. The results are presented in Table 1.

One can see that some *P. bursaria* clones are universal recipients: they can be infected with every isolate of *H. acuminata*. Simultaneously, clones resistant to all available *H. acuminata* can be found. Nevertheless, in most cases the clones are resistant to infection by some *H. acuminata* isolates, but can be infected by others. As all experiments were repeated several times this result cannot be attributed to experimental error. It actually reflects compatibility of the symbiont and host with specific genotypes.

Undoubtedly, syngen infection specificity exists. This appears to very clear for syngen 2; each clone from this syngen is easily infected with *H. acuminata* from *P. bursaria* of syngen 2, but never with *H. acuminata*, isolated from syngen 1. Clones of syngen 3 also cannot be infected with SB isolated from another syngen (from syngens 1 or 2).

The same rule, i.e., the transition of the symbiont within the limits of it's own syngen, is also evident for *P. bursaria* clones of syngen 1. As a rule they are not infected with *H. acuminata*, isolated from syngen 2 *P. bursaria*. Nevertheless, syngen 1 clones are considerably more heterogeneous than syngen 2 clones, with respect to their ability to be infected with SB. Many of them are resistant to one, two or three SB isolates from the same syngen, and some cannot be infected with *H. acuminata* at all.

Thus, compatible and incompatible partner genotypes exist. It is impossible, however, to say that a particular host is resistant or susceptible, or that a particular symbiont is infectious or not. One can see two-by-two compatibility of host and symbiont. The complex multistage infection process, and prolonged maintenance of symbiont in the host nucleus are evidently regulated by many genes and gene complexes of both host and symbiont. Mutations in corresponding host genes may give rise to resistant genotypes. In turn there may be "complementary" mutations in the endosymbiont, compensating for breakages in the host genome and restoring the ability to form the SB. At the new level of integration between symbiont and host, mutation in one of the partners which breaks the symbiotic relationship, may be "complemented" by mutation in the other, thus restoring the status quo.

Thus, symbiont mutations which restore an infective capacity, will obviously render the same cell non-infective to the host with initial genotype. As a result, resistance and infectivity do not appear to be the properties of separate host and symbiont, but are features of a symbiont-host couple.

The repeated experiments on the infection revealed some interesting peculiarities of syngen 1. Some clones, initially infected with *H. acuminata*, became resistant to SB infection after prolonged cultivation (marked as +/- in Table 1). We have found several dozen symbiont-host couples where such resistance arose. At the same time the reversion, i.e., the restoration of the ability to be infected with *H. acuminata*, does not occurs. Several clones became resistant to SB of two different isolates non-simultaneously: firstly they developed resistance to one, and could still be infected by the other, and then

Table 1

Creation of the symbiotic system between *Paramecium bursaria* clones and isolates of *Holospora acuminata*

Paramecium bursaria		Isolates of *Holospora acuminata*						
		From clones of syngen 1				From clones of syngen 2		
s[*]	clones	E2-10	AS65-8	NP-54	MS-9	DS1-22	KM10-7	AK-1
1	AS62-14	+	+	+	+	+	+	+
	AS63-10	+	+	+	+	+	+	+
	MS3-33	+	+	+	+	+	+	+/−
	ASL1-75a	+	+	+	+	+	+	+/−
	AL3-5	+	+	+	+	+	+	+/−
	EB-2a	+	+	+	+	+	+	+/−
	AL2-10	+	+	+	+/−	+	+	+/−
	AL2-1	+	+	+	+	+	+	−
	88T24-15	+	+	+	+	+	+	−
	88T34-1	+	+/−	+/−	+	+	+	−
	AS62-2	+	+	+	+/−	−	+	−
	88T24-4	+	−	+	+/−	−	+	−
	MS-7	−	+	−	+/−	−	+/−	−
	ET87-2	−	+/−	−	−	−	+/−	−
	88T34-13	+	−	−	−	−	−	−
	NP-18	+	+/−	+/−	+	−	−	−
	GoB-4	+	+	+	+	−	−	−
	GB-4	+	+	+	+	−	−	−
	GL-34	+	+	+	+	−	−	−
	88T24-9	+	+	+	+/−	−	−	−
	88T28-5	+	+/−	+/−	+	−	−	−
	NP-28	−	+/−	+/−	+	−	−	−
	AS62-9	+	+	+	−	−	−	−
	AS63-4	+	+	+	−	−	−	−
	MS1-18	−	+/−	−	+/−	−	−	−
	GB-10	+	−	+	−	−	−	−
	GB-7	+	−	+	−	−	−	−
	GoB-1	+	+	−	−	−	−	−
	88T34-3	+	−	−	+	−	−	−
	MS87-1	+/−	−	−	−	−	−	−
	MS87-6	+/−	−	−	−	−	−	−
	MS87-8	−	−	+/−	−	−	−	−
	88T4-2	+	−	−	−	−	−	−
	88T24-14	+	−	−	−	−	−	−
	NP-2	−	−	−	−	−	−	−
	88T32-3	−	−	−	−	−	−	−
	88T24-6	−	−	−	−	−	−	−
2	DS1-7	−	−	−	−	+	+	+
	Ko3-4	−	−	−	−	+	+	+
	ES9-1	−	−	−	−	+	+	+
	KJ-1	−	−	−	−	+	+	+
	BM6-4	−	−	−	−	+	+	+
	Va1-1	−	−	−	−	+	+	+
	Va1-4	−	−	−	−	+	+	+
	SO4-10	−	−	−	−	+	+	+
	KJ-22	−	−	−	−	+	+	+
	KM10-7-31	−	−	−	−	+	+	−
	KM10-3	−	−	−	−	+	+	−
	Ko3-12	−	−	−	−	+	+	−
	DS1-19	−	−	−	−	+	+	−
	VM-14	−	−	−	−	+	+	−
	VM-3	−	−	−	−	+	+	−
	VM-1	−	−	−	−	+	+	−
	Va1-3	−	−	−	−	+	+	−
3	BP-5	−	−	−	−	−	−	−
	BP-28	−	−	−	−	−	−	−
	BP-39	−	−	−	−	−	−	−
	D12-2	−	−	−	−	−	−	−

Table 1 (continued)

Creation of the symbiotic system between *Paramecium bursaria* clones and isolates of *Holospora acuminata*

Paramecium bursaria clones s*	Isolates of *Holospora acuminata*						
	From clones of syngen 1				From clones of syngen 2		
	E2-10	AS65-8	NP-54	MS-9	DS1-22	KM10-7	AK-1
3 D12-7	–	–	–	–	–	–	–
D12-9	–	–	–	–	–	–	–
D12-12	–	–	–	–	–	–	–
D9-24	–	–	–	–	–	–	–

s*   syngenes

\+   *P. bursaria/H. acuminata* combinations create a symbiotic system.

\-   *P. bursaria/H. acuminata* combinations unable to create a symbiotic system.

+/-   *P. bursaria/H. acuminata* combinations had been able to create a SB , but after long term cultivation the ability to create SS has been lost.

Table 2

Development of resistance of *Paramecium bursaria* to named *Holospora acuminata* isolates

P. bursaria recipient clone	Date of infection						
Isolate *H. acuminata*	April 1985	August 1985	October 1985	December 1985	May 1988	October 1988	April 1990
MS-7/MS-9	+	+	0	+	–	0	0
MS-7/KM-10-7	+	–	0	–	–	0	0
AL2-10/MS-9	+	0	0	0	+	+	–
AL2-10/AK-1	+	0	0	0	–	–	–
ET87-2/AS65-8	+	+	+	0	–	–	0
ET87-2/KM10-7	+	–	–	0	–	–	0

\+ *H. acuminata* able to infect *P. bursaria*.

– *H. acuminata* cannot infect *P. bursaria*.

0 Not tested.

resistance to SB of both isolates appeared (see Table 2). Moreover, the resistance to SB from syngen 2 ("stranger") arises first, and then to SB from syngen 1. It is worth nothing that some syngen 1 clones and all syngen 2 clones are stable in their ability to be infected by, and support *H. acuminata* over 5-9 years of regular testing (Table 1).

These facts may possibly be explained by the peculiar organization of the ciliate nuclear apparatus. The development of Ma is connected with significant nuclear rearrangement (Steinbruck 1986, Görtz 1988). Clone aging probably leads to the loss of some genes or gene complexes. The loss of genes necessary for SB infection and/or support, may be thought of as the reason that resistance has arisen. Non-simultaneous development of the resistance to different *H. acuminata* isolates testifies to the participation of at least two genes in the control of this function.

We have to underline that the appearance of resistance to *H. acuminata* infection is typical only for syngen 1 clones. This may be the reason for comparatively high numbers of incompatible couples of host-symbiont in this syngen.

Hybridological analysis carried out according to our plans, allows us to fully study the mechanisms of genetic regulation of symbiotic relations in this system.

The data obtained shows that the symbiont *H. acuminata* rarely crosses the syngen barrier; they live and are distributed within their "own" syngen. The peculiarity of certain intraspecific host groups means that SB occupied different ecological niches to which they are adapting. Further isolation may strengthen the divergence of different *H. acuminata*, initial stages of which we now see.

The second part of the work was devoted to the determination of certain stages in which the infection process is blocked in the case of incompatible partners, revealed in the first part of this work.

We studied *P. bursaria* clones which were fully resistant to *H. acuminata*, as well as clones resistant to some isolates of SB, but susceptible to infection by
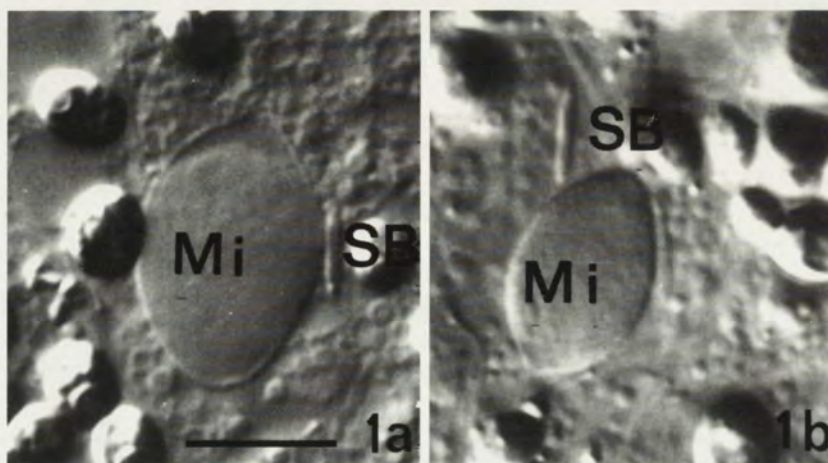

Fig. 1. Symbiont at the Mi. Bacteria are in the cytoplasm, close to the nucleus, but are unable to enter. 1a - *P. bursaria* BP-5/*H. acuminata* DS1-22. 1b - *P. bursaria* BP-5/*H. acuminata* NP-54. Mi - micronucleus, SB - symbiotic bacteria *H. acuminata*. Bar - 5 µm

Table 3

Ability of *Holospora acuminata* to pass particular stages during the infection process

Paramecium bursaria		From clones of syngen 1				From clones of syngen 2		
		E2-10	AS65-8	NP-54	MS-9	DS1-22	KM10-7	AK-1
					infection stages			
syngenes	clones	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
1	AS62-14	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	NP-18	+ + + +	+ + + +	+ + + +	+ + + +	–	–	–
	GG-26	+ + + +	+ + + +	+ + + +	+ + + +	–	–	–
	GoB-4	+ + + +	+ + + +	+ + + +	+ + + +	–	–	–
	AS62-9	+ + + +	+ + + +	+ + + +	± –	–	+ –	–
	AS63-4	+ + + +	+ + + +	+ + + +	–	–	–	–
	GB-10	+ + + +	–	+ + + +	+ –	–	–	–
	MS87-1	+ + + +	+ + – –	–	–	–	–	–
	MS87-6	+ + + +	+ –	–	–	–	–	–
	MS-7	–	+ + + +	–	–	–	–	–
	NP-28	–	+ + + +	–	–	+ –	–	–
	MS1-18	–	–	–	+ + + +	–	–	–
	ET87-2	–	–	–	–	–	–	–
	NP-2	–	–	–	–	–	–	–
	NP-17	–	–	–	–	–	–	–
	88T32-3	–	–	–	–	–	–	–
	88T32-8	–	–	–	–	–	–	–
2	ES9-1	–	–	+ –	–	+ + + +	+ + + +	+ + + +
	KJ-1	± –	+ –	+ –	± –	+ + + +	+ + + +	+ + + +
	BM6-4	+ –	+ –	+ –	+ –[1]	+ +	+ + + +	+ + + +
	Ba1-1	+ –	+ –[1]	+ –[1]	+ –[1]	+ +	+ + + +	+ + + +
	Ba1-4	+ –[1]	+ –[1]	+ +	– + –	+ +	+ + + +	+ + + +
	Ba1-5	+ –[1]	+ –[1]	+ –[1]	+ –[1]	+ +	+ + + +	+ + + +
	SO4-10	+ + –	+ –	+ + –	–	+ + + +	+ + + +	+ + + +
	KJ-22	+ + –	+ –	+ + –	+ + –	+ + + +	+ + + +	+ + + +
	KM10-7-31	–	–	–	–	+ + + +	+ + + +	–
	KM10-3	–	–	–	± –	+ + + +	+ + + +	± –
	DS1-19	+ –	+ –	+ + –	+ –	+ + + +	+ + + +	± –
	BM-14	–	+ –[1]	+ +	– –	+ +	+ + + +	+ + –
	BM-3	+ –[2]	+ –	+ + –	+ + –[2]	+ + + +	+ + + +	± –
	BM-1	+ –	+ –	+ + –	+ –[1]	+ +	+ + + +	+ + –
	Ba1-3	+ –	+ –[1]	+ +	–[1] + –[1]	+ +	+ + + +	+ + –[2]
3	BP-5	+ –	+ –	± –	± –	± –	+ + –	± –
	BP-28	–	+ –	–	–	± –	+ –	–
	BP-39	± –	± –	–	–	± –	+ + –	–
	D12-2	–	–	–	–	–	–	–
	D12-9	–	–	–	–	–	–	–
	D12-12	–	–	–	–	–	–	–
	D9-24	–	–	–	–	–	–	–

Stages:
1 - penetration of *Holospora acuminata* into the micronucleus.
2 - division of euinfective forms of *Holospora acuminata* - chain formation.
3 - division of vegetative forms of *Holospora acuminata*.
4 - maturation of infectious forms of *Holospora acuminata*.

Abbreviations:
+ Existence of SB at the respective stage.
– Absence of SB at the respective stage.
–[1] Rapid lysis of *Holospora acuminata*.
–[2] Delayed lysis of *Holospora acuminata*.
± Small amount of SB penetrate the micronuclei of low number (less than 5%) of *P. bursaria* cells.

others. The universal recipient (clone AS62-14) has
been infected as a control. The entire infection process
has been separated into 4 successive stages, easily iden-
tified cytologically as: SB penetration to Mi, the begin-
ning of SB division in the nucleus - chain formation,
vegetative cell division, and formation of infectious
forms. The results are presented in Table 3.

The syngen specificity of host-symbiont relations
was also revealed at this level. In syngen 1 clones the
resistance in most cases is caused by symbiont inability
to enter the Mi. This stage is the weak spot for
H. acuminata infection with specificity for syngen 1, as
well as for syngen 2.

Sometimes (in the pairs: AS62-9/MS-9, BP-39/E2-
10, BP-39/AS65-8 et al.) symbionts enter the Mi of a
relatively small number of cells (5-10%) and are
eliminated in 1-2 days. Symbiont lysis, typical for some
syngen 2 clones (see later), was never observed.

One symbiont-host couple without symbiont penetra-
tion into the nucleus (MS-7/DS1-22, see Table 3) was
investigated with the help of electron microscopy
(Borchsenius et al. 1992). The symbionts were found to
enter, but not exit, the food vacuole. According to light
microscopy, the exit of the symbiont from the food
vacuole, where the infection process in syngen 1 clones
is blocked in the most cases...(!!)

In syngen 3, as in syngen 1, the infection process is
blocked before symbiont penetration into the Mi, but
one can see some difference when comparing with
syngen 1 clones. In many symbiont-host couples, single
SB enter very few host cells (marked as "+" in Table 3).
Such "rare" events are, however, repeated regularly.
Sometimes we succeeded in observing the symbiont
"attack" on the Mi (Figs. 1a, b): bacteria are present in
the *Paramecium* cytoplasm, close to the nucleus, but
cannot enter. This demonstrates that in these cases

H. acuminata come away from the food vacuole, and
infection is blocked at the stage of symbiont penetration
into the nucleus. The existence of a block at this stage
is of considerable significance. Hence, the exit of SB
from the food vacuole can rarely be determined confi-
dently by the use of light microscopy. Further study of
this stage is required.

In the resistant variants of syngen 2 recipient clones,
SB history differs from that of syngen 1 and 3 clones.
In most cases *H. acuminata* penetrate the Mi with high
intensity and extent. This stage does not differ from the
normal infection process. Nevertheless, in some sym-
biont-host couples (Ba1-4/E2-10, Ba1-1/AS65-8 et al.),
lysis of the bacteria begins 7 -10 hours after symbiont
penetration of the nucleus, ending within 1-2 days (Figs.
2a, b). Lysis has been studied in morphological detail
with both light and electron-microscopy (Skoblo et al.
1990, Ossipov et al. 1993). Bacteria undergo this rapid
type of lysis before they start dividing.

In some cases (BM-3/E2-10, BM-3/MS-9, Ba1-
3/AK-1), bacterial lysis begins 5-7 days after entering
the nucleus. SB destruction during such lysis has been
observed only with a light microscope. Bacteria enter
the Mi as normal but division is impeded, chains are rare
and consist of no more than 2-3 links of different size,
as opposed to the normal with up to 8 links (compare
Figs. 3a and 4a). In Fig. 3 the SB are swelling. Bacterial
destruction ends after 9 days, and then only remains of
the bacterial cell wall can be found in the nucleus (Fig.
3c). Reasons for bacterial lysis are still unknown, both
in terms of the endogenous or exogenous nature of this
process.

From Table 3 it is clear that in many incompatible
couples of *H. acuminata* with syngen 2 clones, SB
penetrates the nucleus and start to divide. Nevertheless,
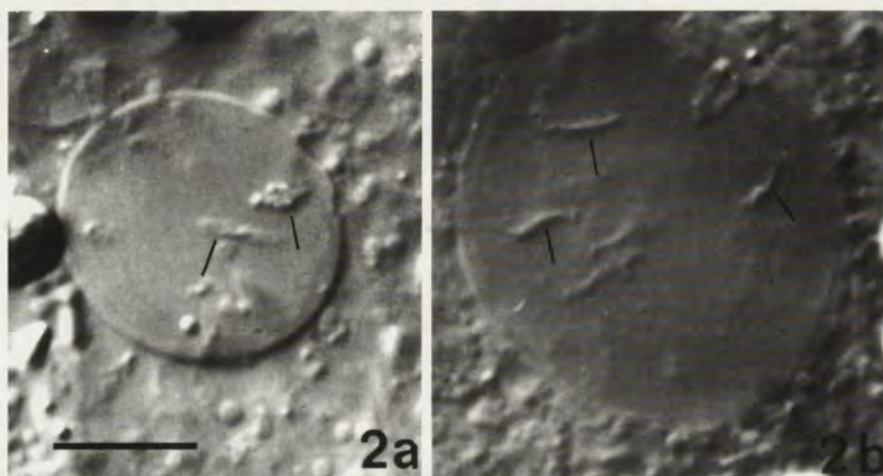the development of infection in the nucleus differs sig-

Fig. 2. The morphology of *H. acuminata*, undergoing rapid lysis. 1a - one day after SB penetration (arrow - destroyed SB). 2b - two days after penetration (arrow - bacterial cell wall remnants). Bar - 5 μm
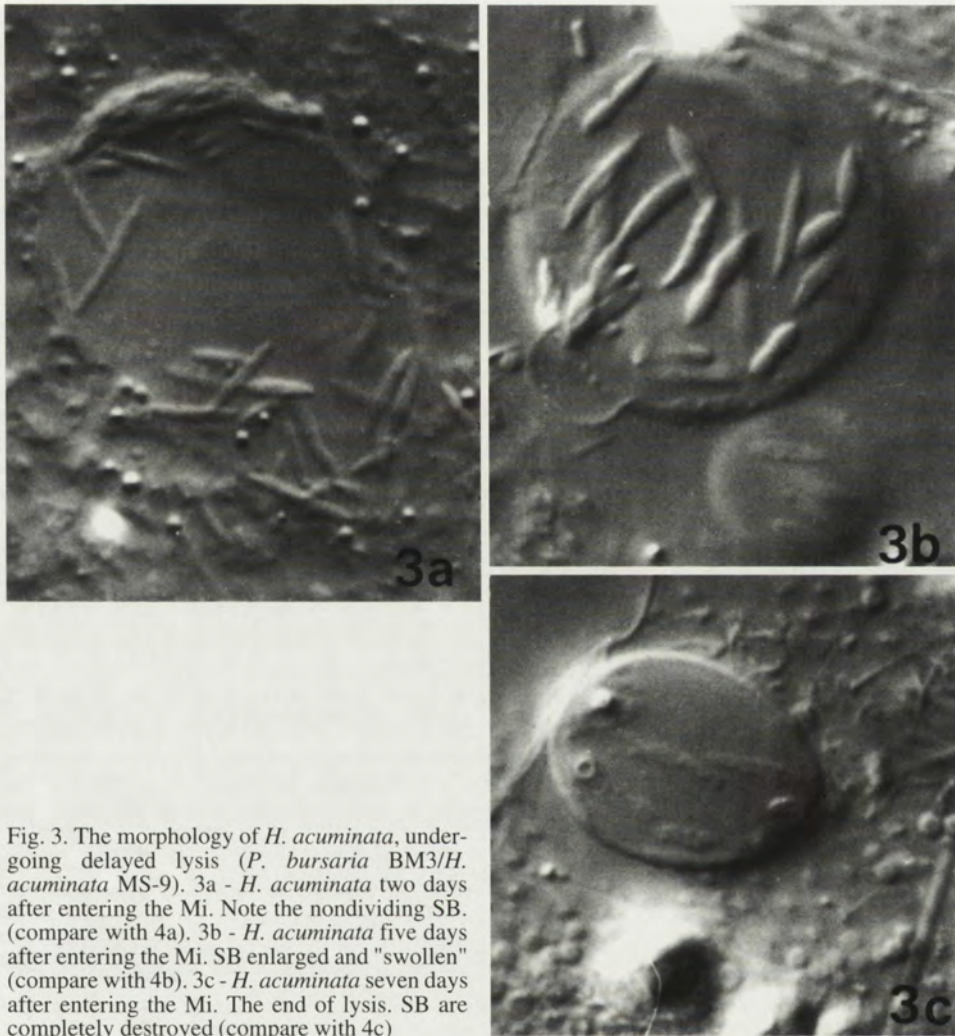
Fig. 3. The morphology of *H. acuminata*, undergoing delayed lysis (*P. bursaria* BM3/*H. acuminata* MS-9). 3a - *H. acuminata* two days after entering the Mi. Note the nondividing SB. (compare with 4a). 3b - *H. acuminata* five days after entering the Mi. SB enlarged and "swollen" (compare with 4b). 3c - *H. acuminata* seven days after entering the Mi. The end of lysis. SB are completely destroyed (compare with 4c)

nificantly from normal. The number of vegetative SB cells does not increase, but decreases gradually without any formation of infectious forms. Uninfected paramecia appear in growing number. The process of gradual Mi deliberation from the vegetative form allows the reproduction process in SB. Probably, the host may lack some specific regulator or substrate to be broken down, necessary for reproduction SB genotype. The first divisions occur due to substances accumulated in the euinfectious body, and further divisions stop, or are sharply slowed. As a result, the number of symbionts and/or total effect of their vital functions in the nucleus does not reach a "critical" level, that necessary for triggering the maturation of infectious forms.

It is interesting to note that within the natural varieties we could never find any symbiont-host combination with a block at the infectious forms at differentiation stage. Damage at this stage is ultimately eliminated in nature.

Results of determining stages where the infection process might be blocked, permit some conclusions to be made. At least 5 crucial points may be distinguished in the formation of this symbiotic system.

(1) SB cannot leave the food vacuole.

(2) SB exits the leave food vacuole to the cytoplasm, but cannot enter the Mi.

(3) SB's penetrate the Mi and are subject to the rapid (1-2 days) lysis without division.

(4) SB enter the Mi and start dividing. Hence, chain formation and vegetative divisions are halted. The process is ended by *H. acuminata* lysis after 7-9 days.

(5) SB's penetrate the nuclei, chains and vegetative cells are formed, but the latter do not reproduce. Host cell division causes a rapid and total decrease in symbiont numbers. Infectious forms do not differentiate in this case.

The frequency of each type of block differs between syngens. Thus, in syngen 1 the most wide spread block
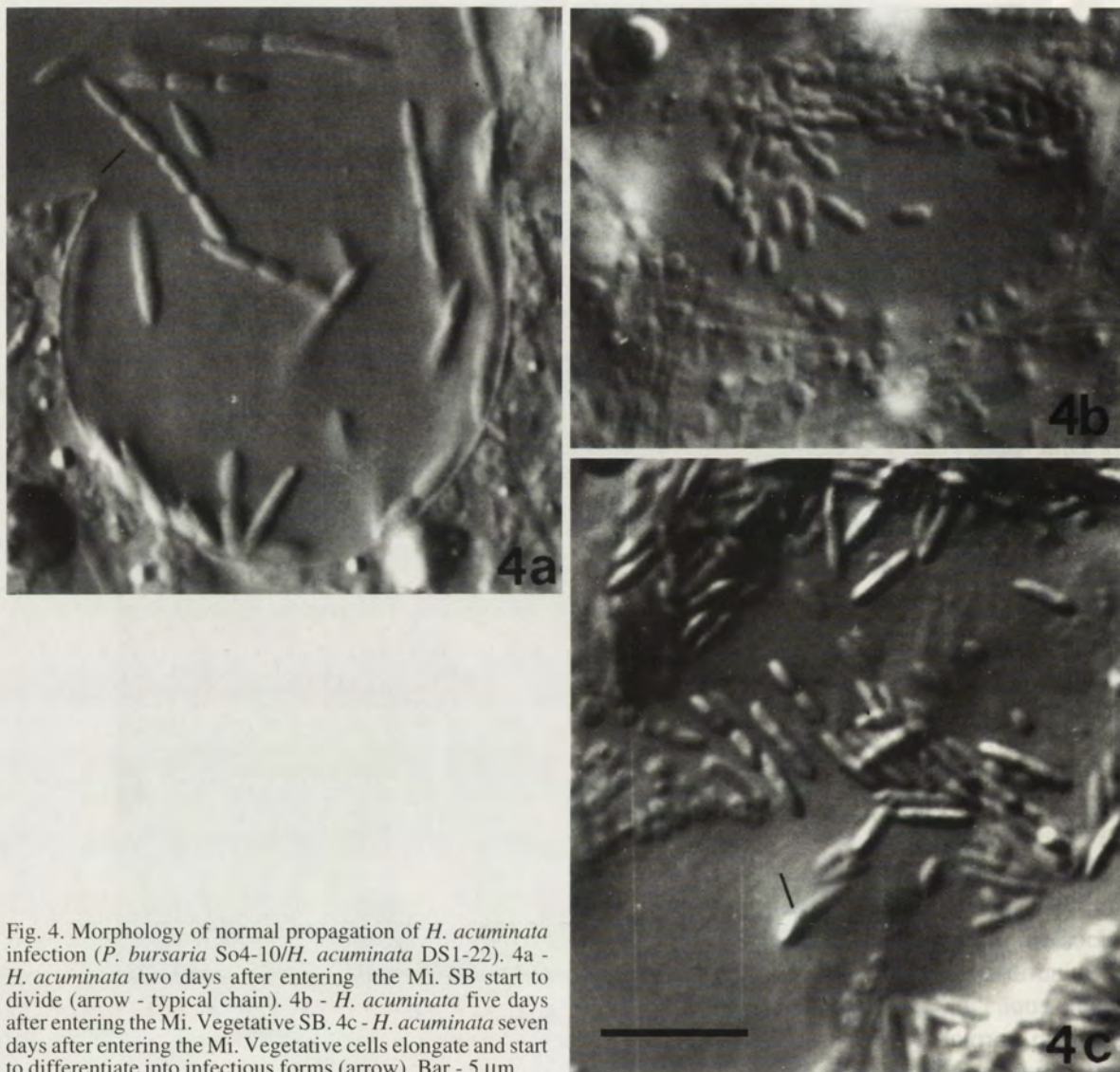
Fig. 4. Morphology of normal propagation of *H. acuminata* infection (*P. bursaria* So4-10/*H. acuminata* DS1-22). 4a - *H. acuminata* two days after entering the Mi. SB start to divide (arrow - typical chain). 4b - *H. acuminata* five days after entering the Mi. Vegetative SB. 4c - *H. acuminata* seven days after entering the Mi. Vegetative cells elongate and start to differentiate into infectious forms (arrow). Bar - 5 μm

is on the first of the mentioned stages, but lysis is never observed. Conversely in syngen 2, lysis (stages 3 and 4) is widely spread, in both the uncoordinated reproduction of symbiont and host (stage 5) occur.

The results of similar work with related SB - *H. obtusa* - differ significantly. During infection of *Paramecium caudatum* with *H. obtusa* no syngen infection specificity has been observed: different *H. obtusa* isolates infect every syngen *P. caudatum*. The number of resistant clones was very small (Rautian et al. 1990). Moreover, analysis of break at various stages of infection showed SB to enter the nucleus in each case. We revealed three stages where the symbiotic relation may be broken:

(1) Symbiont reproduction, when SB do not divide.

(2) Uncoordinated host and symbiont reproduction. At first SB divide intensively, occupying the whole

nucleus, but the rate of divisions decreases and the host is gradually freed of SB. This process may continue for up to one month, occasionally more.

(3) The stage of differentiation of infectious forms.

Such significant differences in relate symbionts and hosts are surprising. They may be explained by the fact that *H. acuminata* is the symbiont of Mi, while *H. obtusa* is that of Ma. The genetic mechanism regulating transport to Mi and Ma may differ significantly. In any case, the breaches, damaging formation of the symbiotic systems *P. caudatum/H. obtusa* and *P. bursaria/H. acuminata* differ qualitatively and quantitatively in natural populations, having been the source of genetic variability in our experiments.

It is interesting to compare our results with those of Fujishima et al.(1991). These workers studied the effect

of various physical and chemical factors on the infection ability of *H. obtusa*. Using a wide range of physical and chemical agents on infectious forms of *H. obtusa* (acids, bases, alcohols, detergents, enzymes, temperature and pH), the authors demonstrated such forms to be rather resistant. Only strong acids, bases and strong detergents - interrupted SB transport to the nucleus. They did not discover any selective agent that permitted separation of transport from propagation of the SB in the nucleus. When SB appear in the nuclei, they start dividing.

The loss of infection ability after SB incubation in α-mannosidase is the most interesting data in the work mentioned. It is one of the tested agents whose effect might be selective enough. Fujishima and co-workers concluded that some component of the *H. obtusa* cell wall, sensitive to α-mannosidase plays a significant role in the process of entering and/or recognition of nucleus. We have demonstrated a block at the stage of exit from the food vacuole. This may be stage that is sensitive to α-mannosidase in *H. acuminata*.

Our work allows at least 5 stages to be elucidated where creation of the symbiotic system might be blocked in the case of host and symbiont genetic incompatibility. We wish to underline that in spite of the syngen specificity discovered in this work, the clones in which the infection process is blocked at different stages are found within every syngen. This opens the way for genetic analysis of the infection process.

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ACTA PROTOZOOLOGICA

Idiocolpoda pelobia gen. n., sp. n., a New Colpodid Ciliate (Protozoa, Ciliophora) from an Ephemeral Stream in Hawaii

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Summary. *Idiocolpoda pelobia* gen. n., sp. n. was discovered in the mud of a dry river bed in Hawaii. Its morphology and infraciliature were studied in live cells using interference contrast optics and in specimens impregnated with silver carbonate and protargol. The new genus, *Idiocolpoda*, belongs to the family Colpodidae and is unique by having the oral apparatus located entirely at the left side of the body. The new species, *I. pelobia*, measures 25-40 x 15-30 μm and has a deep postoral groove which contains 2 specialized kineties. The two lowermost preoral kineties of the left side are distinctly shortened. The small oral apparatus is situated slightly above mid-body in the right half of the left side of the cell. The flat vestibulum is conspicuously scrotum-shaped and unroofed, exposing the oral polykinetids, which are very similar to those known from small species of the genus *Colpoda*, on the surface of the cell. An improved key to the genera of the family Colpodidae is provided.

Key words. Ciliophora, Colpodea, Colpodidae, ephemeral stream, Hawaii, *Idiocolpoda pelobia* gen. n., sp. n., infraciliature.

INTRODUCTION

55 genera with a total of 170 species are currently assigned to the class Colpodea (Foissner 1993). Most colpodid genera are defined by their oral structures which show a puzzling diversity. Some were thus originally misclassified as gymnostomes (e.g. *Platyophrya*), hypostomes (*Pseudochlamydonella*) or heterotrichs (e.g. *Bursaria*). The new genus described here is unique by having the oral apparatus located entirely at the left side of the body. *Idiocolpoda pelobia* thus looks like the mirror image of a small *Colpoda*.

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MATERIAL AND METHODS

Idiocolpoda pelobia was discovered on 26. 06. 1992 in a sample of dry mud collected from the bottom of a temporary stream in Hawaii, North Kohala, where Coastal Road 270 crosses the river bed about 100 m above sea-level (W 155° 50' N 20° 04'). This brooklet rises at Kaunu a Kaleloohie in the Kohala Mountains. It has a steep gradient and was dry when the sample was taken. No data are available as to often and at which times it carries water. The stream bed is full of small and large rock pools many of which have a cylindroid shape indicating that there are often strong water currents. The dry mud, which contained the cysts of *Idiocolpoda pelobia,* was collected from two large, rather flat rock pools (lithotelmes) in the centre of the river bed. The mud consisted of soil particles, plant debris and detritus, and was covered by a greenish algal crust.

In the laboratory, the dry mud was saturated with distilled water according to the non-flooded petri dish method (Foissner 1992a). The rewetted mud had pH 7.1 and was highly saline. *Idiocolpoda pelobia*

appeared 4 weeks after rewetting, when the salinity was already weaken because some soil water had been removed and replaced by distilled water during this time. Attempts to establish pure cultures with the usual methods failed. Thus, all results are based on material taken from the raw culture described above.

Cells were carefully studied in vivo using a high-power oil immersion objective and interference contrast (Foissner 1992b). The silver carbonate method, as described in Foissner (1992c), was used to reveal the infraciliature; it yielded excellent preparations. Biometry and drawings were done on protargol impregnated specimens (Foissner 1992d, protocol 1). However, the results with this method were rather mediocre because cells were covered by a thin, protargol-affine layer. Unfortunately, material was too scant to apply the Chatton-Lwoff silver nitrate method; the dry silver nitrate technique did not work, possibly because the material was saline too. Thus, no data can be provided on the silverline system.

Counts and measurements on silvered specimens were performed at a magnification of x 1,000. In vivo measurements were conducted at a magnification of x 250 - 1,000. Although these provide only rough estimates, it is convenient to give such data as specimens usually shrink in preparations or may even contract during fixation. Standard deviation and coefficient of variation were calculated following textbooks on statistics. Drawings of impregnated specimens were made with a camera lucida.

DESCRIPTION

Data shown in Table 1 are not repeated in the description, which follows the pattern used by Foissner (1993) in his monograph on colpodid ciliates.

Idiocolpoda gen. n.

Diagnosis: Small, bacteria feeding Colpodidae having oral apparatus located at left side of body. Vestibulum small, calotte-shaped, left wall projects over right. Right oral polykinetid composed of few, curved kineties.

Type species: *Idiocolpoda pelobia* sp. n.

Derivatio nominis: Composite of "idio" (peculiar, unusual) and "colpoda" (bosom); both Greek. Feminine.

Idiocolpoda pelobia sp. n. (Figs. 1-18, Tab. 1)

Diagnosis: In vivo about 25-40 x 15-30 μm. 12 somatic kineties, 2 of which distinctly shortened preorally on left side. 2 specialized postoral kineties in deep diagonal (postoral) groove. Vestibulum scrotum-shaped and unroofed, exposing oral polykinetids on surface of cell. Left polykinetid composed of 8, right of 4 kineties.

Type location: Temporary stream in Hawaii, North Kohala, W 155° 50', N 20° 04'.

Type specimens: A holotype and a paratype of *I. pelobia* as two slides of protargol impregnated cells have been deposited in the collection of microscope slides of the Oberösterreichische Landesmuseum in Linz, Austria.

Table 1

Morphometric characterization of *Idiocolpoda pelobia*[*]

| Character	x̄	M	SD	SDx̄	CV	Min	Max
Body, length	30.8	31.5	3.7	1.0	11.9	23	36
Body, width	16.6	16.5	2.8	0.7	16.8	13	21
Distance anterior end to vestibular opening	7.7	8.0	0.7	0.2	9.4	6	9
Distance anterior end to posterior end of vestibular opening	12.4	13.0	1.2	0.3	9.8	9	14
Distance anterior end to macronucleus	12.6	12.5	2.9	0.8	23.3	5	17
Macronucleus, length	5.4	5.0	0.7	0.2	13.9	4	7
Macronucleus, width	4.8	5.0	0.8	0.2	16.7	4	6
Micronucleus, length	2.7	2.5	0.3	0.03	11.8	2	3
Left oral polykinetid, length	3.4	3.0	0.5	0.1	14.8	3	4
Somatic kineties, number	12.4	12.0	0.6	0.2	5.1	11	13
Postoral kineties, number	2.0	2.0	0.0	0.0	0.0	2	2
Kineties in left oral polykinetid, number	7.6	8.0	0.5	0.1	6.5	7	8
Macronucleus, number	1.0	1.0	0.0	0.0	0.0	1	1
Micronucleus, number	1.0	1.0	0.0	0.0	0.0	1	1

[*] All data based on 14 randomly selected, protargol impregnated and mounted specimens. All measurements in μm. Abbreviations: CV – coefficient of variation in %, M – median, Max – maximum, Min – minimum, SD – standard deviation, SDx̄ – standard deviation of the mean, x̄ – arithmetic mean.

Derivatio nominis: "pelobia" is Greek and means living in mud.

Description: Colourless and rather hyaline. Size and shape highly variable, slenderly to broadly reniform
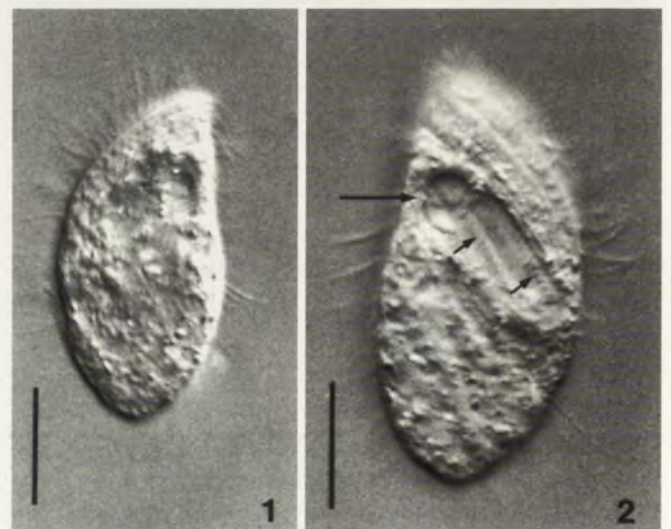
Figs. 1, 2. *Idiocolpoda pelobia*, interference contrast micrographs of live specimens. 1 - right lateral view of typical specimen; 2 - left lateral view of typical specimen showing scrotum-shaped vestibular opening (large arrow) and postoral kineties (small arrows) in deep diagonal groove extending obliquely from vestibulum to dorsal side. Bars - 10 μm
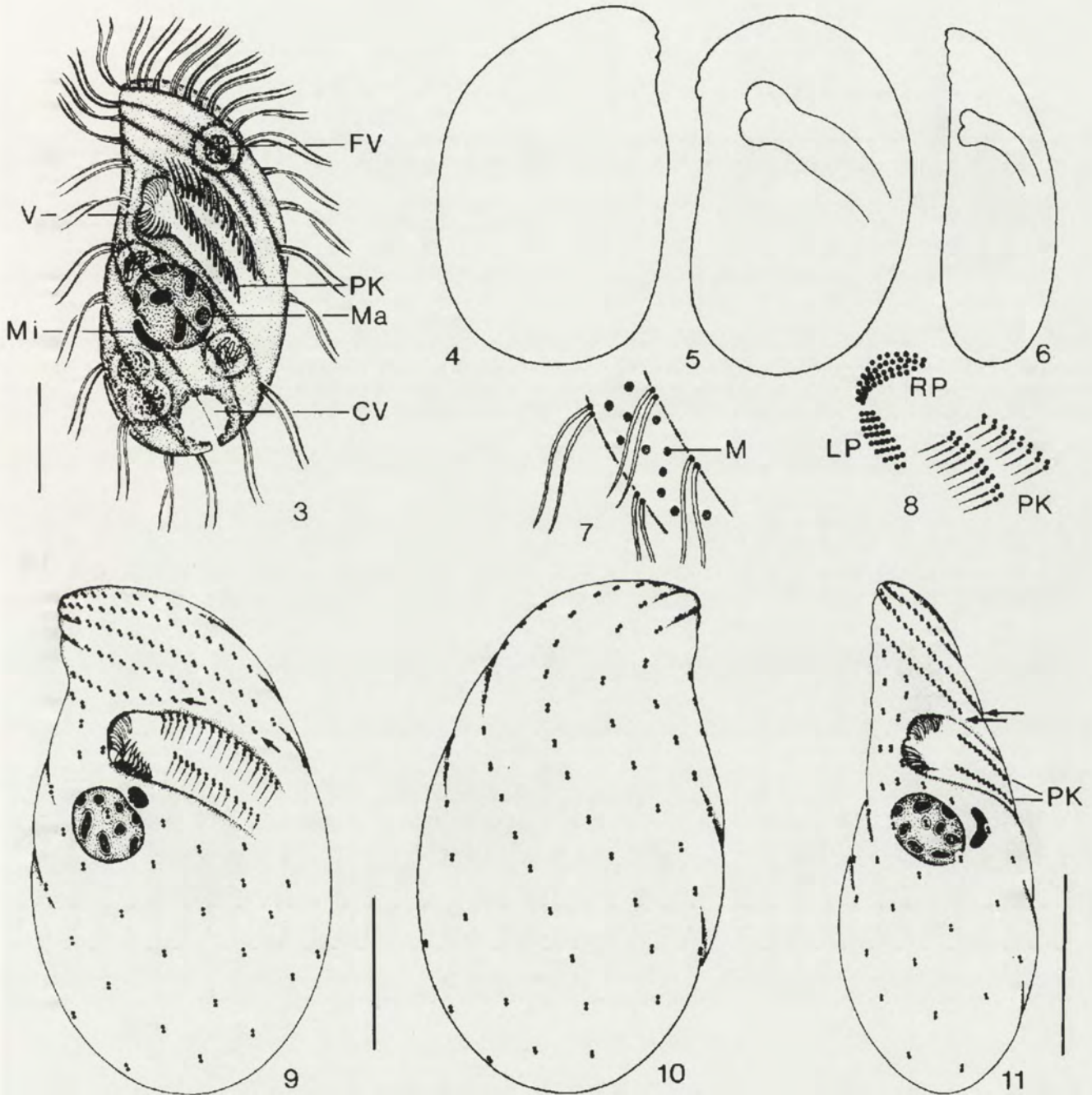
Figs. 3 - 11. *Idiocolpoda pelobia* from life (3-7), after silver carbonate impregnation (8), and protargol staining (9-11). 3 - left lateral view of typical specimen; 4, 5 - right and left lateral view of broad specimens; 6 - left lateral view of slender specimen; 7 - surface view showing loosely arranged cortical granules, very likely mucocysts; 8 - oral infraciliature; 9, 10, 11 - infraciliature of left, right, and ventral side. Arrows mark shortened preoral kineties. CV – contractile vacuole, FV – food vacuole, LP – left oral polykinetid, M – mucocyst, Ma – macronucleus, Mi – micronucleus, PK – postoral kineties in diagonal groove, RP – right oral polykinetid, V – vestibular opening. Bars – 10 μm

(Figs. 1-6). Dorsal side distinctly convex, ventral side flat or slightly concave, rarely convex. Anterior end sharply tapered to bluntly pointed, keel usually distinctly serrate by preoral kineties. Posterior end broadly rounded. Well-nourished cells almost circular in transverse view, theronts flattened up to 2:1. Ventral and dorsal view broadly to slenderly wedge-shaped, no post-

oral sack (Fig. 11). Postoral groove very conspicuous, extends diagonally from vestibulum across left side, gradually flattening and disappearing at dorsal side (Figs. 2, 3, 5, 9).

Macronucleus globular, near centre of cell, contains many ellipsoid nucleoli. Micronucleus loosely attached to macronucleus, conspicuous as main axis half as long
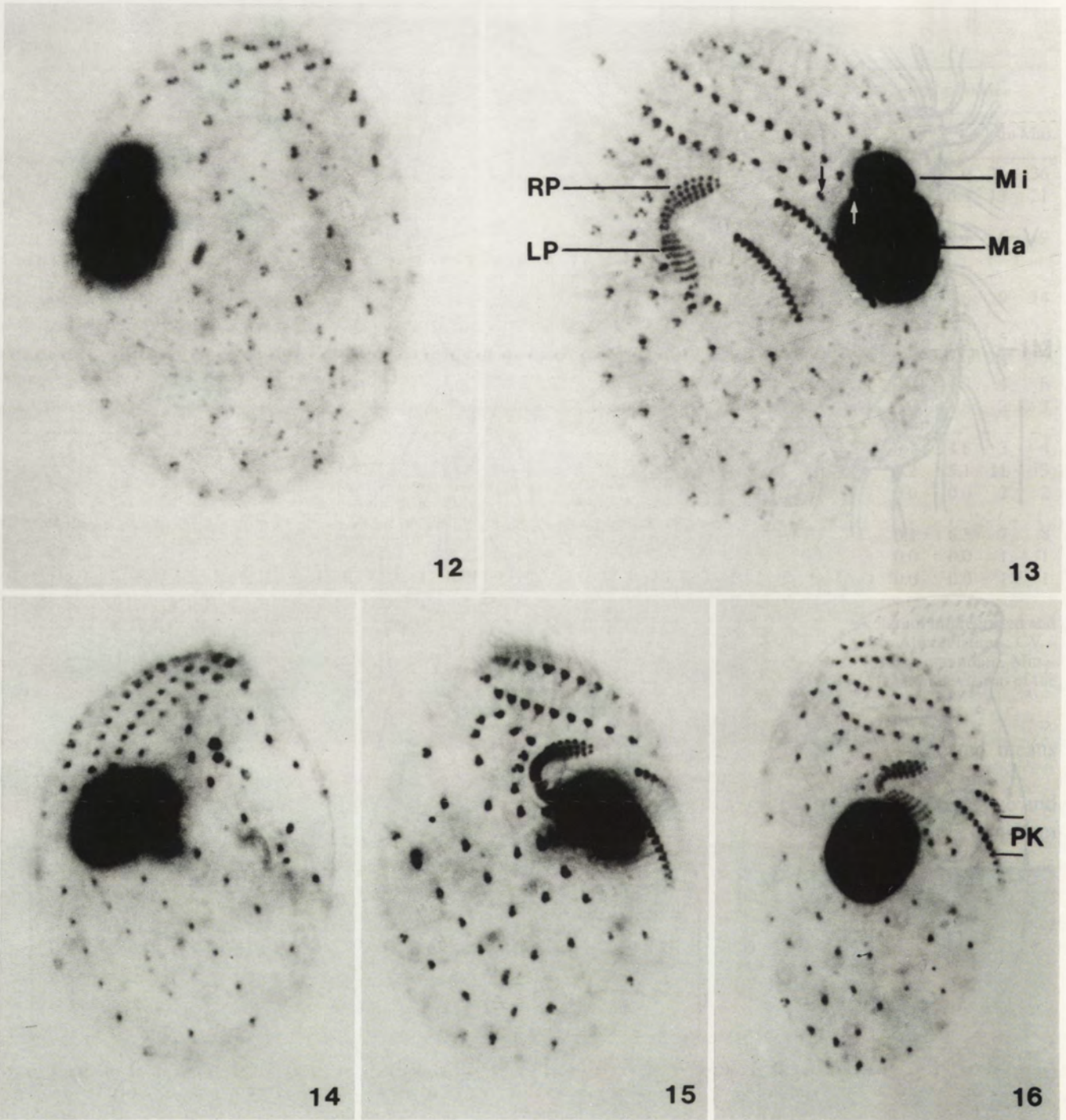
Figs. 12 - 16. *Idiocolpoda pelobia* after silver carbonate impregnation. 12, 13 - infraciliature of right and left side. Arrows mark shortened preoral kineties; 14, 15 - infraciliature of right and ventral side; 16 - infraciliature of ventral side. LP – left oral polykinetid, Ma – macronucleus, Mi – micronucleus, PK – postoral kineties, RP – right oral polykinetid. Figures purposely without scale bars since the applied staining technique leads to inavoidable distortions of the cells which would give meaningless measurements

as macronucleus, lenticular to slightly thorn-shaped, highly refractive in vivo but usually weakly stained by protargol (Figs. 13, 17). Contractile vacuole at dorsal posterior end, i.e. slightly left of median if cell is viewed from left side, releases content via single excretory pore. Cortex thin, flexible, in anterior half of cell distinctly furrowed by somatic kineties, contains loosely arranged, bright granules about 0.3 µm in diameter between kineties (Fig. 7); these granules are very likely muco-cysts since protargol impregnated cells are coated by a thin (1 µm), homogenous layer which often obscures the infraciliature. Cytoplasm with few to many food

species is easily confused. Usually, *I. pelobia* glides shakily on the right side but, like all colpodids, it can also slowly swim in wide spirals by rotation about its long axis.

Somatic cilia about 7 µm long, paired throughout, closely spaced in anterior portion of kineties, especially on left side of cell. Ciliary rows course distinctly spirally from keel to posterior end. Lowermost preoral kineties of left side shortened, terminate at dorsal side (Figs. 9, 11, 13, 17). Kineties of right and left side abut at acute angles preorally; leftmost 2 kineties of right side abut at lowermost preoral kinety of left side (Figs. 11, 16). One to two sparsely ciliated kineties commence at vestibular opening and at posterior edge of diagonal groove. Two conspicuous postoral kineties extend obliquely from left border of vestibulum across left side of cell in diagonal groove. Dikinetids very closely spaced in postoral kineties, each possessing distinct fibre extending obliquely posteriad for a distance of about 5 µm (Figs. 8, 9, 18).

Oral apparatus sligthly above mid-body in right half of left side, small, but distinctly marked by deep postoral groove. Vestibulum flat, right wall conspicuously scrotum-shaped, left wall passes into postoral groove without distinct border. Vestibular roof absent or very inconspicuous, oral polykinetids thus exposed on surface of cell (Figs. 2, 3, 5, 6, 9, 17). Oral polykinetids small, abut at an angle of about 45°. Left polykinetid at bottom of vestibulum, elliptical, composed of regularly spaced rows of monokinetids. Right polykinetid at right vestibular wall, crescentic, composed of 4 regular rows of basal bodies; no dikinetidal row at its dorsal border (Figs. 8, 18).
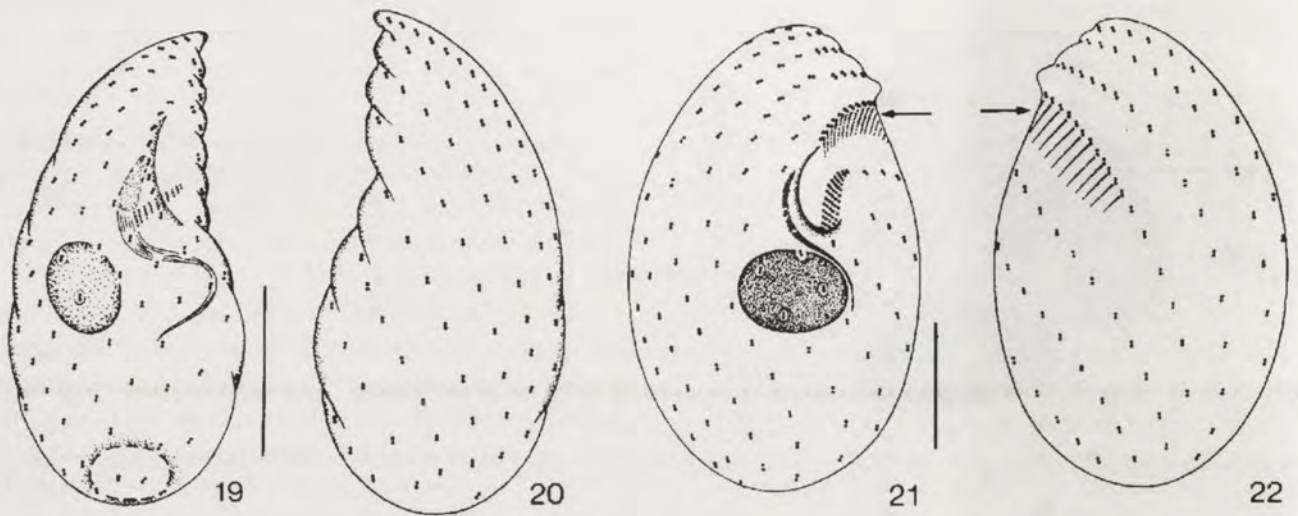
No data available on resting cysts and morphogenesis.

Occurrence: As yet found only at type location, together with 18 other ciliate taxa, some of which are new species too. A complete species list will be published at a later date.

DISCUSSION

Classification and comparison of *Idiocolpoda pelobia* with related taxa

Idiocolpoda pelobia has the typical characteristics of members of the family Colpodidae as defined by Foissner (1993), i.e. the vestibular opening is in the anterior half of the body and the right and left oral polykinetid are approximately equal in length. There are

Figs. 17, 18. *Idiocolpoda pelobia* after silver carbonate impregnation. 17 - infraciliature of left side. Arrows mark shortened preoral kineties; 18 - high magnification of oral infraciliature. Large arrows mark shortened preoral kineties. Small arrow marks pharyngeal fibres. F – conspicuous fibres extending posteriad from dikinetids of postoral kineties, LP – left oral polykinetid, Ma – macronucleus, Mi – micronucleus, PK – postoral kineties, RP – right oral polykinetid. Figures purposely without scale bars since the applied staining technique leads to inavoidable distortions of the cells which would give meaningless measurements

vacuoles about 5 µm in diameter and many tiny (1 µm) crystals recognizable only in interference contrast by their silvery shimmer. Feeds on small bacteria.

Movement highly characteristic, viz. slow and trembling, like certain large flagellates with which this

Figs. 19, 20. *Colpoda edaphoni* (from Foissner 1993), infraciliature of right and left side. Note that vestibular opening is on right ventro-lateral side and not on left side as in *Idiocolpoda pelobia* (Fig. 3). Bar - 10 μm

Figs. 21, 22. *Apocolpoda africana* (from Foissner 1993), infraciliature of right and left side. Arrows mark specialized lowermost preoral kinety which is very similar to the specialized postoral kineties found in *Idiocolpoda pelobia*. The vestibular opening of *A. africana* is, however, on the right side. Bar - 10 μm

thus now seven well-defined genera in the family Colpodidae, four of which are monotypic (Fig. 23). These genera comprise 39 species (Foissner 1993). There is no indication that the family is polyphyletic, i.e. there is no firm character known which would suggest assigning the genera to two or more families.

Most colpodids have the oral apparatus located more or less distinctly at the right side of the body, so that at least the right vestibular wall or the paroral membrane extends on the right side of the cell (Fig. 23). The only known exceptions are a few members of the family Marynidae which have the oral apparatus shifted to the rear end of the body and to the left side of the preoral suture (Foissner 1993). *Idiocolpoda pelobia* is the first member of the family Colpodidae which has the oral apparatus located entirely on the left body surface. This causes a highly characteristic appearance of the live cells, which look like mirror-imaged, small Colpodas. In fact, I first classified *I. pelobia* as "*Colpoda aspera* (?)". It was only its curious trembling movement on the wrong (right) side that induced me to look at it in more detail.

Idiocolpoda pelobia lacks the right vestibular wall and the oral polykinetids are thus exposed on the surface of the cell. Such specializations are unknown in other members of the Colpodidae but occur in some species of the related family Hausmanniellidae. However, this similarity is very likely a convergence because the right oral polykinetid of *I. pelobia* is much more similar to those known from small *Colpoda* species (e.g.,

C. aspera, C. steinii, C. maupasi) than to those known from hausmanniellid colpodids.

Other remarkable characteristics of *I. pelobia* are the distinct diagonal (postoral, somatic) groove and the two specialized kineties extending within. Such specializations occur in most of the larger *Colpoda* species but are absent in the smaller members of the genus (Figs. 19, 20). There is, however, one small species, viz. *Apocolpoda africana* Foissner, 1993, which has similar characteristics (Figs. 21, 22). It is thus reasonable that *I. pelobia* and *A. africana*, which are known from the tropics only, are rather closely related.

The two specialized postoral kineties of *I. pelobia* are very likely displaced portions of the two lowermost preoral kineties which are shortened by about the number of dikinetids found in the postoral kineties. No other member of the order has shortened preoral kineties.

Idiocolpoda pelobia is easily recognizable, even in vivo, by the location of the oral apparatus and the scrotum-shaped vestibular opening. There is no other species known which has these characteristics [see Foissner (1993) for a detailed account of the whole class].

Key to the genera of the family Colpodidae (Fig. 23)

The following key is an adapted and refined version of that published by Foissner (1993), which includes a detailed discussion of all genera.
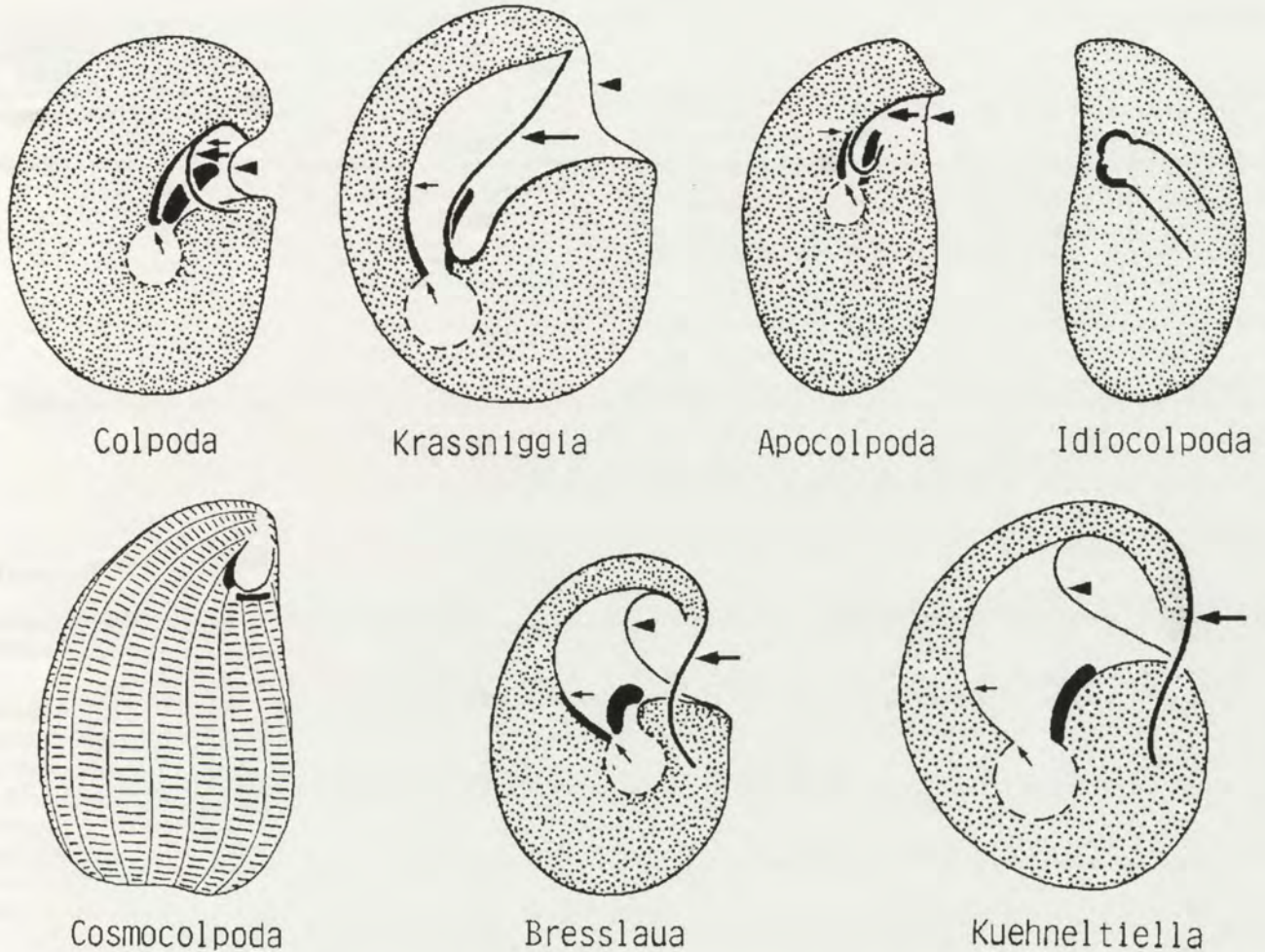
Fig. 23. Genus distinction in the family Colpodidae by the location of the oral apparatus (at left or right side of cell), the structure of the cortex (conspicuous ridges in *Cosmocolpoda*), the shape of the vestibulum (funnel-shaped in *Colpoda, Apocolpoda, Idiocolpoda* and *Cosmocolpoda*, cave-like in others), the shape of the right (large arrows) and left (arrowheads) vestibular wall, and the right oral ciliary field (small arrows), which consists of a single row of dikinetids in *Kuehneltiella* (from Foissner 1993; supplemented). Vestibulum and descending food vacuole unstippled, oral polykinetids shaded black, ends of right oral polykinetid marked by small arrows

1 Vestibulum large, occupies front half of cell which thus has conspicuous, bright patch anteriorly. Rapacious carnivores 5
– Vestibulum inconspicuous, i.e. usually small compared to size of cell. No bright patch anteriorly. Microphages usually feeding on bacteria.................2
2 Right wall of vestibulum and oral opening on right or right ventro-lateral side of cell 3
– Right wall of vestibulum and oral opening on left side of cell .... *Idiocolpoda*
3 Surface smooth or with tooth-like processes 4
– Surface ornamented ladder-like by conspicuous cortical crests............................ *Cosmocolpoda*
4 Anterior cell half helmet-like due to distinct preoral ridge, the proximal edge of which forms the right vestibular wall and is aligned with a membranoid

structure produced by the lowermost left lateral somatic kinety...*Apocolpoda*
– Anterior cell half not helmet-like (usually rounded or acute) and without preoral ridge .... *Colpoda*
5 Right wall of vestibulum overhangs semicircularly notched left vestibular wall; cell thus possesses conspicuous, bright, circular patch anteriorly 6
– Right wall of vestibulum extends utricle-like posteriad on right side of cell, i.e. does not overhang left vestibular wall .... *Krassniggia*
6 Right oral polykinetid composed of many short, disordered kineties (use silver carbonate impregnation) ....*Bresslaua*
– Right oral polykinetid inconspicuous, composed of single row of dikinetids (use silver carbonate impregnation)................................... *Kuehneltiella*

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Ciliates in the Plankton of the River Itchen Estuary, England

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Summary. The abundance of ciliates in a region of the river estuary where the salinity ranged between 20 and 30‰ showed an annual cycle with highest ciliate numbers (16.9 ml^{-1}) in the summer, when the salinity was also high, and lowest numbers (down to < 0.2 ml^{-1}) in the winter, when the salinity was low. At another site in the estuary, where the salinity of collected samples varied between 1.5 and 8.4‰, the ciliate populations were usually low (about 0.2 ml^{-1}), but a population exceeding 5 ml^{-1} was present in July. The ciliate community at both sites was generally dominated by non-loricate oligotrichs, with other planktonic forms, *Mesodinium rubrum*, tintinnids and *Didinium*, in decreasing order of abundance, as well as a seasonally varying selection of benthic hypotrichs, hymenostomes and gymnostomes. Nanoflagellates that could provide a food supply for the filter-feeding ciliates were especially numerous in the summer, but the numbers of bacteria were low, raising questions about the nutrition of the heterotrophic flagellates.

Key words. Ciliates, estuary, plankton.

INTRODUCTION

The tidal region of the River Itchen extends from the entry of its estuary into Southampton Water at Weston Point (Fig. 1) for a distance of some 6.5 km to a weir at Woodmill. A freshwater flow that normally ranges between 2 and 6 m^{3}s^{-1} continually enters the estuary at Woodmill, and saline water enters at Weston Point with a tidal cycle of about 12.5 h. Although the tidal pattern is roughly sinusoidal, the peak of high water is prolonged, and on the spring tides there is a double high water (a "stand"), with two peaks about 2 h apart separated by a shallow trough. (This complex pattern is related to the location of the mouth of Southampton Water near to a 'node' in the tidal pattern of the English Channel). The tidal range at Weston Point is about 2 metres during neap tides and about 4 metres during spring tides. As the tide rises the saline water moves up the Itchen estuary, mixing with the fresh water from the river and also (being more dense) tending to flow beneath the fresh water. At high tide the lower part of the estuary is filled with water that has entered from Southampton Water, and the water in this region typically has a salinity near 30‰; towards the top of the estuary the water becomes much more dilute, with much less penetration of saline water. During the low water of spring tides the upper part of the estuary retains only a fresh-water river at its centre, but the lower estuary is deep enough to retain a significant amount of saline water during the lowest tides.

A typical marine planktonic community of ciliate protozoa is found in Southampton Water (Leakey et al.

1992, 1993), but true planktonic ciliates are not represented among the protozoans of the River Itchen above Woodmill, since the ciliates found in the water column of the fresh-water river are benthic and epiphytic forms swept from their normal habitat by the current (Baldock et al. 1983, Sleigh et al. 1992). Where the fresh and saline waters meet in the estuary, the two communities will mix, and at some parts of the estuary the salinity will vary very widely through the tidal cycle, especially during spring tides. Ciliates will be carried with the water flows and may be mixed into water of higher or lower salinity that is beyond their tolerated physiological range, when they will die. From a single transect comprising 7 samples from a depth of 1m taken at intervals down the length of the estuary in March 1986, Leakey (1989) reported that the abundance of ciliates was very low (around 200 l^{-1}) at the top of the estuary near Woodmill, but rose to a maximum of about 10^4 ciliates l^{-1} near the middle of the estuary before falling back to about 2000 ciliates l^{-1} near to the mouth of the estuary at Weston Point. The numbers of protozoans have been observed to reach exceptionally high levels in estuarine regions of water mixing (Burkill, personal communication), perhaps because of the increased levels of organic matter entering from the rivers, including drifting organisms that die when they encounter the seawater.

In this study we chose two convenient sampling sites, one in the lower estuary, 2 km from the mouth, and the other in the upper estuary, about 5 km from the mouth. We followed the changes in abundance of ciliates in near-surface waters at the high water of spring tides throughout one year of sampling, and followed changes in community structure to learn more of the characteristics of the elevated populations that Leakey had detected in the estuary. We wished to discover whether these ciliates were marine forms which found especially suitable conditions here, or whether there was any evidence for specific estuarine types. We also estimated the abundance of flagellates and bacteria and followed changes in chlorophyll *a* to gain some understanding of the trophic relationships of the ciliate communities in the estuary.

MATERIAL AND METHODS

Sampling was carried out from two floating pontoons, permanently moored in the estuary of the River Itchen. One, situated some 300 m north of Cobden Bridge (Nat. Grid Ref. 437144), is used for landing and temporary boat mooring, and permits samples to be taken 5 m or so from the bank; at this point the estuary is about 100 m wide. The
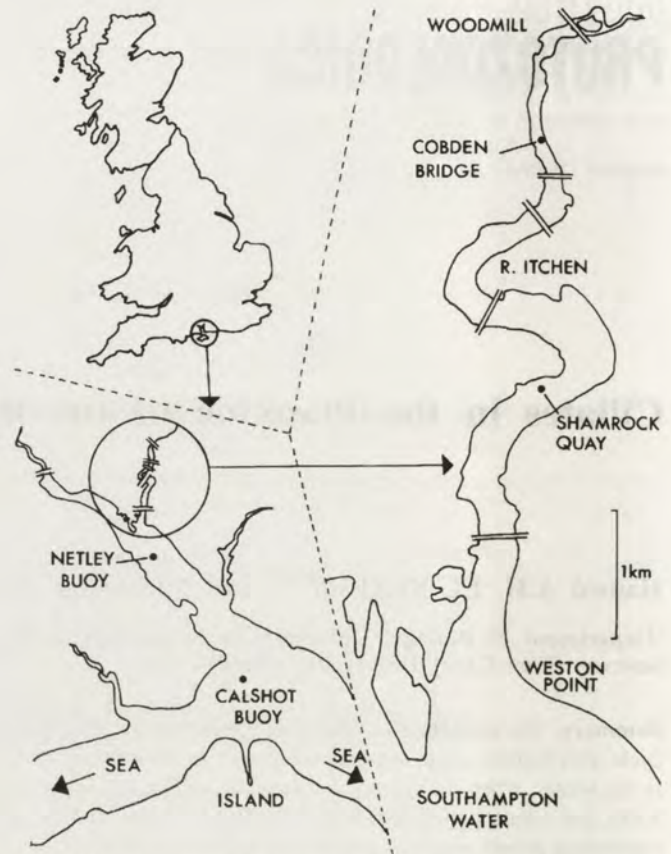

Fig. 1. Maps showing the estuary of the River Itchen and the position of the two sampling sites in relation to Southampton Water and the entrances to the English Channel

other, at Shamrock Quay (Nat. Grid Ref. 437124), is a permanent boat mooring which permits samples to be taken 15-20 m from the bank; here the estuary is about 300 m wide. The main current of the estuary sweeps along and beneath both pontoons. The depth of water beneath the pontoon at Cobden Bridge reaches 2-3 m during high spring tides, and during low water of spring tides this pontoon is left stranded on the mud. The depth of the water at the Shamrock Quay site varied between about 4 and 8 m during spring tides. Samples were collected from each site during the stand of high water during spring tides, once a month during winter and once every two weeks during spring, summer and autumn.

On each sampling visit three separate samples of 800 ml each were collected from a depth of about 15 cm in glass jars closed by plastic tops. The temperature of the water was immediately measured with a thermometer calibrated to 0.1°C. The samples were taken directly to the laboratory and within 0.5 to 1 h subsamples were fixed for enumeration of microorganisms. A volume of 180 ml was taken from each bottle, after gentle but thorough mixing, and fixed with Lugol's iodine solution (Throndsen 1978) to a final concentration of 1%. A further 100 ml was taken from each bottle of well-mixed sample and fixed with 10 ml of 25% glutaraldehyde (filtered through a 0.22 μm filter). Fixed sub-samples of both types were stored in the dark at 4°C.

The live ciliates in subsamples from each sample were counted as soon as possible after collection, and always within 2 h of return to the laboratory. Three subsamples of 6 ml each were taken from each bottle after mixing to ensure even dispersal of the ciliates. Each
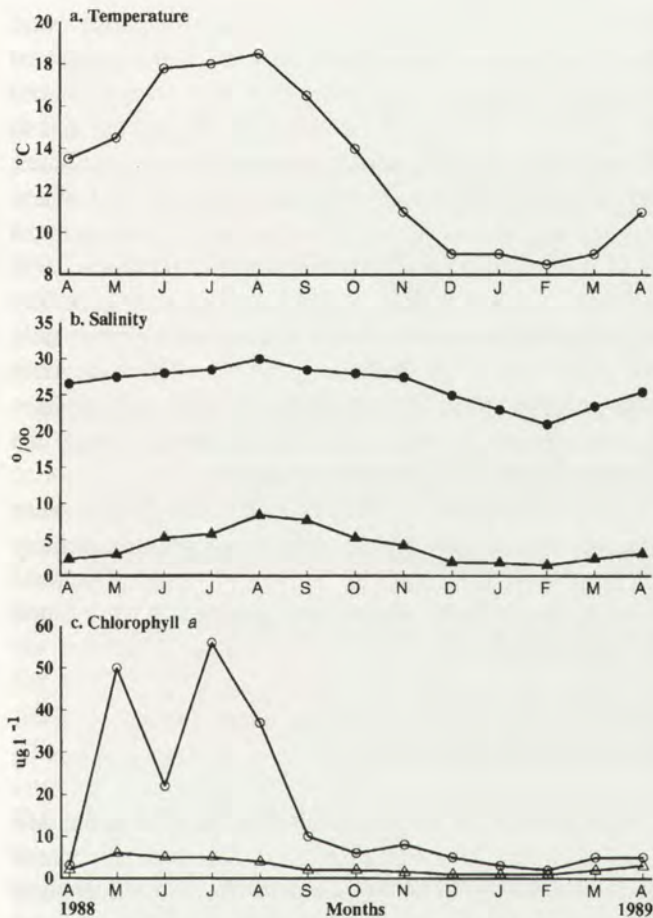
Fig. 2. Measurements made on samples collected at Shamrock Quay (circular symbols) and Cobden Bridge (triangular symbols) in the period from April 1988 to April 1989. a - the mean temperature recorded from water samples collected from both sites in each month. b - the mean salinity values measured in samples from the two sites in each month. c - the mean concentrations of chlorophyll *a* measured in samples from the two sites in each month

subsample was placed in a Bogorov counting chamber, and the number of live ciliates in the chamber was counted under a stereomicroscope at a magnification of x 25-50, without any attempt at further identification.

Ciliates in the Lugol's-fixed samples were identified after settlement by the Utermöhl method (Utermöhl 1958). The preserved samples were gently stirred and poured into three 65 ml settling cylinders, each mounted on a shallow circular trough whose base was formed from a cover glass 25 mm in diameter. The samples were left to stand for at least 12 h before the supernatant was carefully removed and the base was transferred for examination of the settled plankton with a Wild M40 inverted microscope (with phase contrast) at a magnification of x 400. The whole settled sample was scanned and all ciliates were identified to genus level and recorded. With three subsamples examined per bottle and three bottles per collection, there were nine replicate counts per site sampled; the counts were expressed as numbers per litre of water.

Two 2 ml subsamples of each thoroughly mixed glutaraldehyde-fixed water sample were taken to estimate the numbers of bacteria and flagellates. Each subsample was separately mixed with 0.6 ml of 0.3% DAPI (4'6-diamidino-2-phenylindole (Sigma)) stain (Porter and Feig

1980), which had been filtered through a 0.22 μm filter to remove particles. The mixture was kept in the dark for 7 min before being filtered onto a 0.2 μm pore diameter black polycarbonate filter, using a filter pressure of no more than 10 mm Hg. The filter was mounted on a slide with Gurr's Uvinert immersion oil (BDH, Poole, England) and examined at x1000 with an Olympus BH-2 microscope fitted with a reflection fluorescence attachment. Bacteria and the nuclei of nanoplanktonic flagellates emitted a blue fluorescence with DAPI, and could be distinguished easily from one another, while autotrophic flagellates showed a red fluorescence from chloroplasts. These three categories of organisms were counted in 20 microscope fields on each filter to estimate the number of each type per ml of original sample. There were 6 replicate filters per site on each sampling visit.

The chlorophyll *a* content of the water was also measured. Two 100 ml water samples (250 ml in winter) from each site were filtered separately through 25 mm diameter Whatman glass fibre filters (GF/F). Chlorophyll pigments were extracted from the homogenised filters with 90% acetone. The extract was centrifuged at 2000 rpm for 5 min and the supernatant was made up to 25 ml with 90% acetone. The fluorescence of the extract was measured with an Aminco fluorometer that had been calibrated against standard concentrations of chlorophyll *a* (Parsons et al. 1984), and the chlorophyll concentration in the water samples was calculated.

The salinity of samples was routinely measured by titration against standard silver nitrate and periodically cross-checked with a salinometer bridge (MCS, Electronic Switchgear, London).

RESULTS

Temperature, Salinity and Chlorophyll in Surface Water

Water temperatures dropped to 8-9°C between December and March, and rose to 17.5-18.5°C between June and August (Fig. 2a). The salinity at each site varied with the seasons in the same general manner as the temperature, but dependent largely upon seasonal changes in rainfall and its subsequent run-off in the river catchment. The range of salinity in high-tide samples at Shamrock Quay ranged between 20 and 30‰, and at Cobden Bridge between 1.5 and 8.5‰ (Fig. 2b).

Measurements of chlorophyll *a* concentrations in water samples from the two sites varied seasonally as shown in Fig. 2c. The levels of chlorophyll were consistently higher at Shamrock Quay than at Cobden Bridge, the highest levels at both sites being found between May and August, when chlorophyll concentrations were 5-10 times higher at Shamrock Quay than at Cobden Bridge.

Ciliate Populations

The annual cycle of variation in abundance of ciliates in the surface waters at the two sites is shown in Fig. 3.
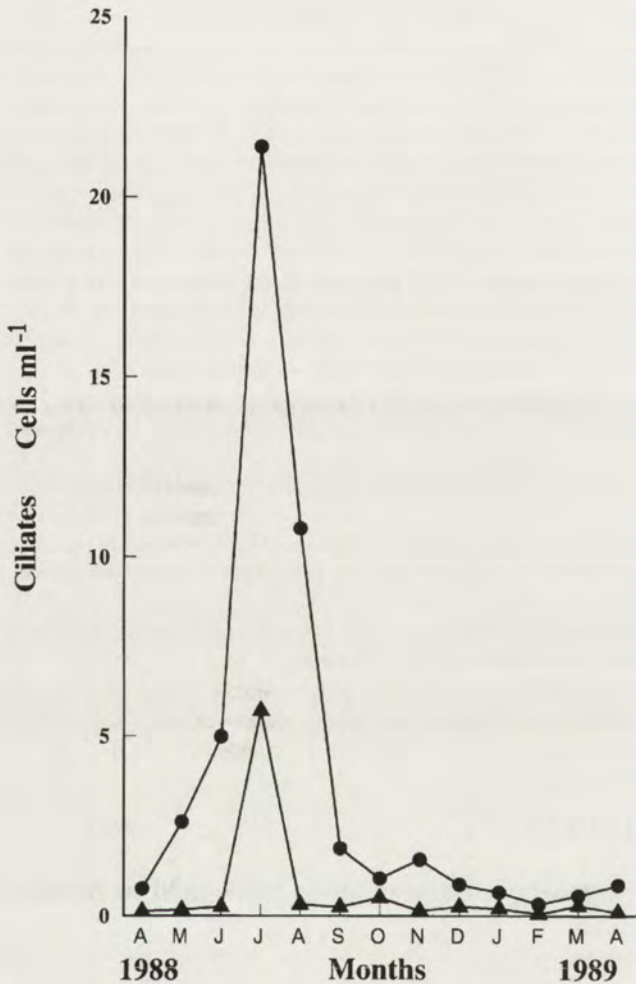
Fig. 3. The mean population density of ciliates in samples from the surface waters at Shamrock Quay (circles) and Cobden Bridge (triangles), in each of the thirteen months of the study. The standard error ranged from <1 cell ml^{-1} for small values to 4 cells ml^{-1} on highest values

Live counts and those from fixed samples taken from the same collection were similar throughout, in most cases differing by less than 5%. The populations at Shamrock Quay were consistently higher than at Cobden Bridge, with highest values between June and August; only in July did the population density of ciliates in samples from Cobden Bridge exceed 1ml^{-1}.

The relative concentrations of different categories of ciliates in monthly samples from Shamrock Quay are shown in Fig. 4. In almost every month the non-loricate oligotrich ciliates *Lohmaniella* and *Strombidium* (in similar numbers) formed the largest group. The autotrophic ciliate *Mesodinium rubrum* was common through most months of the year. The predatory *Didinium* formed a significant proportion of the community in several months, but its populations varied erratically. Loricate tintinnids (mainly *Favella* and *Tintinnopsis*, with

some *Helicostomella* and a few *Stenosemella*) were present in almost every sample; they reached their highest numbers in June and July, when they were overshadowed by the high numbers of non-loricate oligotrichs, and in January they were the most numerous category of ciliates. Hypotrichs (*Euplotes, Uronychia, Diophrys* and a few *Aspidisca*), hymenostomes (*Paramecium, Uronema* and a few *Colpidium*) and Gymnostomes (*Dileptus* and *Litonotus*), more typical of the benthos, were generally present in these samples, but never reached high numbers; the abundance of the different genera tended to be more seasonal than that of the true planktonic types, e.g. *Aspidisca* was present in winter but not in summer, while the reverse was true of the other hypotrichs.

The populations of ciliates at Cobden Bridge very seldom included any loricate forms; a population density of 30 tintinnids l^{-1} was the highest recorded. The non-loricate species were of the same genera that were found at Shamrock Quay.

Bacteria and Flagellates

Fluctuations in the abundance of bacteria at the two sites through the year (Fig. 5a) followed the same general trends as those of ciliate numbers or chlorophyll concentrations. There was less difference between the bacterial population densities at the two sites than there was for these other parameters, the summer peak was more prominent at Shamrock Quay than at Cobden Bridge, and both sites (especially Cobden Bridge) showed a minor autumn peak.

The abundance of heterotrophic nanoflagellates in summer and autumn samples from both sites was very similar, in the range between 1.0-2.5 x 10^4 ml^{-1} (Fig. 5b), but their numbers were lower at Cobden Bridge than Shamrock Quay in the winter. The numbers of autotrophic flagellates at Shamrock Quay were at least an order of magnitude lower than the numbers of heterotrophic forms at the same site (Fig. 5c), and very low numbers of autotrophic flagellates were found in samples from Cobden Bridge.

DISCUSSION

The abundance of ciliates at the two sites in March was similar to that found at the same regions of the estuary in the earlier single transect study by Leakey (1989), whose data suggest that a population peak would

have occurred near the mid-point of the estuary, between the two sites sampled in this study. It seems clear that the ciliate populations at Cobden Bridge were strongly reduced by the freshwater input, and so it is probably pointless to compare them quantitatively with data from other studies. The peak population density of heterotrophic ciliates found at Shamrock Quay in July 1988 (18.5 ml^{-1}) was a little greater than that found at NW Netley Buoy in the main part of Southampton Water (Fig. 1) in June 1986 (16 ml^{-1}) (Leakey et al. 1992), but the Shamrock Quay samples contained much lower populations of heterotrophic ciliates (< 1 ml^{-1}) in the months between December and April, whilst the populations at Netley generally remained above 3 ml^{-1}. It is interesting that the ciliate abundance at Calshot Buoy, near the entrance to Southampton Water (Fig. 1), was less than at Netley, the population density only exceeding 4 ml^{-1} during one month (May 1987) of a 13 month survey by Leakey et al. (1992). There was a tendency for the populations of heterotrophic ciliates to reach a peak later in the year at Shamrock Quay than at Netley. The abundance levels of ciliates at Shamrock Quay were similar to those recorded in other estuaries, e.g. the Damariscotta estuary (Maine, USA) (Revelante and Gilmartin 1987), but in the Kiel Bight (Smetacek 1981) the population densities of ciliates were up to five times higher. The annual cycle of ciliate population changes, with a summer maximum and a winter minimum, is typical of temperate coastal waters (e.g. Verity 1987).

The taxonomic composition of the heterotrophic ciliate community found at Shamrock Quay was similar to that found in other comparable coastal sites (e.g. Smetacek 1981, Revelante and Gilmartin 1987, Verity 1987), with *Tintinnopsis* and *Favella* as the dominant tintinnid genera and *Lohmaniella* and *Strombidium* as the dominant non-loricate genera. Clearly the general character of the ciliate community at this level of the estuary has much in common with that in Southampton Water, with no evidence of specifically inner estuarine forms. The proportion of tintinnids and their diversity were both less at Shamrock Quay than at Netley (Leakey et al. 1993), and it is interesting that the proportion and diversity of tintinnids were found by Leakey et al. (1993) to be even greater at Calshot than at Netley. A higher diversity of non-loricate oligotrichs was also reported from the two more saline sites in Southampton Water by Leakey et al. (1993), but there was a greater proportion of benthic forms (hypotrichs etc.) at Shamrock Quay, probably because of the greater turbulence and lesser depth of water at this site than the other two. Large
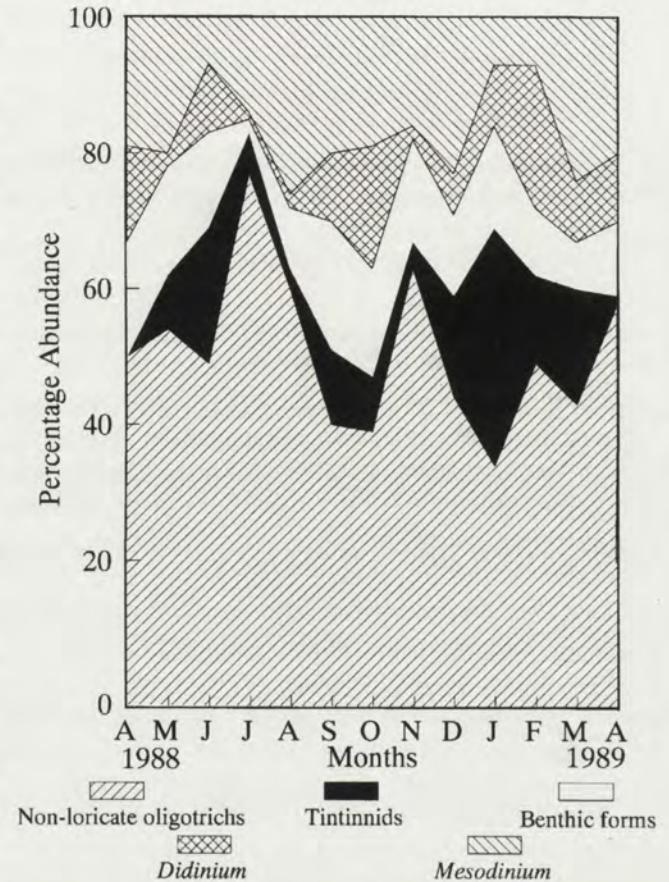

Fig. 4. The percentage contribution of non-loricate oligotrichs, tintinnids, benthic ciliates, *Didinium* and *Mesodinium* to the ciliate community at Shamrock Quay through the annual cycle

predatory ciliates occasionally became numerous at Shamrock Quay (e.g. *Dileptus* reached a density of 200 l^{-1} in August), while predators like *Cyclotrichium* and *Rhagadostoma*, were common in summer at Netley (Leakey et al. 1993), but none of these predators was found at the other site; *Didinium*, which was found in every month at Shamrock Quay, was only occasionally recorded at Netley.

The mouthless autotrophic ciliate *Mesodinium rubrum* was found in every collection at Shamrock Quay, reaching a population density of around 3 ml^{-1} in July and August. Summer blooms of this ciliate occur in most years in Southampton Water, resulting in concentrations that may exceed 1000 ml^{-1} (Antai 1989, Crawford and Purdie 1992), and several reports that it is often the dominant photosynthetic organism present during the summer months in the middle and upper regions of Southampton Water. While Leakey (1989) estimated mean populations of *Mesodinium* from live counts at Netley of 366 ml^{-1} in August 1986 and 285 ml^{-1} in June 1987, the abundance of this ciliate recorded at

Calshot in the same study was less than at Shamrock Quay, seldom exceeding 1 ml⁻¹. The relatively high numbers of *Mesodinium* present at Shamrock Quay in July and August may have been responsible for the elevated chlorophyll levels in those months, while the May peak was probably due to diatoms. *Mesodinium* appears to be able to photosynthesise efficiently at low light intensities (Smith and Barber 1979), and appears well adapted to estuarine waters with their high concentrations of suspended particles, as well as flourishing at the high nutrient levels present in estuaries and in upwelling areas (Packard et al. 1978). This ciliate also appears to possess a behavioural adaptation allowing it to avoid being flushed out of estuaries with the ebb tide (Crawford and Purdie 1992). It is not clear whether *Didinium* preys on *Mesodinium*; it is sometimes present in densities exceeding those of *Mesodinium*, so it seems unlikely to be restricted to this diet, even if it can capture this very fast-swimming ciliate (Lindholm 1985).

Bacterial population densities at Shamrock Quay are higher than those at Cobden Bridge, in spite of the high input of organic matter from freshwater sources. These bacterial numbers are lower than those found at sites in Southampton Water by Leakey (1989) and Antai (1989); the latter recorded bacterial populations of over 10^7 ml⁻¹ in July 1988, at Netley, following a large *Mesodinium* bloom. The numbers of heterotrophic nanoflagellates at Shamrock Quay, and to a lesser extent at Cobden Bridge, are high compared with those found in Southampton Water by Antai (1989), who recorded populations between about 1×10^3 and about 9×10^3 flagellates ml⁻¹ in surface waters at Netley during winter and summer, respectively. Literature data on the abundance of microorganisms in coastal marine waters indicate average values of around 10^6 bacteria ml⁻¹ and 10^3 heterotrophic flagellates ml⁻¹. For example, in his studies on the Limfjord, Fenchel (1982) found bacterial concentrations of between 1.5 and 3×10^6 ml⁻¹ and nanoflagellate concentrations of between 2×10^2 and 3×10^3 ml⁻¹ during August and September. However, rather lower concentrations of bacteria (2.3-6 $\times 10^5$ ml⁻¹) have been found in the English Channel by Linley et al. (1983), and rather higher concentrations of bacteria in estuaries, e.g. 6.4×10^6 ml⁻¹ in summer and 13.3×10^6 ml⁻¹ (many of them attached) in winter in surface waters of the Humber estuary by Goulder (1977) and 2-18.4 $\times$ 10^6 ml⁻¹ in the top 2 metres of the Newport River estuary (USA) by Palumbo and Ferguson (1978). Caron (1983) reported populations of heterotrophic nanoflagellates between about 2×10^2 and 3×10^3 ml⁻¹, with lower values
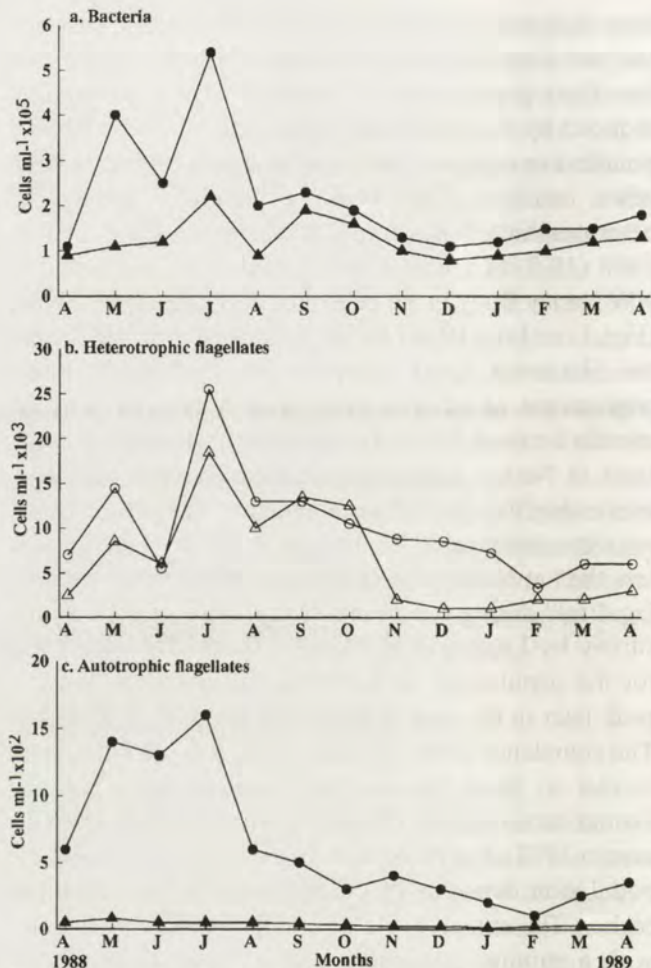

Fig. 5. Mean values for the abundance of non-ciliate microorganisms in each month at Shamrock Quay (circular symbols) and Cobden Bridge (triangular symbols). a - bacteria; b - heterotrophic flagellates; c - autotrophic flagellates

in open sea and higher values in inshore waters, while Coffin and Sharp (1987) found heterotrophic flagellate concentrations between 4×10^2 and 9×10^3 ml⁻¹ in the Delaware estuary (USA). Clearly the bacterial populations we encountered in the Itchen estuary are lower than expected, whilst the heterotrophic flagellate populations are somewhat higher than those usually recorded. Autotrophic nanoflagellates were 10 to 30 times as abundant at Shamrock Quay as at Cobden Bridge, but even at the former site they comprised generally less than 10% of the total nanoflagellate numbers; these autotrophs are probably a minor component of the food web, but the mixotrophs among them may have an advantage in the sediment-laden waters of the estuary. They are less numerous in the Itchen estuary than in the nearshore waters sampled by Caron (1983).

These smaller microorganisms were enumerated to gain some indication of the food resources for ciliates.

In this connection the filtration activities of the heteroflagellates and ciliates are compared with the population densities of bacteria, flagellates and filter-feeding ciliates in Table 1, drawing data from collections at Shamrock Quay in January and July. Filtration rates from the literature are used to calculate the potential rate of capture of prey of the different categories, and the time required for the whole water body to be filtered by flagellates and ciliates.

If the high populations of nanoflagellates do indeed filter the whole body of water every 4 h in summer, the bacteria must be reproducing quickly to maintain their populations, or there must be continuous very active recruitment of bacteria into suspension from sediments and surfaces. However, at the observed relative population densities of flagellates and bacteria, the number of bacteria that may be caught per flagellate per hour is very small, even in July, and is so low in January that the flagellate population would surely not be able to maintain itself, let alone multiply. Fenchel (1982) calculated a maximum ingestion rate of 27-254 bacteria/flagellate/hour and Sherr et al. (1983) found ingestion rates of 10-75 bacteria/flagellate/hour; minimum ingestion rates for survival were not given, but Fenchel (1987) noted that starvation could be withstood temporarily by reducing respiration. It might be suggested that estuarine bacteria could be larger, so that fewer would be needed to sustain the flagellates, but Palumbo et al. (1984) found that estuarine bacteria were no larger than those from neritic and coastal waters. It is conceivable that flagellates could catch more bacteria by increasing the filtration rate, since Sherr et al. (1983) showed that the clearance rates of microflagellates increased at low bacterial concentrations. If this were the case, it only increases the difficulty of explaining how the bacterial population may be maintained in the face of very intense grazing pressure by the flagellates.

The ciliates could also be consuming bacteria, but flagellates are a more likely food source, and presumably a more "attractive" one, in view of the much greater nutritive value of each cell and the coarser filter required. The population of filter-feeding ciliates in July is large enough to filter the whole water body twice a day, and a ciliate could surely catch enough flagellates to provide for growth and maintenance of an average oligotrich. The possible food capture rate per ciliate in January gives less scope for growth, but the balance must depend on the relative sizes of the organisms involved. Rassoul-zadegan et al. (1988) found that the tintinnids consume nanoplankton in the 2-20 μm size range, the smaller

Table 1

A comparison of the bacterial, total nanoflagellates (TNAN), heterotrophic nanoflagellates (HNAN) and filter-feeding ciliate populations at Shamrock Quay in July 1988 and January 1989, with estimates of the clearance rates and potential rates of food capture by these flagellates and ciliates

	July 1988	January 1989
Bacteria numbers (l^{-1})	5.5x10^8	1.2x10^8
Flagellates		
TNAN numbers (l^{-1})	2.7x10^7	7.4x10^6
HNAN numbers (l^{-1})	2.5x10^7	7.2x10^6
Volume cleared/HNAN/hour* (l h^{-1})	10^{-8}	10^{-8}
Bacteria encountered/HNAN/hour (h^{-1})	5.4	5.2
Volume cleared by HNANs in each litre in an hour (l)	0.25	0.075
Time for HNANs to filter whole water body (h)	4	13
Ciliates		
Filtering ciliate numbers (l^{-1})	18600	525
Volume filtered/ciliate/hour** (l h^{-1})	5x10^{-6}	5x10^{-6}
Bacteria filtered/ciliate/hour (h^{-1})	2750	600
TNAN filtered/ciliate/hour (h^{-1})	135	37
Volume filtered by ciliates in each litre in one hour (l)	0.093	2.6x10^{-3}
Time for ciliates to filter whole water body (h)	10.75	385

* Mean value from Fenchel (1982)

** Mean value for a range of tintinnids and non-loricate oligotrichs in data by Heinbokel (1978), Heinbokel and Beers (1979), Verity (1985) and Jonsson (1986)

oligotrichs (μm) ate more picoplankton (0.2-2 μm size range) than nanoplankton, whilst the diet of larger oligotrichs (50 μm) consisted almost exclusively of nanoplankton.

It is clear from the high populations of flagellates and ciliates that the water column at Shamrock Quay is productive, especially in summer, but the basis of this productivity is not proven. Chlorophyll levels are fairly high, and it will be necessary to find out what contributes to these; the numbers of *Mesodinium* and autotrophic flagellates can only be part of the answer, and the relatively high turbidity will surely restrict photosynthesis. The production of bacteria will presumably be the more important component at the base of the food chain at this site. Materials, and indeed the whole microbial community, move up and down the estuary with the tide, so this planktonic community is not isolated either from adjacent water masses or from the benthic community beneath. The situation at Cobden Bridge is clearly even less stable, and the erratic admixture of freshwater may be assumed to restrict the normal metabolism and growth of the marine ciliates found at that site.

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ACTA PROTOZOOLOGICA

Comparative Morphometry of *Giardia* Trophozoites From Man and Animals

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Summary. Thirty axenic *Giardia* isolates of the *G. duodenalis* morphological-type were investigated: 22 were isolated in Poland from humans with symptomatic or asymptomatic giardiasis, 5 from animals in Poland (primates and rodents), and three from human, cat and guinea-pig in the USA. *G. muris* trophozoites were obtained directly from the intestine of a spontaneously infected mouse. The overall length (L) and the maximum width (B) of the cells and the length of adhesive disk (SL) were measured both in live as well as in fixed and stained trophozoites. The major measurements and ratios were analyzed statistically by using Duncan test and by clustering of average measurements by single linkage or nearest neighbor method and group average strategy in two- or three-dimensional space. Our study proved the existence of statistically significant differences of the three measurements in the trophozoites of different *Giardia* isolates. The most reliable seems to be the analysis done by clustering of all dimensions in three-dimensional space; the nearest neighbor method groupped all the *Giardia* isolates into six groups and the group average strategy into eight groups. *G. muris* trophozoites constituted a separate morphological group in every statistical analysis. The differences made possible the dividing of separate *Giardia* groups partially correlating with molecular patterns of the isolates. However, the results of our study also made us aware that morphometrics cannot constitute a sole reliable criterion for *Giardia* speciation.

Key Words. *Giardia* sp., morphology, morphometry, taxonomy.

INTRODUCTION

With all the characteristics of *Giardia* populations described recently, and a great advance in our knowledge on the organization and function of this parasite, there exist no generally accepted criteria for species defining within this genus, and for the transmission of the parasite. The main reason of this situation is the confusion concerning the taxonomy of *Giardia* genus.

According to the concept of rigid host specificity, *Giardia* from each host species are considered as separate species; as a result of this concept over 40 *Giardia* species, mostly morphologically indistinquishable were described (Kulda and Nohynkova 1978). Recently, more and more widely Filice's proposal (1952) of the existence of three morphological groups with the type species of *G. agilis, G. muris,* and *G. duodenalis,* is accepted. However, there still is much controversy as concerns the *G. duodenalis* - type species which comprises most of *Giardia* populations, a.o. the parasite from man, *G. intestinalis.* The observed heterogenicity in the characters of *Giardia* isolates suggests that either

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Table 1

List of *Giardia* isolates from humans and animals

Isolate	Date of isolation	Geographic source	Host	Clinical course
P-1	1976	USA	Human	Symptomatic
HP-10	1982	Poland	Human	Symptomatic
HP-34	1982	Poland	Human	Symptomatic
HP-42	1982	Poland	Human	Asymptomatic
HP-50	1982	Poland	Human	Asymptomatic
HP-53	1983	Poland	Human	Asymptomatic
HP-63	1983	Poland	Human	Asymptomatic
HP-88	1983	Poland	Human	Symptomatic
HP-89	1983	Poland	Human	Symptomatic
HP-92	1983	Poland	Human	Symptomatic
HP-94	1983	Poland	Human	Symptomatic
HP-98	1983	Poland	Human	Asymptomatic
HP-99	1983	Poland	Human	Asymptomatic
HP-100	1983	Poland	Human	Asymptomatic
HP-101	1983	Poland	Human	Symptomatic
HP-102	1983	Poland	Human	Symptomatic
HP-103	1983	Poland	Human	Symptomatic
HP-104	1983	Poland	Human	Asymptomatic
HP-105	1983	Poland	Human	Symptomatic
HP-106	1984	Poland	Human	Symptomatic
HP-107	1984	Poland	Human	Asymptomatic
HP-108	1985	Poland	Human	Symptomatic
HP-109	1986	Poland	Human	Symptomatic
CAT-1	1970	USA	*Felis domesticus*	
GP-1	1976	USA	*Cavia porcella*	
SLP-111	1988	Poland	*Nycticebus coucang*	Asymptomatic
GGPRP-114	1988	Poland	*Cricetomys gambianus*	Asymptomatic
SP-115	1988	Poland	*Symphalangus syndactylus*	Asymptomatic
LSLP-116	1988	Poland	*Nycticebus pygmaeus*	Asymptomatic
CP-117	1988	Poland	*Galea musteloides*	Asymptomatic

G. duodenalis represents one species with a high level of intraspecies variability or *G. intestinalis* constitutes a complex species which comprises several species morphologically identical. To obtain as many as possible data for taxonomic consideration, thorough morphological and morphometrical studies on different *Giardia* populations (isolates) are necessary.

The aim of the study was to measure and compare the major dimensions of trophozoites from axenic *Giardia* cultures of the *G. duodenalis* morphologic type isolated from human and animals, as well as of *G. muris* morphologic type trophozoites obtained from the intestine of a spontaneously infected laboratory mouse. The inclusion of *G. muris* in that morphometric study was of great value for the comparison of the two morphologic types of *Giardia* populations.

MATERIAL AND METHODS

Parasites

Axenic isolates of *G. duodenalis*-type. Thirty axenic *Giardia* isolates of the *G. duodenalis* morphological-type were investigated (Table 1): 22 were isolated from humans with symptomatic or asymptomatic giardiasis (Kasprzak and Majewska 1983, 1985), 5 isolates were from animals (primates and rodents) (Majewska and Kasprzak 1990), and three were well known laboratory strains isolated from human, cat and guinea-pig in the USA (Meyer 1970, 1976, Fortess i Meyer 1976).

G. muris **trophozoites**. Becasue all attempts at establishing axenic *G. muris* cultures has as yet failed, the parasites were obtained directly from the intestine of a spontaneously infected BALB/c mouse.

Culture method

The axenic *Giardia* isolates were maintained on BI-S-33 medium (Diamond et al. 1978) supplemented with 0.075% bile, and additional l-cysteine-HCl (total 0.2%), without the vitamin-Tween 80 mixture (Keister 1983, Visvesvara 1980). The medium was adjusted to pH 7.0 to 7.2 with 1 N NaOH and sterilized by filtration. Following filtration the medium was supplemented with 10% of heat inactivated bovine serum. For routine maintenance of the cultures *Giardia* trophozoites were re-inoculated every seven days into fresh medium to a final concentration of 1×10^4 trophozoites per ml. The cultures were incubated at 37°C in a slant.

Preparation of trophozoites

Trophozoites from axenic culture. For morphometric studies only actively growing trophozoites (attached to the tube wall) from the logarithmic phase of growth were used. After 3 days of incubation the sediment and the supernatant with free-swimming trophozoites were removed and the chilled PBS (pH 7.2) was added to the tubes containing only the attached cells. Then the tubes were immersed in an ice water-bath for 20 min, shaken in a micro-shaker, and centrifuged at 600 g for 10 min at 4°C. The supernatant was discarded and the pellet with trophozoites re-suspended in 2 ml of PBS (pH 7.2). The suspension of trophozoites was dropped on slides. After the cells had attached to the surface of the slides (microscopic control), they were wet fixed in Schaudinn's solution, stained with iron-hematoxylin after Heidenhain's long method, and mounted using Caedax (Merck). Wet mount slides with live trophozoites were prepared from the same suspension of cells used for permanent stained smears.

G. muris **trophozoites from the intestine of the mouse**. The mouse was killed by an overdose of ether, the terminal segment of small intestine was removed, dissected longitudinally, and placed in culture tube filled with BI-S-33 medium. After 3 h of incubation at 37°C, the intestine segment, the supernatant and the sediment were discarded. The tubes containing only the trophozoites attached to the wall, were filled with chilled PBS (pH 7.2), immersed in an ice water-bath for 20 min, shaken in a micro-shaker, and centrifuged at 600 g for 10 min at 4°C. The supernatant was discarded, the pellet with trophozoites re-suspended in 2 ml of PBS (pH 7.2). Fixed and stained preparations, as well as wet mount slides were made in the same way as the trophozoites from axenic culture.

Measurement of fixed and stained trophozoites

Trophozoites were measured with an ocular micrometer (PZO) by light microscopy (Reichert) under oil immersion at a total magnification of x 1,500. The ocular micrometer was calibrated with a stage micrometer with the smallest division of 0.01 mm; 100 divisions of the ocular micrometer were equal to 79 μm. The overall length (L) and the maximum width (B) of the cells and the length of adhesive disk (SL), as the dimensions accepted in differentiation of *Giardia* species, were measured according to Kulda and Nohynkova (1978). For every *Giardia* isolate 100 undistorted trophozoites with well-marked morphology were measured, taking also into account the presence and the shape of median bodies.

Measurement of live trophozoites

To evaluate the influence of fixative and stain on the dimensions of the cells, overall length and maximum width of 100, well-defined, attached to the surface of slides, live trophozoites of CP-117 *Giardia* isolate were measured. Trophozoites were measured by light microscopy, as described above, at a total magnification of 675 x; 100 divisions of the ocular micrometer were equal to 172 μm.

Statistics

From the average of all dimensions the L/B and SL/L ratios were calculated. For statistical analysis individual measurements and ratios were analyzed by using multiple range analysis (Duncan test); $P \leq 0.05$ was considered significant for statistical observations. The similarity of 31 *Giardia* isolates was also determined by clustering of average measurements by single linkage or nearest neighbor method and group average strategy in two- or three-dimensional space of features. The influence of fixative and stain on the size of the cells was analyzed by comparing the dimensions of live and fixed and stained trophozoites and using Student's t-test (a $P \leq 0.05$ was considered significant for statistical observations).

RESULTS

Median bodies, which were typical in shape for *G. duodenalis* and *G. muris* morphological groups, were found in most of the cells; only five isolates (HP-34, HP-63, HP-42, HP-100 and GGPRP-114) were lacking the median bodies in substantial although different percentage of the trophozoites (from 10% to 38%). The major dimensions of trophozoites (L, B, SL) with median bodies were compared with those of the cells of the same isolates without median bodies. In the trophozoites without median bodies significant differences ($P \leq 0.05$) were found in most dimensions when Student's t-test was used; in 3 of 5 isolates the

Table 2

Trophozoites morphometric and morphological data of *Giardia* isolated from humans and animals (n = 100)

Giardia isolate	L $\bar{x} \pm SD$	B $\bar{x} \pm SD$	SL $\bar{x} \pm SD$	MB+
P-1	13.98±0.71	7.19±0.40	5.85±0.40	100
HP-10	12.89±0.82	7.30±0.51	5.80±0.64	96
HP-34	12.40±0.79	6.40±0.55	5.06±0.55	62
HP-42	13.75±0.87	7.74±0.40	6.16±0.55	77
HP-50	14.62±1.26	8.14±0.47	6.79±0.87	94
HP-53	14.62±0.79	7.43±0.55	6.48±0.47	97
HP-63	11.21±0.84	6.26±0.46	5.17±0.49	75
HP-88	13.51±0.95	7.74±0.63	6.32±0.55	96
HP-89	14.62±0.79	7.58±0.40	6.24±0.40	96
HP-92	14.62±0.87	7.58±0.79	6.40±0.40	98
HP-94	15.17±0.95	7.74±0.32	6.40±0.40	100
HP-98	13.87±0.79	7.85±0.49	6.62±0.52	96
HP-99	14.54±0.95	7.82±0.79	6.24±0.47	91
HP-100	13.35±1.03	7.27±0.55	6.00±0.55	78
HP-101	14.30±1.03	7.74±0.55	6.48±0.55	94
HP-102	14.85±0.71	7.74±0.24	7.19±0.24	99
HP-103	13.67±0.87	7.35±0.47	6.40±0.47	99
HP-104	13.72±0.62	7.57±0.37	5.90±0.57	98
HP-105	13.90±1.34	7.27±0.47	6.40±0.47	91
HP-106	14.46±0.63	7.82±0.71	6.24±0.32	98
HP-107	15.80±0.55	8.53±0.32	6.87±0.47	100
HP-108	15.01±1.19	8.14±0.55	7.03±0.47	99
HP-109	15.22±1.10	7.97±0.46	6.68±0.69	93
SLP-111	14.44±1.50	7.89±0.45	5.82±0.68	95
GGPRP-114	14.51±1.71	7.24±0.94	5.73±0.66	90
SP-115	15.45±1.16	7.54±0.65	5.91±0.64	96
LSLP-116	15.01±1.61	7.14±0.85	5.87±0.74	95
CP-117	16.63±1.50	8.21±0.59	6.94±0.68	92
CAT-1	15.64±0.87	8.06±0.40	7.19±0.47	95
GP-1	13.83±0.87	7.43±0.63	6.16±0.47	99
G. muris	8.85±0.55	6.72±0.40	5.61±0.40	99
CP-117*	19.09±0.87	9.97±0.52	n.d.	n.d.

L - length of trophozoites; B - width of trophozoites; SL - length of adhesive disc; MB+ - number of trophozoites with median body; * live trophozoites; n.d. - not done.

trophozoites without median bodies were smaller (data not shown).

The comparison of the overall length and maximum width of live as well as fixed and stained trophozoites of CP-117 *Giardia* isolate showed significant differences in the dimensions; the fixed and stained cells were reduced to 82.3% of normal length and to 87.1% of normal width.

Table 2 shows the results of 9500 measurements of axenic *G. duodenalis* - group isolates from 23 humans and from 7 different animals (non-human primates, rodents, carnivores) and of one *G. muris* isolate from a spontaneously infected mouse.

Among the differences between the isolates analysed by using Duncan test in every dimension (L, B, SL) the
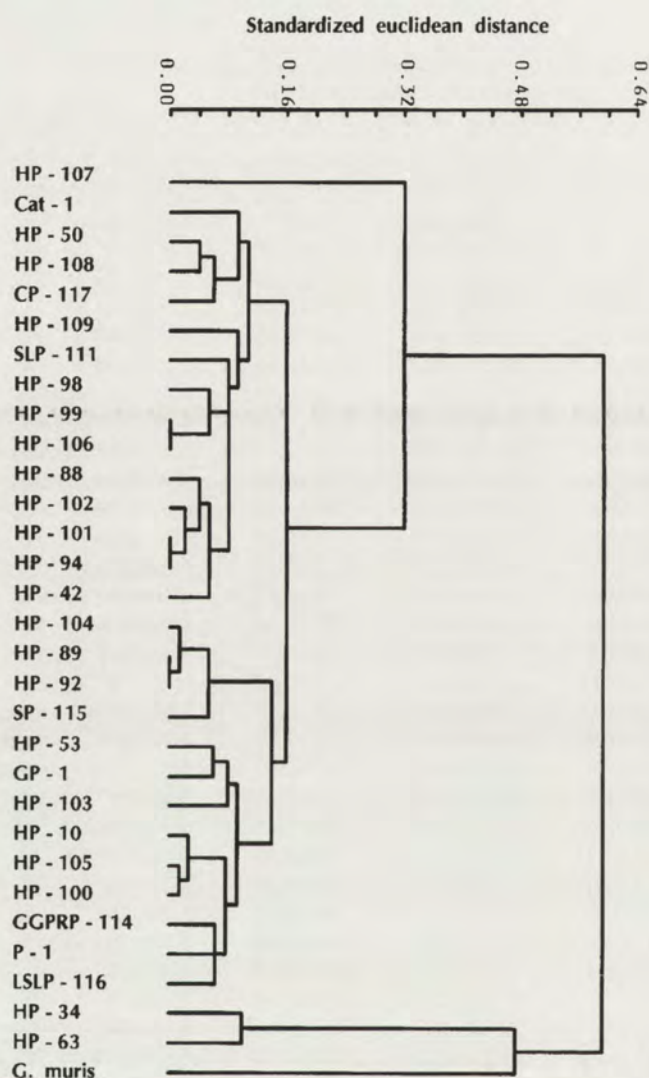
Fig. 1 Phenogram of euclidean distances among isolates of *Giardia* clustered by nearest neighbor method concerning SL/L (adhesive disc length to overall length of trophozoite) and B (maximum width of trophozoite).

most pronounced was the overall length; however, the most stable was the length of adhesive disk. In the group of the smallest organisms, as concerns every dimension, were *G. muris* and the human HP-34 and HP-63 isolates. Most of the trophozoites of *G. duodenalis* - type isolated from animals were marked by a short adhesive disk.

The analysis of the average measurements of L, B and SL by using the Duncan test showed the existence of different groups of isolates (19, 10, and 12 respectively).

From the analysis of the overall length to maximum width ratio (L/B), being indicative of the cell's shape, it appears that the trophozoites of *G. duodenalis*-type isolates are very similar; the ratio ranged from 1.75 to 2.10.

On the contrary, the *G. muris* trophozoites are distinctly different; the overall length to maximum width ratio (L/B) was 1.32.

The analysis of the length of the adhesive disk to the overall length ratio (SL/L) by using the Duncan test shows the existence of two groups. The first was composed of all *G. duodenalis* - type isolates (the adhesive disk in the trophozoites did not exceed half of the body length and the differences between the isolates were statistically not significant) and the second was represented by *G. muris* trophozoites (the adhesive disk was longer than a half of the cell length).

Because the statistical analysis of every individual measured and calculated major dimension showed varrying scheme of differentiation of *Giardia* isolates studied, we performed an analysis of two dimensions simultaneously (SL/L and B). That statistical analysis performed both by the nearest neighbor method and by the group average strategy in two-dimensions space of features divided the *Giardia* isolates by the d_{crit} 1.43, into five groups (Fig. 1): Group 1 - HP-107; Group 2 - Cat-1, HP-50, HP-108, CP-117, HP-109, SLP-111, HP-98, HP-99, HP-106, HP-88, HP-102, HP-101, HP-94, HP-42; Group 3 - HP-104, HP-89, HP-92, SP-115, HP-53, GP-1, HP-103, HP-10, HP-105, HP-100, GGPRP-114, P-1, LSLP-116; Group 4 - HP-34, HP-63; Group 5 - *G. muris*.

Using other statistical analysis for the determination of the similarity among all studied *Giardia* isolates (analysis of three dimensions by the nearest neighbor method or by the group average strategy in three-dimensional space) different numbers of groups were found; 6 (Figure 2) and 8 respectively (data not shown).

DISCUSSION

A critical review of the studies on the taxonomy of *Giardia* revealed that the problem is currently unsettled in spite of various criteria used in differentiating the species of this genus. One may accept that the failure of correlation between several criteria is due to the fact that the establishing of species taxonomic criteria is more difficult for agamous organisms morphologically very similar.

As early as in 1922 Hegner proposed to compare different dimensions of the cell to differentiate *Giardia* species. Later other authors (Filice 1952, Georgi et al. 1986, Grant and Woo 1978, Sogayer and Correa 1984,

Soloviev 1975) measured trophozoites of few *Giardia* populations both from the same and different hosts, and showed statistically significant similarities as well as differences in their dimensions. Thus, although morphology was one of the first classification tools used for *Giardia* speciation, it is still disputable and by some authors recognized as a conservative taxonomic approach. One of the reasons of these doubts was that in these morphological studies the trophozoites obtained directly from host's intestines were used. Therefore it is doubtful whether the differences in cell morphology represent evolutionary divergence or are due to differences in the intestinal environment of hosts (Owen 1980). Using *Giardia* trophozoites from the same controlled conditions of a in vitro axenic cultures is therefore of fundamental value. Morphometric data based on comparison of axenic cultures of *Giardia* trophozoites were presented so far by Bertram et al. (1984) and Kiorpes et al. (1987), but both studies were conducted on a small number of isolates. During the present study morphometric data were for the first time conducted and compared on trophozoites from numerous axenic in vitro *Giardia* isolates from different hosts species. Only *G. muris* was isolated directly from the intestine of a mouse because of difficulties in establishing axenic culture of the isolates representing *G. muris* morphological group. However, including this isolate to present morphometric study actually makes possible the defining of the range of differences between *Giardia* isolates from two morphological groups.

According to the findings of many authors (Bertram et al. 1984, Filice 1952, Grant and Woo 1978, Soloviev 1975) the trophozoites with median bodies are larger than those without. The authors supposed that the lack of median body may be related to trophozoites which have recently divided or excysted. The comparison of the cells with and without median body in a preliminary study confirmed this relation in most isolates investigated; therefore, in the final analysis of *Giardia* isolates similarity by clustering of all three dimensions in three-dimensional space only the measurements of the trophozoites with median bodies were taken in account.

Among all *Giardia* axenic isolates investigated in the present study the three American isolates (P-1 from human, GP-1 from guinea pig and CAT-1 from a cat) were also examined by Bertram et al. (1984). Therefore we compared the results of measurements of Bertram et al. and of the present studies (length and width of trophozoites) by statistical analysis (Student's t-test), and found statistically significant differences either for
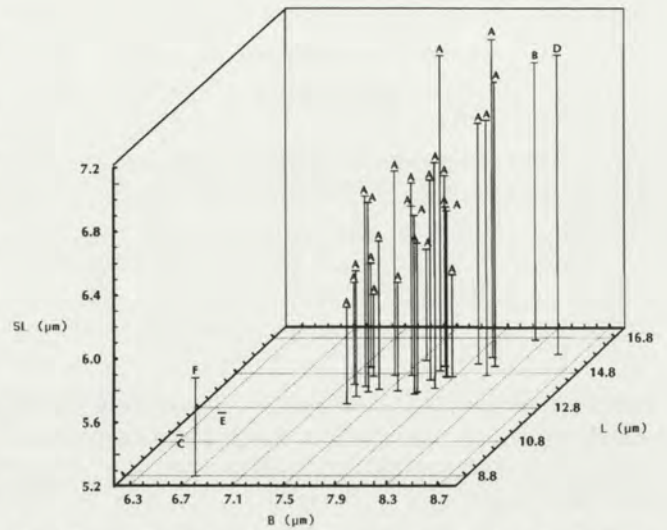
Fig. 2 Three-dimensional plot (SL - adhesive disc length, B - maximum width and L - overall length of trophozoite) showing the similarity of *Giardia* isolates by the nearest neighbor method.
A - HP-10, HP-104, HP-98, HP-109, SLP-111, GGPRP-114, SP-115, LSLP-116, P-1, HP-42, HP-50, HP-53, HP-88, HP-89, HP-92, HP-94, HP-99, HP-100, HP-101, HP-102, HP-103, HP-105, HP-106, GP-1, and CAT-1 *Giardia* isolates; B - CP-117 *Giardia* isolate; C - HP-63 *Giardia* isolate; D - HP-107 *Giardia* isolate; E - HP-34 *Giardia* isolate; F - *G. muris* isolate.

both measurements (isolate P-1) or for one measurement (GP-1 and CAT-1). We suppose that these differences are due to the different methods of fixation and staining used in the two studies.

In the past some authors identified *Giardia* species basing on minor morphological differences or even on one dimension of the cell. More recently Bertram et al. (1984) interpreted the results of their study more cautiously despite greater difference in the dimensions of length and width they found in trophozoites belonging to the same or different species. During the present study even greater differences in length and width of trophozoites of the *G. duodenalis* morphological group were found. However, it should be accepted that even these marked differences in the dimensions could not be considered as the proper criterion for differentiation of *Giardia* species.

Several authors attempted the differentiation of *Giardia* populations on the ground of simple length to width ratio and by using simple statistical analysis. As a result, it was not possible to differentiate *Giardia* populations within the same morphological group (Sogayar and Correa 1984, Soloviev 1975). On the other hand, Grant and Woo (1978), also considering the length to width ratio, found significant differences between *Giardia* species. However, the authors emphasized that a large number of trophozoites from numerous hosts should be

examined to obtain measurements representative of the species as a whole. The results of the present study, conducted on large material by using rigorous methods of statistical analysis, testify to the results of Grant and Woo's study and prove the inefficiency of such simple observation in differentiation of *Giardia* isolates.

In most studies performed hitherto the length of adhesive disk was not taken into account, despite the opinion of some early authors (Filice 1952, Hegner 1922a, 1922b) on the importance of this dimension in differentiation of *Giardia* species. The results of the present study distinctly showed that the average lenght of adhesive disc - contrary to the average lenght and width of the trophozoites - is the most stable feature and that measuring only the length and width of the trophozoites is not sufficient for a genuine differentiation of *Giardia* isolates.

The statistical analysis of trophozoite length/width ratio, and adhesive disc length/trophozoite length ratio by Duncan test divided the parasite's populations into two groups (*G. duodenalis* and *G. muris*), but did not differentiate them within each individual group. From this, it is evident that the analysis of the ratio between any two morphometric dimensions will not be sufficient for differentiation of *Giardia* populations. Consequently for a further evaluation of the similarity between *Giardia* isolates the cluster analysis of the adhesive disk length to total length ratio and the maximal width of the trophozoites in two-dimensional space of features was introduced. This analysis resulted in the separation of two large groups of *G. duodenalis* - type isolates comprising the parasites from humans and from animals, *G. muris*, and of three individual human isolates.

The most reliable seems to be the analysis of *Giardia* isolates similarity by clustering of all three major dimensions (length and width of the cell, and length of adhesive disk) in three-dimensional space. The nearest neighbor method groupped all the *Giardia* isolates into six groups and the group average strategy into eight groups.

The largest group, clustered by the first method, comprises the majority of *G. duodenalis* - type isolates independently from the host (human or animals) from which they were isolated, and from the clinical course of the infection (symptomatic or asymptomatic). In our previous study we found that there were two profiles of restriction fragment polimorphism in the animal *Giardia* identical to two profiles in isolates of human origin, and that isoenzyme and total protein patterns divided the strains into the same two groups. One group involves

the isolate from slow loris (SLP-111) and human isolates from different geographic origin and the second group comprises the four other animal isolates (GGPRP-114, SP-115, LSLP-116 and CP-117) and some of the Belgian *Giardia* isolates (De Jonckheere et al., 1990). Also the hybridization with pGI7 (1.8 kb rDNA fragment of human *Giardia* cloned by De Jonckheere et al. 1989) confirmed the homogeneity of the animal *Giardia* isolates and their resemblance to the human isolates. The present morphometric study correlates in part with the results of our molecular analysis. Similarity determination by clustering of average measurements using every method in two- or three-dimensional space of features, groupped the SLP-111 animal isolate together with human isolates, and the group average strategy method in three-dimensions space separated the four other animal isolates from human isolates (the present study das not involved the human Belgian *Giardia*). It is worthy of noticing that this large group included the isolates from the same individuals obtained from different material (faeces and duodenal fluid); this fact testified to the morphological similarity of the trophozoites population in the same host.

Smaller groups were formed by some isolates both from humans and animals; different methods of statistical analysis most often clustered the same human (HP-34, HP-63, HP-107), or animal (CP-117) *Giardia* isolates. The populations of both HP-34 and HP-63 human isolates were composed of smallest trophozoites, and clustered in the two- and three-dimensions space of features very close to *G. muris*. On the contrary, the trophozoites of human HP-107 and of animal CP-117 isolates constituted the populations of largest cells. In our previous investigations we found that some of these individual axenic *Giardia* isolates differed in a distinctive way; e.g. the Southern blot of DNA from *Giardia* HP-63 and HP-34 human isolates probed with pG17 showed an aberrant hybridization pattern (De Jonckheere et al. 1989). Besides, the trophozoites of HP-63 isolate - solely of all investigated isolates - exhibited most pronounced carbohydrate-specific lectin binding (WGA) in micro-agglutination system (Kasprzak and Majewska, in press). It is worth noticing that *G. muris* trophozoites constituted a separate morphological group in every statistical analysis.

Our study proved the existence of statistically significant differences of the three measurements in the trophozoites of different *Giardia* isolates. The differences made possible the dividing of separate *Giardia* groups partially correlating with molecular patterns of

the isolates. The resemblance of the animal *Giardia* to the human isolates permits to speculate on the epidemiological significance of this observation. However, the results of our study also made us aware that morphometrics cannot constistute a sole reliable criterion for *Giardia* speciation.

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