

COMPARTMENTALIZATION OF THE
DEVELOPING MACRONUCLEUS
FOLLOWING CONJUGATION IN
STYLONYCHIA AND *EUPLOTES*

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ABSTRACT

The development of the macronucleus following conjugation in the hypotrichous ciliates *Euplotes* and *Stylonychia* has been examined with the electron microscope. Banded polytene chromosomes can be seen in thin sections of the macronuclear anlagen during the early periods of exconjugant development. As the chromosomes reach their maximum state of polyteny, sheets of fibrous material appear between the chromosomes and transect the chromosomes in the interband regions. Individual bands of the polytene chromosomes thus appear to be isolated in separate compartments. Subsequently, during the stage when the bulk of the polytenic DNA is degraded (1), these compartments swell, resulting in a nucleus packed with thousands of separate spherical chambers. Individual chromosomes are no longer discernible. The anlagen retain this compartmentalized condition for several hours, at the end of which time aggregates of dense material form within many of the compartments. The partitioning layers disperse shortly before replication bands appear within the elongating anlagen, initiating the second period of DNA synthesis characteristic of macronuclear development in these hypotrichs. The evidence presented here suggests that the "chromatin granules" seen in the mature vegetative macronucleus represent the material of single bands of the polytene chromosomes seen during the earlier stages of macronuclear development. The possibility is also discussed that the degradation of DNA in the polytene chromosomes may be genetically selective, which would result in a somatic macronucleus with a different genetic constitution than that of the micronucleus from which it was derived.

INTRODUCTION

During the vegetative growth of ciliated protozoa, cellular functions are controlled by genes residing within the "polyploid" macronucleus (11, 13, 21). The diploid micronucleus is considered to be a generally inert germ nucleus, dividing mitotically with each cell fission. However, under certain conditions (such as a dwindling food supply) ciliates become sexually active, and animals of

complementary mating type unite to form conjugal pairs. Within each conjugant, the micronucleus undergoes meiotic divisions. Most of the resulting nuclei degenerate, but two haploid nuclei are retained, the stationary (female) pronucleus and the migratory (male) pronucleus. Following the reciprocal exchange of male pronuclei and the fusion of each of these with the

stationary pronucleus of the respective partner to form the diploid synkaryon, or zygote nucleus, the conjugants separate. The synkaryon then divides at least once; of the resulting division products, which are genetically identical, one (or more, depending on the species) retains the diploid condition as the new definitive micronucleus (or micronuclei), while one (or more) of the remainder, the macronuclear anlage(n), is converted in a series of steps to the new macronucleus (macronuclei) of the cell. The old macronucleus eventually degenerates completely.

Recent studies of macronuclear differentiation following conjugation in a number of ciliates have revealed the presence of long cross-banded chromosome-like structures within the macronuclear anlagen at early stages of exconjugant development (1, 4, 6, 15, 17, 18). Because the width of such strands increases during an initial period of DNA build-up within the macronuclear anlagen, and because DNA is concentrated in the banded regions, these strands have been interpreted as polytene chromosomes similar to those found in larval dipteran tissues. Although a specificity of banding pattern along the macronuclear chromosomes has not yet been established, some of the bands have been reported to resemble the "puffs" found on dipteran giant chromosomes (12, 18). In the most carefully studied cases, the hypotrichs *Stylonychia mytilus* (1) and *Euplotes woodruffi* (18), it has been found that following the period of maximum polyteny, the giant chromosomes break down and the bulk of their DNA is degraded (1-3, 18). After an interval of several hours, the remaining DNA of the anlagen replicates many times, without the formation of discernible chromosomes, to form the "polyploid," RNA-producing macronucleus of the vegetative cell.

An electron microscopic study of macronuclear development in *Euplotes* and *Stylonychia* has disclosed unusual structures within the anlagen (9). These structures, which may be referred to as "intranuclear partitions," appear to segregate portions of the individual polytene chromosomes, and may help explain the enigma of the build-up and subsequent destruction of the polytene state.

METHODS AND MATERIALS

Culture Methods and Procedures for Obtaining Conjugants

Conjugation and macronuclear development are basically similar in *Euplotes woodruffi* and *Stylonychia mytilus*, with the exception that anlagen development

to the stage of maximum polyteny requires 30-40 hr in *S. mytilus* (measured from the time of conjugant separation) and only 10-12 hr in *E. woodruffi* (at 21°C.). The mating stocks of *E. woodruffi* were originally collected by M. V. N. Rao, and are maintained in wheat infusion containing *Chilomonas* and *Tetrahymena* as food organisms. Mixing cultures of different mating types soon after they have exhausted their food supply results in the formation of large numbers of conjugating pairs, which are then isolated for further study, as described by Rao (14, 16).

Cultures of a larger *Euplotes* species, probably *E. eurytomus* (obtained from Carolina Biological Supply Co., Burlington, N. C.) were maintained in dilute Cerophyl solution inoculated with *Enterobacter aerogenes* and containing *Tetrahymena* as food organisms. Different mating types of this strain were not isolated, so that large numbers of precisely timed exconjugants could not be obtained. However, starvation induces intracolonial conjugation to varying degrees in different cultures, so that small numbers of exconjugants could be fixed and compared with the more reliably staged exconjugants of *E. woodruffi* and *Stylonychia*.

Exconjugants of *S. mytilus* at various stages of development were provided by Dieter Ammermann, who has described in detail the culture conditions and the methods for obtaining conjugation used with these organisms (1).

Preparation of Animals for

Electron Microscopy

Chromosome development in *Euplotes* and *Stylonychia* can be monitored by examining acetic acid spreads of animals at various intervals following separation of the conjugants (cf. 18). At appropriate stages of exconjugant development, groups of several hundred animals were concentrated and fixed for 30 min in cold 3% OsO₄, buffered to pH 7.4 with 0.01 M ϵ -collidine-HCl and containing 1 mg/ml CaCl₂. In other cases, animals were concentrated in a small drop of culture fluid and fixed by exposure to OsO₄ vapor for 5 min at room temperature. Following a collidine buffer rinse, the animals were dehydrated in cold ethanols, washed in propylene oxide, and embedded in an Epon-Araldite mixture (Mollenhauer's No. 2; 10). Some groups of organisms were concentrated by centrifugation following each preparative step; other groups were embedded in agar prior to dehydration and embedding, according to Flickinger (5). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM-200 electron microscope operated at 60 kv.

RESULTS

Thin sections of *Stylonychia* exconjugants during early macronuclear anlage development (before

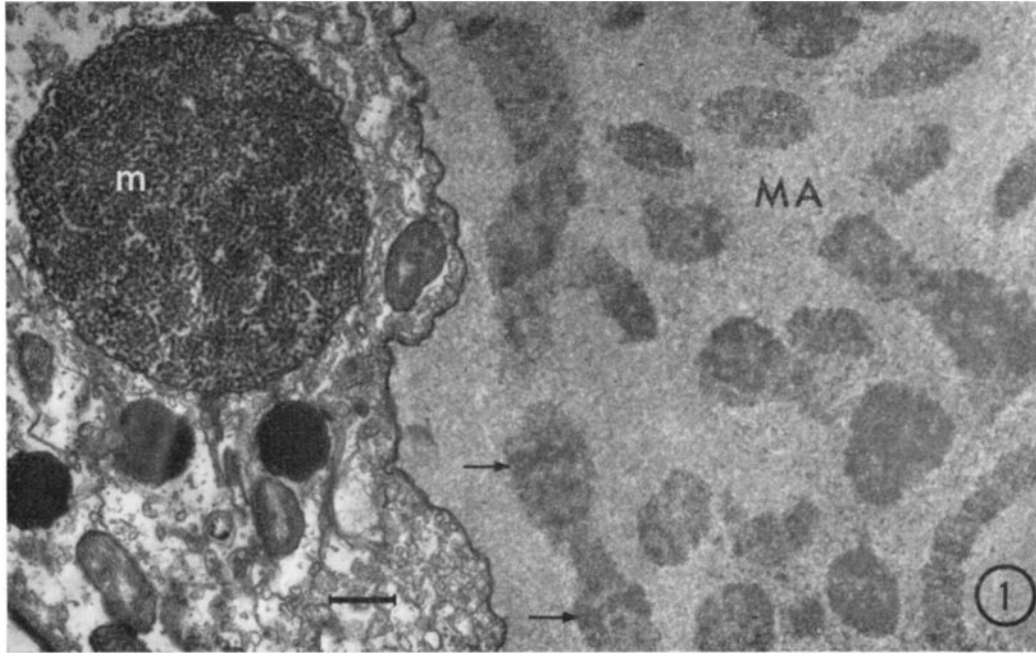


FIGURE 1 *Stylonychia mytilus* exconjugant during the period of increasing polyteny, showing a micro-nucleus (*m*) with its reticular chromatin, and the developing macronuclear anlage (*MA*) with sections of several chromosomes. Only slight indications of chromosomal banding can be seen, as at the arrows. Scale in this and the remaining figures is 1μ . $\times 8900$.

the animals have reached the period of peak polyteny) reveal discrete chromosomes, composed of compact masses of granular and fibrillar material, lying within the nucleoplasm (Fig. 1). The chromosomes at this stage display only suggestions of banding along their lengths. As the anlagen approach the maximum degree of polyteny, the distinct chromosomal banding pattern seen with the light microscope (cf. 1) is also readily observed with the electron microscope (Fig. 2). Upon closer examination, it can be seen that condensations of dense material have formed between the chromosomes, and also transect the chromosomes in the interband regions (Figs. 2 and 3), thus enhancing the banded appearance. Since these dense condensations present a linear image regardless of the plane of section, they must be interpreted as *sheets* of material, with the result that the individual bands of the polytene chromosomes are apparently isolated within separate compartments at this stage of macronuclear development. A similar partitioning of the polytene chromosomes is seen in *Euplotes eurystomus* (Fig. 4).

The arrangement of chromosomal material in

early *Euplotes woodruffi* anlagen is more difficult to interpret. Amorphous aggregates of moderately dense granular material and a few rodlets of much greater density are found clumped in the center of the nucleus (Fig. 5). When acetic acid spreads are made of *E. woodruffi* anlagen at this stage, elongated chromosomes are clearly seen; thus it can be assumed that the granular material seen in thin sections represents chromatin, and that the individual masses are very likely linearly connected in the form of intertwined chromosomes. Rao and Ammermann (17) have pointed out that the giant chromosomes in this species are very tightly packed within the anlage. At slightly later stages of macronuclear development, as the DNA content of the anlagen increases, sheets of fibrous material appear within the nucleus. These sheets, which wind through the central chromosomal mass and surround individual clumps of chromatin (Fig. 6), are interpreted as being analogous to the partitioning layers seen in *Stylonychia* and *E. eurystomus*.

The most remarkable appearance of the macronuclear anlagen in all three species is found when the exconjugants have passed the stage of increas-

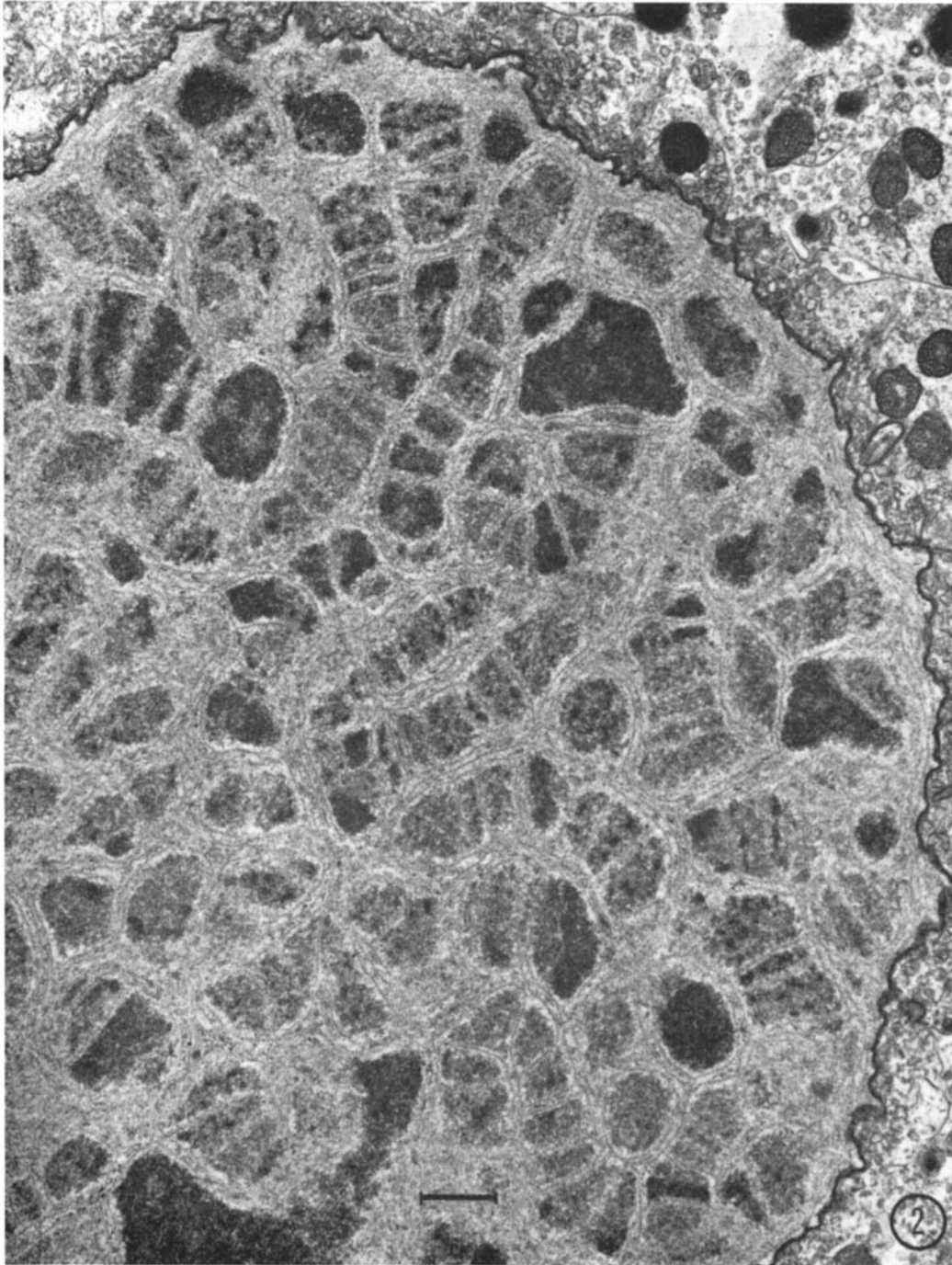


FIGURE 2 Macronuclear anlage of a *Stylonychia* exconjugant, with the banded polytene chromosomes at their largest. At this stage lamellar partitions can be seen among the chromosomes and passing between the bands of individual chromosomes. $\times 10,900$.

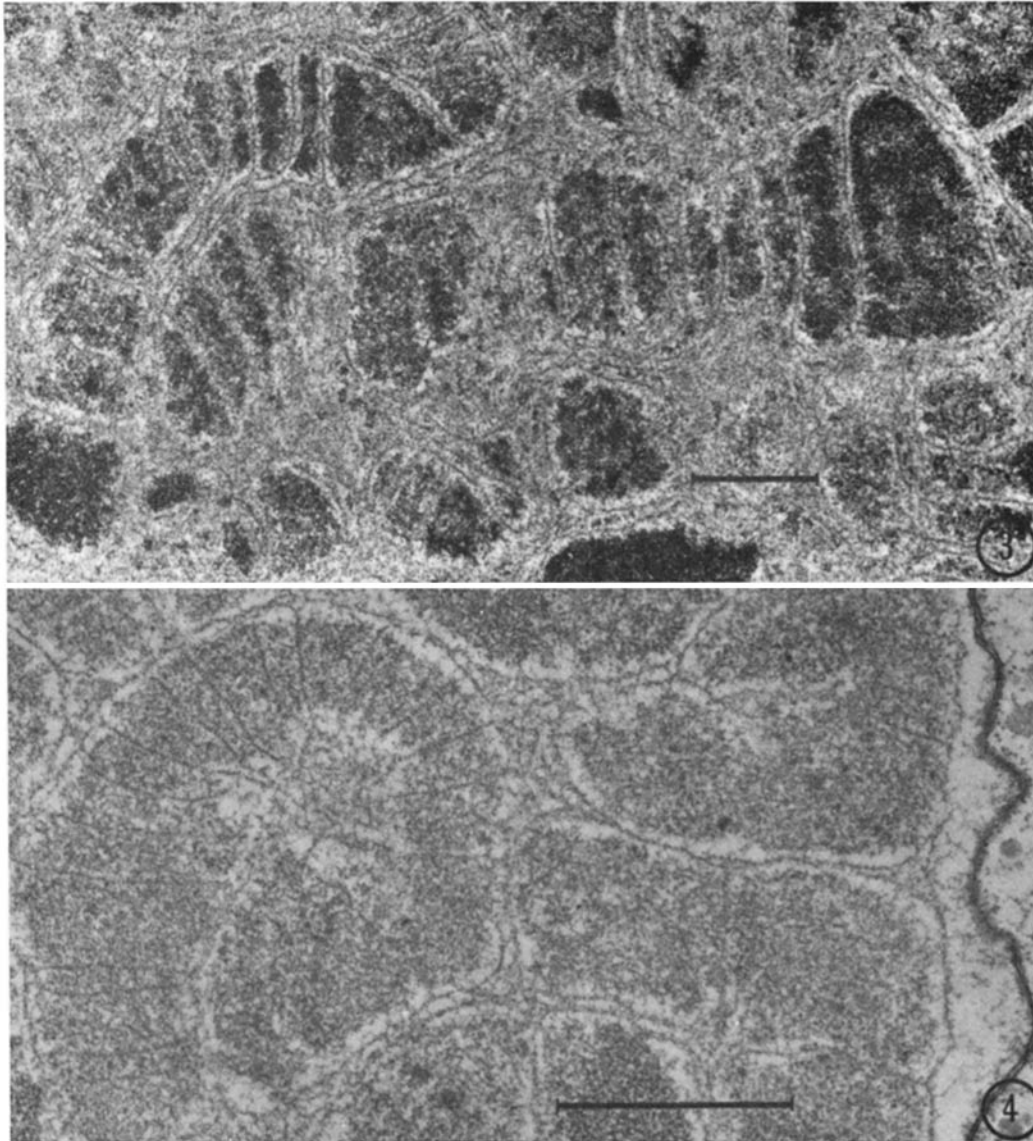


FIGURE 3 Section through the same nucleus as in Fig. 2, showing more clearly the intranuclear partitions traversing the polytene chromosomes. $\times 16,300$.

FIGURE 4 Macronuclear anlage of a *Euplotes eury stomus* exconjugant, showing the chromosomal partitioning in this species. $\times 31,000$.

ing polytenization and have begun the chromosome degradation and DNA loss demonstrated by Ammermann (1) and Rao and Ammermann (18). The segmental compartments, which had enclosed the material of individual chromosomal bands (Figs. 3 and 4), swell until the nucleus is tightly

packed with globular chambers or "corpuscles" 1-2 μ in diameter, each separated from the others by one or more lamellar partitions (Fig. 7). From this stage onward, individual chromosomes are no longer discernible in any of the animals.

The macronuclear anlagen retain this "com-

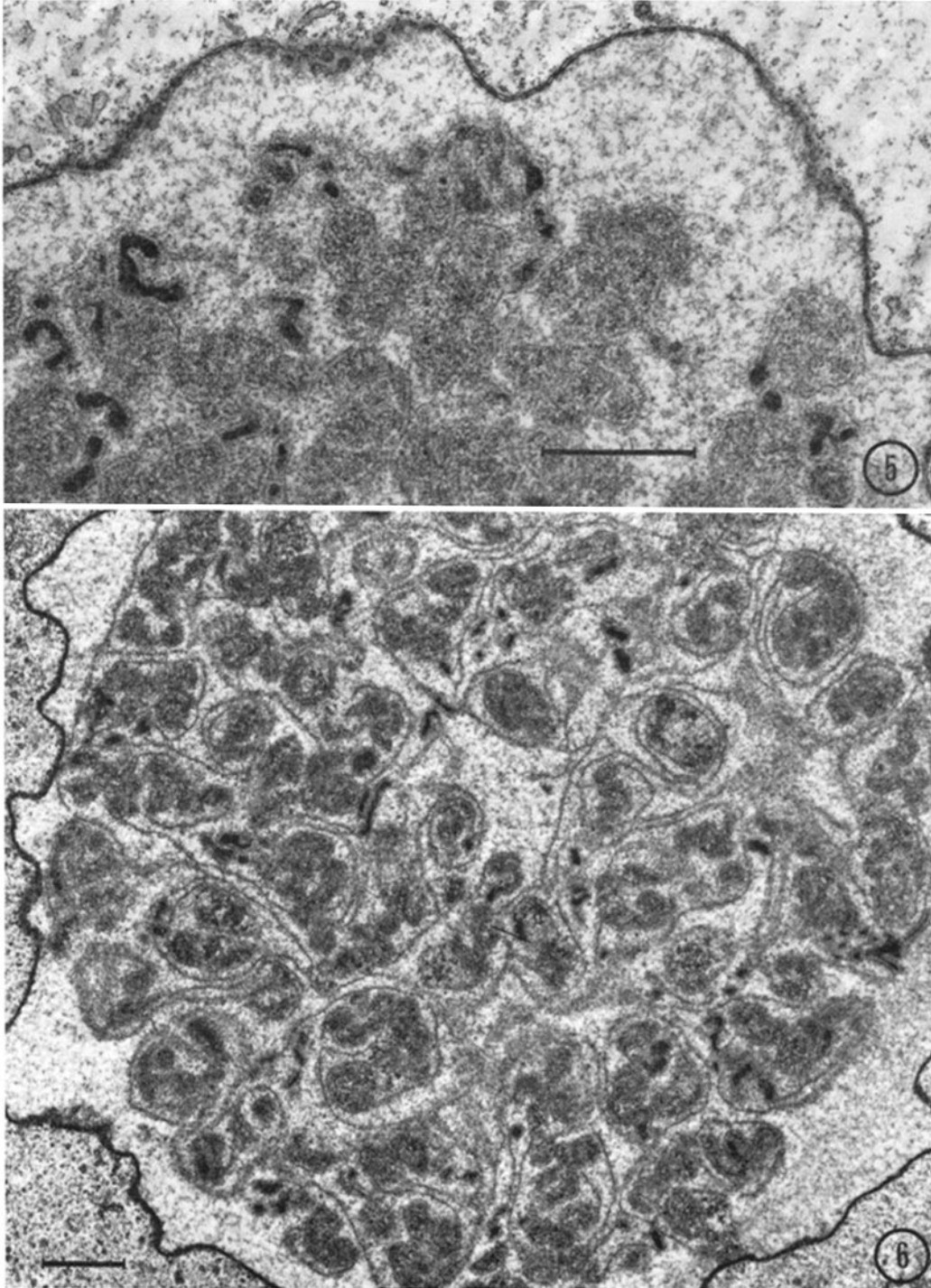


FIGURE 5 Early *Euplotes woodruffi* exconjugant, showing the compact masses of chromatin present in the anlage during the initial polytenization in this species. The composition of the irregular electron-opaque rodlets is not known. $\times 22,000$.

FIGURE 6 A later stage of macronuclear anlage development in *E. woodruffi*, showing tortuous lamellae winding among the chromatin masses. These lamellar layers are interpreted as being analogous to the partitioning layers seen in the other two species investigated (Figs. 3 and 4). $\times 11,600$.

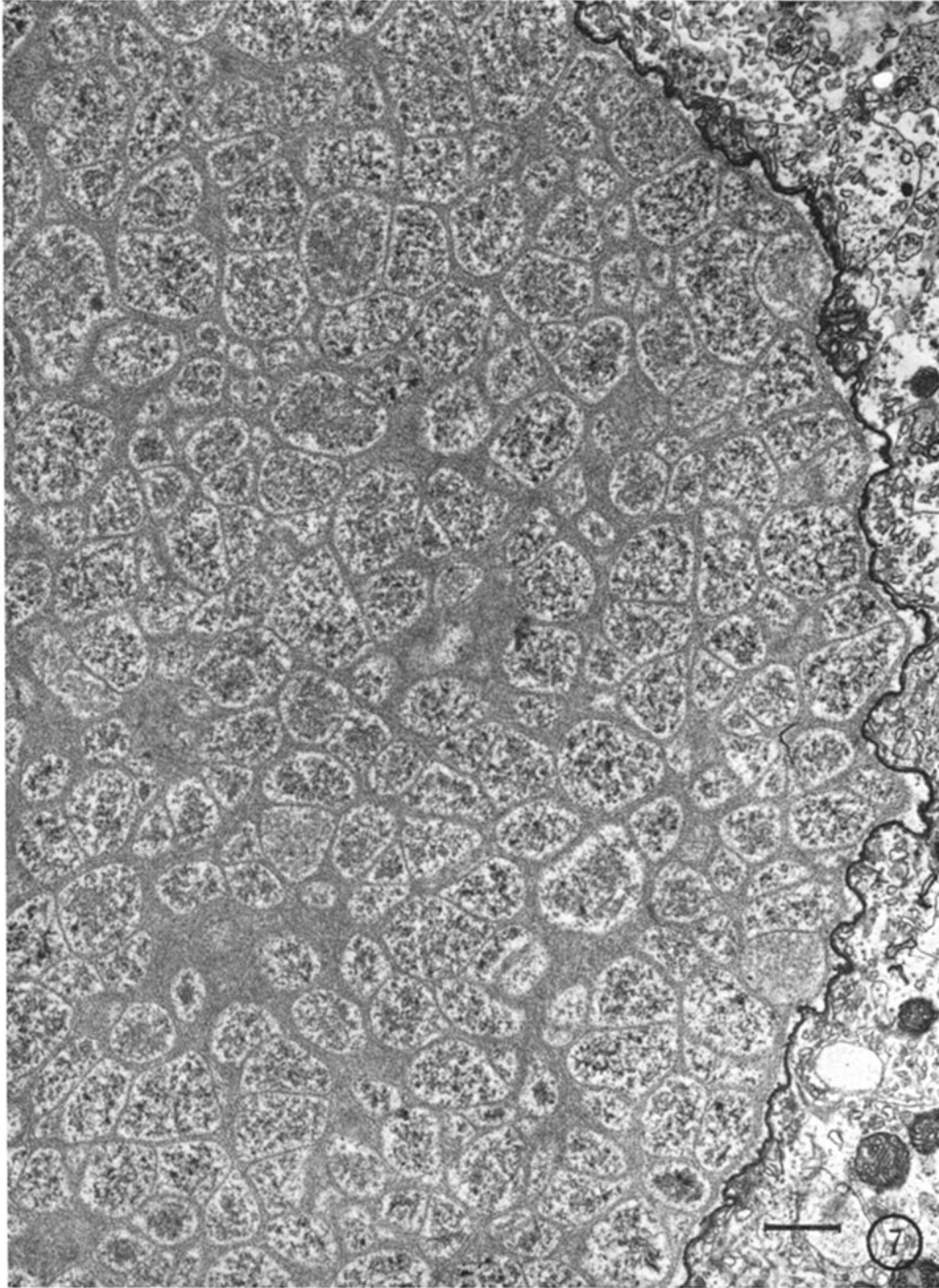
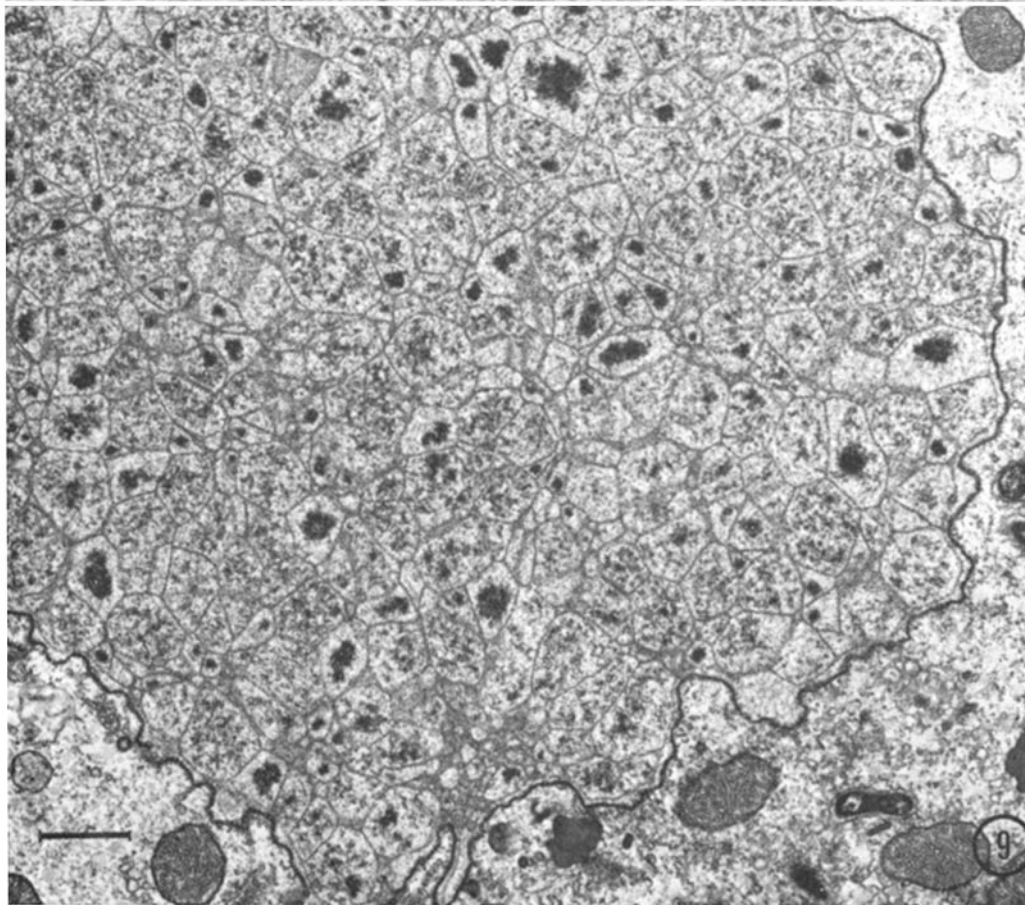
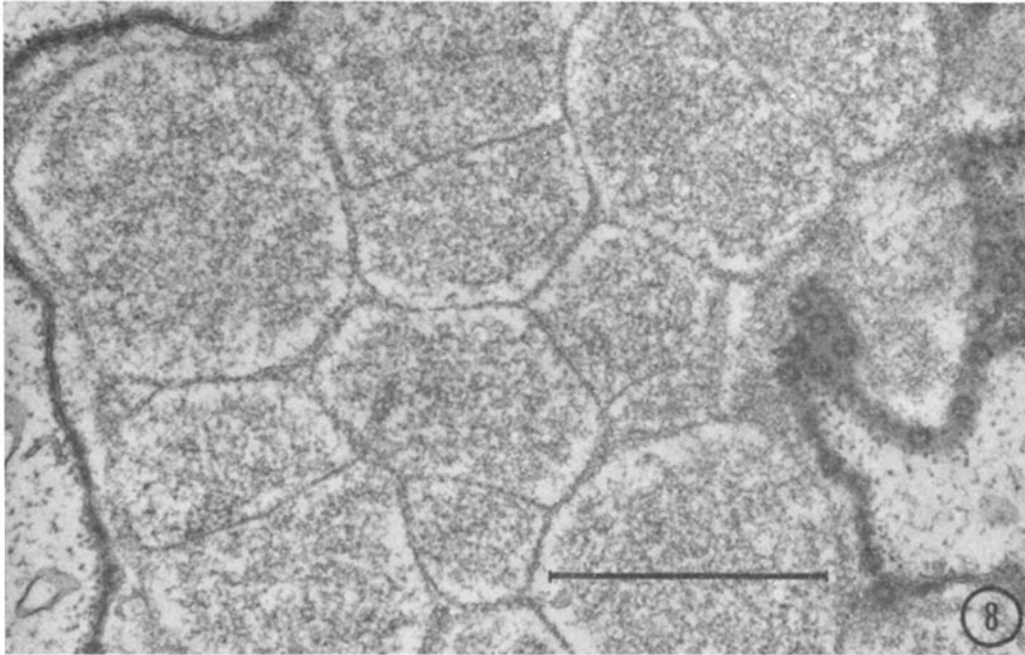


FIGURE 7 Compartmentalized macronuclear anlage during the DNA-breakdown or DNA-poor ("achromatic") stage in *Stylonychia*. Individual chromosomes are no longer discernible, although each of the swollen compartments is interpreted to contain material from what had been, at an earlier stage, an individual band of a polytene chromosome. $\times 10,900$.



partmentalized" condition for periods ranging from 8–14 hr in *E. woodruffi* to 24–30 hr in *Stylonychia*. During most of this time the contents of the nuclear compartments remain rather finely granular, in the case of the *Euplotes* species (Fig. 8), and more coarsely granular in *Stylonychia*. Near the end of this period, condensations of dense material appear within many of the compartments (Fig. 9). The fibrous material comprising the lamellar partitions subsequently disperses and disappears as the anlagen continue to elongate. Each nucleus at this stage then contains a number of small dense chromatin masses embedded in a granular nucleoplasm (Figs. 10 and 11). Nucleolar bodies are first seen clearly at this time, although the possibility remains that these bodies, which are characteristic of the vegetative macronucleus (19), begin to form at an earlier point in the scheme of anlagen development.

The number of chromatin granules and nucleoli then increases as the macronuclei undergo an additional rise in DNA content, this time by means of replication bands of the type seen in vegetative macronuclei (19). After a number of pairs of replication bands have passed through it, the anlage is indistinguishable from a mature macronucleus (Fig. 12). In confirmation of earlier work with many ciliates (cf. Raikov, 13), chromosomes are never seen during the course of the final DNA synthetic phase.

DISCUSSION

There are two features of macronuclear development in these hypotrichs that seem particularly puzzling. The first is the finding (1, 18) that the DNA build-up in the anlage occurs in two steps: initially by an apparent polytenization of prominent chromosomes, and later, following a period of DNA breakdown, by means of replication bands similar to those found in vegetative macronuclei.

The initial polytenization might be thought to occur because of a large requirement for RNA synthesis during early macronuclear development. However, Ammermann (2), was unable to detect

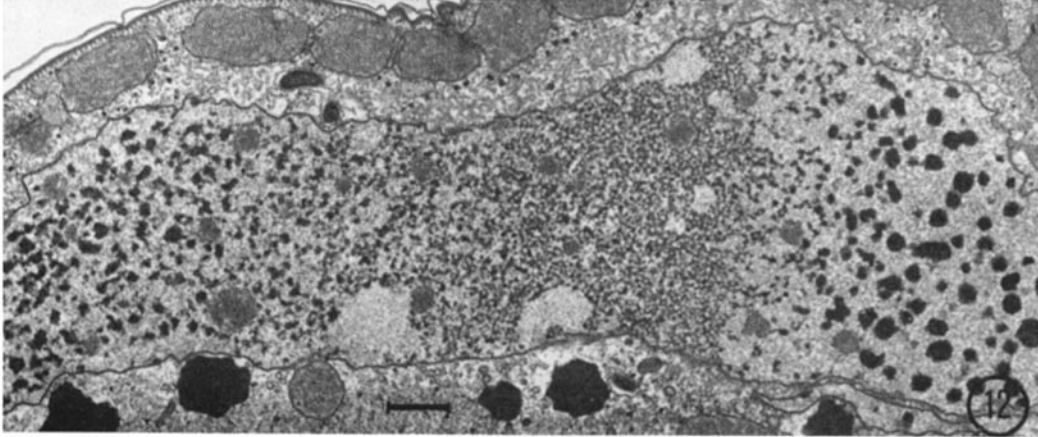
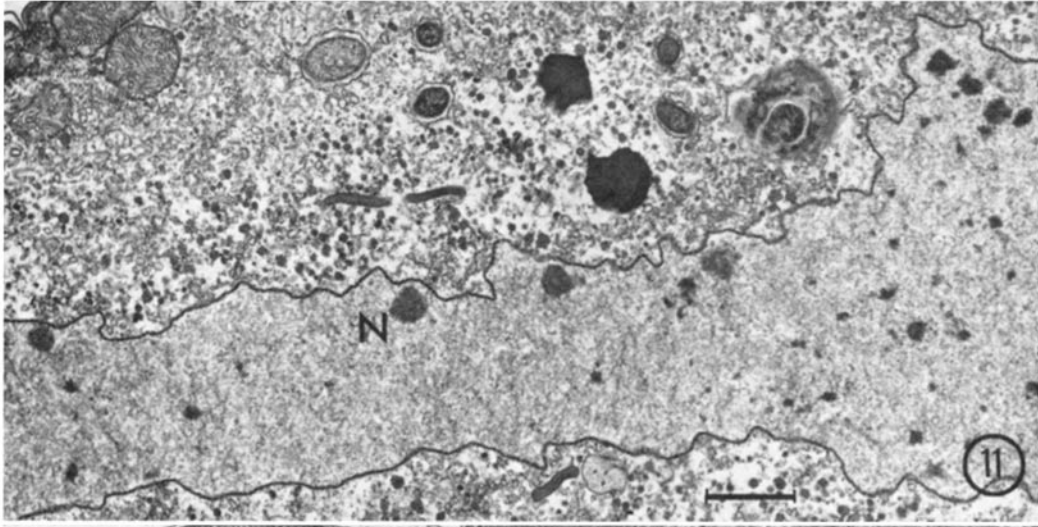
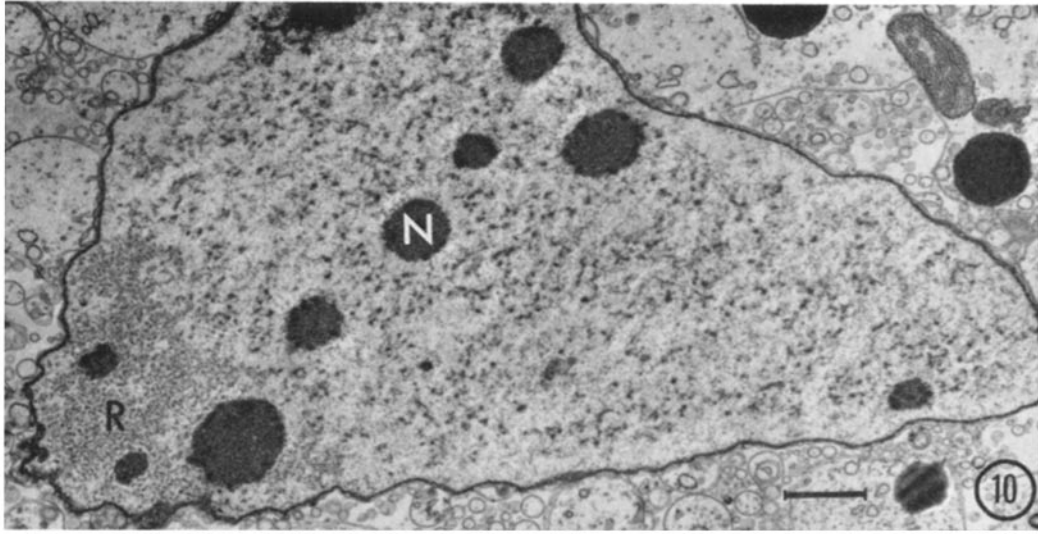
RNA synthesis within the developing anlagen of *Stylonychia mytilus* during the polytene stages, while Rao (16) and Rao and Ammermann (18) could demonstrate only low levels of anlagen RNA labeling in radioautographs of *Euplotes woodruffi* and *E. eurytostomus* exconjugants at similar stages of macronuclear development. Moreover, it was demonstrated (17, 18) that the limited RNA incorporation detected in the case of *Euplotes* was not restricted to certain bands (puffs) as in dipterans. Thus the polytenization does not appear to enhance the RNA-synthetic capacity of the macronuclear anlagen.

The second remarkable feature of macronuclear development in *Euplotes* and *Stylonychia* is the "partitioning" of the polytene chromosomes and the "compartmentalization" of the anlagen during the DNA-breakdown and DNA-poor stages demonstrated in the electron micrographs presented here. Within a single thin section there may be hundreds of separate intranuclear chambers. Thus the entire anlage must contain several thousand compartments, many more than would be present if each corresponded to a (diploid) "subnucleus" of the type proposed by Sonneborn (21). To reinforce the notion that these compartments should not be thought of in terms of subnuclei, one need only realize that they are present in later anlagen in which the *entire* DNA content is equivalent only to approximately the diploid amount, at least in the case of *Stylonychia* (1).

Ringertz (19) has estimated that the mature macronucleus of *Euplotes* contains 11,000–23,000 chromatin granules. This is the same order of magnitude as the number of separate chambers in the compartmentalized macronuclear anlage, suggesting that the core or "nucleus" of a chromatin granule may form within each compartment (Fig. 9). If the origin of the intranuclear compartments is traced back to the partitioning of discrete chromosomes, it becomes reasonable to propose that the material contained in a mature chromatin granule may represent a rather restricted portion of the entire genome, i.e., a portion that had been

FIGURE 8 Higher magnification view of the compartmentalized macronuclear anlage following polytene chromosome breakdown in *E. eurytostomus*. $\times 37,000$.

FIGURE 9 Macronuclear anlage of *E. woodruffi* as it begins to elongate just prior to breakdown of the intranuclear partitions. Note the condensations of dense material that have formed within many of the compartments. $\times 11,600$.



localized in a single band of a polytene chromosome. However, it has been noted (19) that the number of chromatin granules fluctuates greatly during the passage of replication bands through the macronucleus. Thus it may be more realistic to think of the chromatin granules as transient rather than static structures (19, 23).

It is apparent that additional information on the molecular events occurring during macronuclear development is needed to clarify the relationship between the structural features described here and the organization of the chromosomal material in the mature macronucleus. For example, the idea that the lamellar partitions function to segregate different gene sequences during the DNA-breakdown stage is based at present solely on morphological evidence. It is not yet known whether the linear integrity of the DNA comprising the polytene chromosomes is actually interrupted by these partitions. Nevertheless, it can be noted in this regard that when acetic acid spreads are made of *Euplotes* during the earlier stages of exconjugant development, long banded chromosomes are clearly seen with the light microscope. At later stages, similar preparations reveal that the anlagen are full of apparently unconnected granules, indicating that the chromosomes have indeed fragmented. Further verification of this point will be sought by electron microscopic examination of the contents of compartmentalized anlagen that are isolated and lysed on an air-water interface, as performed on isolated *Paramecium* macronuclei by Wolfe (23).

The nature of the material comprising the intranuclear partitions is not known at present. At the thinnest (presumably the closest to true cross-section) each of the partitioning lamellae is approximately 100 Å in thickness; this dimension is difficult to determine precisely because of the diffuse

borders of the partitions. These lamellae are presumed to be protein, although the results of cytochemical tests currently in progress will be needed to confirm this.

Present theories of macronuclear structure (cf. 13) assume that the "chromosomes" supposed to be present in the macronucleus are somehow linked together in haploid or diploid sets ("composite chromosomes," 7; "subnuclei," 21) in order to achieve a "segregation of genomes" during macronuclear division, and prevent the loss of chromosomes through random segregation. A mechanism by which chromosomes might be held together in sets is not known. Similarly, if there is a fragmentation of the polytene chromosomes accompanying DNA breakdown, as the evidence presented here suggests, presumably there must be a mechanism of organizing the fragments in such a way as to prevent the loss of genetic elements during amitosis. Although DNA-containing fibrils are sometimes seen with the light microscope in vegetative macronuclei, and are often interpreted as chromosomes (13), Wolfe (23) has shown in electron microscopic whole mounts that these fibrils may actually be granules of chromatin interconnected in a rather complex network.

It has been proposed that the DNA loss in *Euplotes* and *Stylonychia* anlagen may be genetically selective (9, 17, 18). Ammermann (1) found that there is approximately a diploid amount of DNA remaining in the anlage following the destruction of the polytene chromosomes in *Stylonychia*. However, this result could be fortuitous, since a diploid amount of DNA is not necessarily synonymous with a complete diploid genome. Recent evidence (18) indicates that *Euplotes woodruffi* does not reach a "second diploid" stage during macronuclear development; rather, the DNA content of the macronucleus is reduced temporarily to about half the

FIGURES 10-12 Later stages of macronuclear development, following dissolution of the intranuclear partitioning layers.

FIGURE 10 Elongating *Stylonychia* anlage, showing many small chromatin granules and a few large nucleolar bodies (*N*). A replication band (*R*) is seen in one corner of the nucleus. $\times 10,300$.

FIGURE 11 Elongate anlage of *E. woodruffi*, containing relatively few chromatin granules and a small number of nucleoli (*N*). $\times 11,400$.

FIGURE 12 Later anlage of *E. woodruffi*, after several pairs of replication bands have passed through it (one of these is seen in the center of this section). Dense chromatin granules and less densely staining nucleoli have greatly increased in number; the morphology is very similar to that seen in mature vegetative macronuclei. $\times 8000$.

amount present at peak polyteny, indicating that several times the diploid DNA content remains. Certainly it is difficult to understand why *Stylonychia* exconjugants should synthesize the extensive amount of DNA involved in the production of polytene chromosomes, and then degrade the excess DNA to produce a nucleus genetically identical to that present before the polytenization began. If it proves to be correct that the intranuclear septa do indeed isolate individual bands of the giant chromosomes, the many nuclear compartments thus produced could provide the structural basis for a differential breakdown of portions of the polytene genome. Certain regions of some chromosomes might be preferentially maintained in enhanced ploidy, while other regions could be partially or completely eliminated. The morphological differences in the material seen within the nuclear compartments shortly before the partitioning layers disperse and the first replication bands appear (Fig. 9) may indicate that such a selective process has occurred during the period of DNA degradation. As an obvious result of such a process, the DNA of the mature vegetative macronucleus would *not* be genetically identical to that of the micronucleus (and polytene anlage) from which the macronucleus is derived. Although the ciliate macronucleus is generally assumed to contain multiple sets of the complete micronuclear genome (7, 11, 13, 21), there is little direct evidence to support this assumption. Furthermore, since ciliate micronuclei and macronuclei are regarded as analogous to the germ nuclei and somatic nuclei in multicellular organisms (11, 13), a precedent for this hypothesis of selective DNA loss during macronuclear development is provided by the phenomenon of chromatin diminution in the somatic cells of such organisms as *Ascaris* and the sciarid and cecidomyid flies (22).

At the present one can only speculate as to how widespread this phenomenon of macronuclear anlage compartmentalization may be, since in only one other case has the fine structure of a developing ciliate macronucleus been reported. Jurand et al. (8) examined the postconjugant development of *Paramecium aurelia*, and at no stage were intranuclear partitions found within the anlage. This may be correlated with the microspectrophotometric data of Woodard et al. (24), who found that the DNA content of the macronuclear anlage in this species increases steadily through the first three postconjugant divisions, rather than showing the biphasic DNA build-up reported for *Stylonychia*

and *Euplotes*. Similarly, Seshachar (20) reported a single phase of macronuclear DNA increase following conjugation in the primitive holotrich *Chilodonella*. Thus it appears that the more elaborate mode of macronuclear development described here may be restricted to the spirotrichs. It may be no coincidence, therefore, that it is only in this group that the appearance of polytene chromosomes during macronuclear development has been reported, i.e., both in the heterotrich *Nyctotherus* (6) and in the hypotrichs used in the present study.

Raikov (13) has pointed out that an "achromatic" stage (i.e., weak Feulgen stainability of the anlage) following an initial polyploidization is a rather consistent feature of macronuclear development in ciliates. This may be due in some cases to an extreme despiralization and dilution of the chromatin elements within the enlarging nucleus; nevertheless, it seems very likely that if more spirotrichs were given careful cytophotometric study during this stage, many more instances of DNA loss would be revealed, perhaps accompanied by the distinctive morphological features found in *Euplotes* and *Stylonychia*.

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