

Macronuclear Duplication in the Ciliated Protozoan *Euplotes**

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ABSTRACT

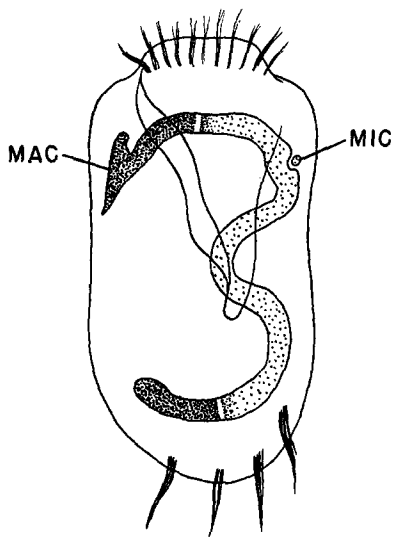
The ribbon-like macronucleus of *Euplotes eurystomus* pinches in half amitotically at each cell division. Several hours before the actual division two lightly staining duplication bands (reorganization bands) appear at the ends of the nucleus and approach each other slowly, finally meeting near the middle. Distal to the bands, that is, in regions through which the bands have already passed, the concentration of DNA (Feulgen) and "histone" (alkaline fast green) is greater than in the central zone. These facts suggest the hypothesis that DNA-histone synthesis takes place in a sequential fashion starting at the tips of the nucleus and proceeding to the middle. That this hypothesis is correct is shown by autoradiographic and photometric observations. Tritium-labelled thymidine is incorporated only in a limited region immediately distal to the bands. The average amount of Feulgen dye bound by the nucleus rises as the duplication bands approach each other, and is double the presynthesis value by the time the bands meet. A similar rise in the alkaline fast green dye is seen in duplicating nuclei, although no completely post-synthesis values were obtained in this study. The quantitative data are consistent with the assumption that the macronucleus contains a number of DNA-histone "units," presumably chromosomes, each of which duplicates once and only once.

No other animals display such a diversity of nuclear phenomena as the Protozoa, and consequently, this group has long held the attention of cytologists and geneticists alike. A persistent problem concerns the nature of the ciliate macronucleus which, as is well known, plays an important role in the determination of the phenotype (29) and yet divides "amitotically." The present study deals with the mechanism of macronuclear duplication in the hypotrich ciliate, *Euplotes eurystomus*, and gives particular attention to the synthesis of deoxyribose nucleic acid (DNA) and histone. As we shall see, amitosis in this case follows an exceedingly precise course (*cf.* reference 18).

In common with other ciliates *Euplotes* possesses an inconspicuous micronucleus and an enormous, densely staining macronucleus. In this form the macronucleus is a C- or W-shaped band

extending mainly along one side of the dorso-ventrally flattened body (Text-fig. 1). Cell division involves a transverse fission of the body and is preceded by an intranuclear mitosis of the micronucleus. The macronucleus, on the other hand, pinches in half amitotically. Several hours prior to cell division the macronucleus undergoes remarkable changes in structure and staining characteristics, which are usually referred to collectively as "reorganization." The beginning of reorganization is indicated by the appearance at each end of the nucleus of a narrow, transverse band which stains weakly with the usual nuclear dyes. Over a period of several hours the two "reorganization bands," as they are called, move slowly toward one another and finally meet somewhere near the middle of the nucleus (Figs. 1 to 7). Before reorganization the nuclear contents are finely punctate and moderately basophilic, but distal to the reorganization bands, that is, in those parts of the nucleus through which the bands have already passed, the chromatin is coarsely granular and stains

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TEXT-FIG. 1. Oral or ventral view of *Euplotes eurystomus*, showing the small micronucleus (MIC) located in a notch in the W-shaped macronucleus (MAC). Near the ends of the macronucleus may be seen two duplication bands ("reorganization bands") that slowly move toward the middle. The regions distal to the bands have completed the duplication of DNA (Feulgen) and histone (alkaline fast green).

intensely with basic dyes. These general features have been known for many years, an excellent account being that of Turner (35). The species described by Turner, although called *Euplotes patella*, was in all probability the same used in this study, namely *E. eurystomus* (23). In any case macronuclear reorganization bands are found throughout the hypotrichs (30), and only minor differences would be expected among different species of the same genus.

The significance of the reorganization bands has remained obscure, despite two clues which, in retrospect, give the story away: (a) reorganization invariably precedes fission, and (b) it is accompanied by an over-all increase in basophilia. These facts suggest the simple hypothesis that *DNA duplication takes place in a sequential fashion starting at the tips of the nucleus and proceeding toward the middle*. Evidence of two sorts, photometric and autoradiographic, will be presented to demonstrate the essential correctness of this hypothesis. The observations show that the DNA content of the macronucleus doubles during reorganization and that DNA synthesis takes place only at or near the reorganization bands.

Furthermore, there is reason to believe that a basic protein component, presumably histone, is duplicated simultaneously with the DNA.

Materials and Methods

Stocks.—Cultures of *E. eurystomus* were obtained originally from the General Biological Supply House, Chicago; later Dr. Francis Yow kindly supplied specimens. They were maintained in timothy hay infusions in mixed culture with *Aerobacter aerogenes* and *Chilomonas* which served as food organisms.

Autoradiography.—Tritium-labelled thymidine was purchased from Schwarz Laboratories, Mount Vernon, New York. In the isotope experiments it was added directly to the culture medium in amounts ranging from about 1 to 10 $\mu\text{c./ml}$. Two different lots of thymidine were used, one having a specific activity of 118, the other 360 $\mu\text{c./mm}$. After varying times in the isotope solution the animals were concentrated by centrifugation. In some cases they were allowed to dry directly from the living state on conventional microscope slides. In others they were fixed a few minutes in 4 per cent formaldehyde buffered to pH 7.0 or in Carnoy's mixture of 3 parts ethanol and 1 part acetic acid, and squashed beneath a coverslip. The coverslips were removed by the dry ice technique (9). Some slides were next stained by the Feulgen reaction using a 5 minute hydrolysis in 1 N HCl at 60°C. followed by about an hour in leucofuchsin; in other cases staining was deferred until after development of the autoradiographs. The slides were covered with autoradiographic stripping film AR-10 (Kodak, Ltd.) according to the technique of Doniach and Pelc (10). Exposures were for approximately 2 weeks, the slides being kept in a refrigerator during this time. Further processing of the Feulgen slides followed the instructions given by Taylor (33). When staining was carried out after development of the films the following schedule was used. The films were hardened as usual and treated to remove excess fixer, after which they were washed briefly in distilled water and allowed to dry thoroughly. Next they were placed in azure B at pH 4 (13) for about 30 minutes, rinsed quickly in pH 4 buffer, and placed in distilled water just long enough to remove the stain from the film. Once again the preparations were air-dried. Such slides may be observed by placing immersion oil directly on the film, or they may be passed through xylene and mounted under a coverslip as usual.

Photometry.—Slides were stained by the Feulgen reaction for DNA following the details given by Swift (31), and by the alkaline fast green technique for histone described by Alfert and Geschwind (3). In both cases the specimens were mounted in an oil of refractive index 1.568 in order to reduce scattered light. Estimates of the dye content of the nuclei were made with a microspectrophotometer modelled after that of Pollister and Ris (24). Basically it consists of a tungsten filament as

source, a Bausch and Lomb grating monochromator with a 38.8 mm. exit lens, a 0.25 N.A. objective used as the microscope condenser, and a 1.25 N.A. objective projecting an enlarged image of the specimen onto a photocathode of the 1 P21 type. Aperturing of the measured area was carried out at the plane of the final image. The Feulgen dye was measured at 532 $m\mu$, the fast green at 625 $m\mu$.

Estimation of the dye content of the nucleus seemed at first to pose serious problems due to the large and irregular shape of the nucleus and particularly to the noticeably inhomogeneous distribution of dye. Assuming the absorber to follow the Beer-Lambert relationship, the total dye content could be calculated by summing the extinctions from a large number of very small regions in the nucleus. Unfortunately the instrument available for these measurements was not sufficiently versatile for such an integrating technique. As an alternative, the specimens were squashed as thoroughly as possible, thereby reducing the previously cylindrical nucleus to a thin ribbon, and causing flattening and coalescence of the larger granules. Such a procedure results in a considerably more homogeneous specimen with transmissions in the favorable range of about 0.3 to 0.7. Twenty transmission measurements were then made along the length of each nucleus, care being taken to space the readings so that equal areas in the projected image of the nucleus received approximately equal numbers of readings. The mean of these twenty readings was used as an estimate of the average transmission of the nucleus. Relative areas were obtained by printing pictures of the measured animals at a constant magnification, cutting out the nuclei, and weighing them. The dye content (in arbitrary units) was then obtained by multiplying the area of a nucleus by its extinction, calculated as the $-\log$ of the average transmission.

The above procedure results in values uncorrected for errors introduced by inhomogeneous dye distribution, and so is directly comparable to the "plug" method commonly used with smaller nuclei (32). Errors will be small if (a) the twenty individual readings are included within a small range, and (b) the dye distribution within each area is relatively uniform. The magnitude of the first error is readily estimated by taking the $-\log$ s of the twenty readings individually and averaging these instead of the transmissions. Such a corrected extinction was calculated for several of the most inhomogeneous nuclei and in no case did the corrected value differ by more than 2 or 3 per cent from the uncorrected. Since the sampling error in estimating the average transmission is itself probably this great, the extra computational work was dispensed with. The second source of error could be circumvented by using the two wavelength method described by Ornstein (20) and Patau (21). Several specimens were tested for inhomogeneity of dye distribution by this method; in only a few individual measurements did the dye distribution cause

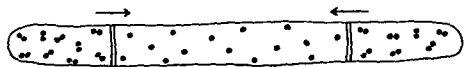
errors of more than a few per cent in the estimated transmissions.

RESULTS

Autoradiography.—The general question to which we seek an answer is the following: Does DNA synthesis take place throughout the entire macronucleus at once, or is it confined to an area near the reorganization band? Since the pattern of precursor incorporation should give rather direct evidence on this point, animals were grown for varying periods in solutions of H^3 -labelled thymidine.

In the first experiment rapidly growing animals were placed for 8 hours in culture medium to which was added 5.5 $\mu c.$ H^3 -thymidine per ml. (specific activity 118 $\mu c./mm$). They were squashed in acetic-alcohol, stained by the Feulgen reaction, and covered with autoradiographic film. In the developed autoradiographs, activity was found only in those organisms possessing reorganization bands, and in every case activity was limited to the region distal to the bands (15). Typical nuclei from this group are shown in Figs. 8 to 10. Thymidine is generally assumed to be a specific precursor for DNA (11, 14, 25), although at least one report (8) suggests that it may be diverted into RNA. In the present case, however, the activity is not in RNA, since this compound is removed by the Feulgen hydrolysis. In subsequent experiments slides were treated with boiling 5 per cent trichloroacetic acid for 15 minutes to remove both types of nucleic acid, and in these cases no radioactivity was bound in any organism.

The first experiments establish a relationship between the reorganization band and thymidine incorporation. However, they do not tell us whether incorporation occurs *only* at the reorganization bands, or whether it continues for an indefinite period throughout the entire distal zone through which the band has passed. Shorter term experiments were therefore run, once again using thymidine as precursor. Animals were left for periods of 2, 3, and 4 hours in the isotope solution and autoradiographs made as before. In these shorter experiments many animals were found in which the radioactive zone extended from the reorganization band for only a short distance into the distal area (Figs. 11 and 12). In general the shorter radioactive zones were found in animals left the shorter periods in thymidine. We may conclude, therefore, that incorporation occurs only at or near the reorganization band and does



TEXT-FIG. 2. Diagram of the duplication scheme proposed for the macronucleus of *Euplotes*. The nucleus is assumed to consist of a large number of "units" which duplicate in a sequential fashion starting at the ends of the nucleus and proceeding toward the middle. It is easily shown that under these circumstances g , the total number of units in the nucleus at any moment is related to g_0 , the number of units before the onset of duplication, and r , the fraction of units in the duplicated zones. Thus, $g = g_0 \left(1 + \frac{r}{2 - r} \right)$. The test of this proposal consists of a comparison between the experimentally determined dye contents of duplicating nuclei and the predicted contents, based on the fraction of dye in the duplicated portion (Text-figs. 3 and 6).

not persist in regions through which the band has already passed. As can be seen most clearly in Figs. 9 and 12, the lightly staining reorganization band itself is not radioactive. It is not clear in these experiments whether *Euplotes* takes up the precursor from solution, incorporating it directly into its own nucleic acid, or whether it utilizes radioactive nucleic acids derived from the bacteria and other microorganisms upon which it feeds.

DNA (Feulgen). Photometry.—It is commonly assumed that precursor incorporation occurs only during the duplication of DNA, and this assumption is supported by a great deal of experimental evidence. A word of caution, however, has recently been sounded by Pelc (22) who finds incorporation of adenine into DNA of mouse seminal vesicle nuclei at a time when mitosis and endopolyploidy are not demonstrable. In the case of *Euplotes*, however, we can show that the incorporation of thymidine into DNA is directly correlated with a doubling of the DNA content of the nucleus.

In order to establish a baseline for the study of DNA increase during reorganization, the Feulgen dye content was measured in two sets of nuclei: (a) a group of twelve prior to the appearance of reorganization bands, and (b) a group of 5 in which the bands had already met in the middle. Unfortunately, cells of the latter type are quite rare, since the meeting of the reorganization bands is followed rapidly by macronuclear division and cytokinesis. The dye content in arbitrary units of the nuclei prior to reorganization was 33.4 ± 0.8 (mean \pm standard error of the mean) and for those

after reorganization was 71.5 ± 2.2 . Dividing the second mean by 2, we obtain 35.8 ± 1.1 , which does not differ significantly from the first mean ($t = 1.2$; $p > 0.2$). We can safely say, therefore, that reorganization is accompanied by a doubling of the mean Feulgen dye content, presumably as a reflection of a similar doubling in DNA content. It should be remarked that no independent checks have been made in this study on the validity of Feulgen staining for measurements of relative DNA contents (32). However, the values just reported were obtained from nuclei of quite diverse size and shapes, and differing by factors of 2 to 3 in average extinction (*cf.* Figs. 1, 6, 7). It is perhaps easier to suppose that the dye binding is proportional to the amount of DNA than to postulate compensating errors to account for the rather precise numerical relationships found. Nevertheless, without an independent measure one cannot distinguish errors due to differential dye binding from true variations in DNA content.

Next, a series of fifteen nuclei in various stages of reorganization were measured. The average extinctions in the two regions distal to the bands and in the central zone were measured separately and a dye content calculated for each (*cf.* sample calculation in Table I). When the total dye content was estimated for these nuclei, all but two gave values falling between the previously calculated means for pre- and postreorganization (Text-fig. 3). The two exceptional nuclei had almost completed reorganization, and these yielded values slightly higher than the postreorganization mean. Clearly, then, the Feulgen dye measurements substantiate the hypothesis that reorganization is closely linked to DNA duplication. Throughout the remainder of the paper the term "reorganization band" will therefore be replaced by the more descriptive expression "duplication band."

We have seen that the mean dye contents of nuclei before and after DNA synthesis are in the ratio of 1:2. Furthermore, the spread of individual readings is relatively small, the standard deviation amounting to about 7 per cent. We may reasonably assume that the readings for nuclei in the process of duplication are subject to about this same spread, since all measurements were made under the same conditions. With this degree of accuracy we are encouraged to analyze the data somewhat more closely to gain insight into the course of DNA synthesis.

Let us first set up a simple hypothesis regarding

TABLE I

Sample Data Sheet to Show the Calculation of Observed and Predicted Dye Contents from the Photometric Data and the Morphological Features of the Macronucleus

	Anterior duplicated zone	Middle non-duplicated zone	Posterior duplicated zone
1/Average transmission: 1/T.....	2.70	1.45	2.72
Extinction: log 1/T.....	0.432	0.162	0.434
Area (weight of cutout in mg.): <i>a</i>	22.5	137	30.0
Length (inches on photograph): <i>l</i>	0.9	5.3	1.2
Dye content: <i>a</i> × log 1/T.....	9.7	22.1	13.0

(a) Total dye content of macronucleus: $44.8 = \frac{44.8}{34.1} g_0 = 1.31 g_0$ in which $g_0 = 34.1$, the average dye content before duplication.

(b) Predicted dye content from *r*:

$$r = \frac{\text{Dye in duplicated zones}}{\text{Dye in whole nucleus}} = \frac{9.7 + 13.0}{44.8} = 0.507$$

$$\left(1 + \frac{r}{2 - r}\right)g_0 = 1.34 g_0$$

(c) Predicted dye content from areas:

$$x = \frac{\text{Area of duplicated zones}}{\text{Total area}} = \frac{22.5 + 30.0}{190} = 0.28$$

$$(1 + x)g_0 = 1.28 g_0$$

(d) Predicted dye content from lengths:

$$x = \frac{\text{Length of duplicated zones}}{\text{Total length}} = \frac{0.9 + 1.2}{7.4} = 0.28$$

$$(1 + x)g_0 = 1.28 g_0$$

DNA synthesis in the macronucleus, one which is consistent with what is now known of DNA and chromosome replication. We will assume that the macronucleus contains a very large number of DNA units, which for the present argument could as well be molecules, chromosomes, or whole genomes. At any moment during DNA synthesis the nucleus will consist of a central region in which none of the units have replicated, and of two regions distal to the duplication bands in which each unit has replicated once and only once (Text-fig. 2). Let us say that there are g_0 units before synthesis begins and that during synthesis x is the fraction of units which have already duplicated and $1 - x$ the fraction which have not. We may therefore write,

$$g = 2g_0x + g_0(1 - x) = g_0(1 + x) \quad (1)$$

in which g is the total number of units in a nucleus at any time. We will arbitrarily set $g_0 = 34.1$, which is the weighted mean of 33.4 (pre-

duplication dye content) and 35.8 ($1/2$ postduplication content). The value of x could be estimated from the dye content of either the duplicated, $2g_0x$, or non-duplicated, $g_0(1 - x)$, parts of the nucleus alone; for reasons to be discussed shortly, we shall calculate it from the fraction of dye in the duplicated zones. Thus,

$$r = \frac{\text{Duplicated}}{\text{Whole}} = \frac{2g_0x}{g_0(1 + x)} = \frac{2x}{1 + x}$$

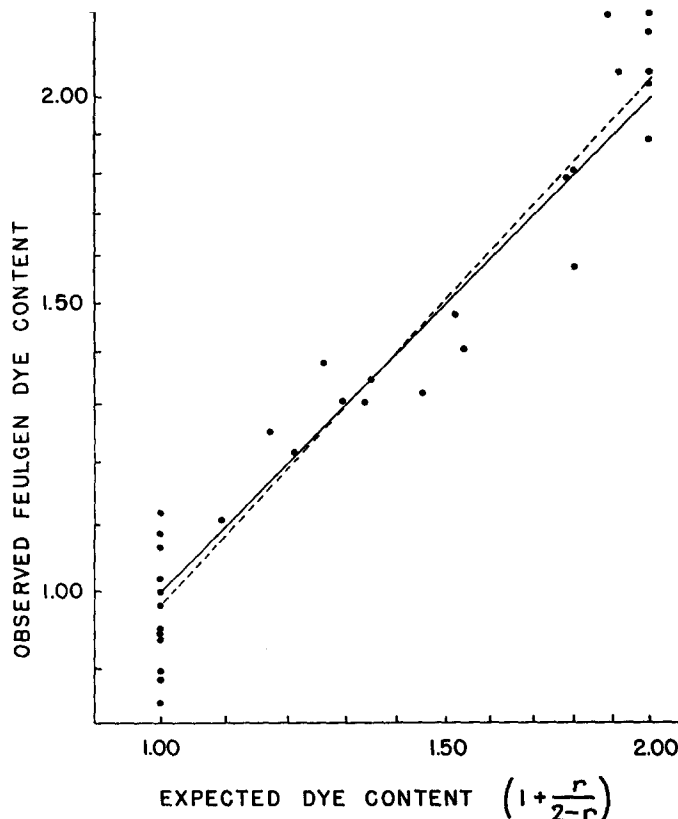
Solving for x ,

$$x = \frac{r}{2 - r}$$

Finally, substituting this value of x in equation (1) we obtain

$$g = g_0 \left(1 + \frac{r}{2 - r}\right) \quad (2)$$

Knowing r , then, we can readily calculate the total amount of dye expected on this theory.

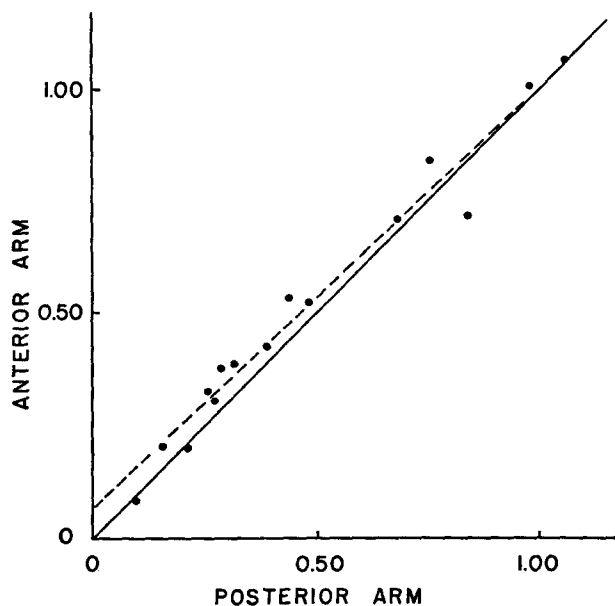


TEXT-FIG. 3. Plot of observed Feulgen dye contents of *Euplotes* macronuclei against expected contents $\left(1 + \frac{r}{2-r}\right)$. The expected contents are predicted from the fraction of dye in the duplicated zones according to the scheme shown in Text-fig. 2. The dashed line is the regression of observed on expected for the fifteen nuclei undergoing duplication. It does not differ significantly from the expected (solid) line. Measured preduplication and postduplication dye contents are included at the expected values of 1.0 and 2.0, respectively.

Values of r for the fifteen duplicating nuclei were estimated from the fraction of Feulgen dye in the distal zones. These values were then used to calculate $\frac{g}{g_0} = 1 + \frac{r}{2-r}$. In determining expected dye contents, $\frac{g}{g_0}$ has been used rather than g so that values for all nuclei will lie between 1.0 and 2.0. In Text-fig. 3 the expected values have been plotted against the observed Feulgen dye contents (arbitrary units divided by g_0). The regression of observed on expected has been calculated and is shown as the dotted line. It has a slope of 1.08 and passes through the points (1.00, 0.98) and (2.00, 2.05). A perfect fit of observed to expected would of course have a slope of 1.00 and pass through (1.00, 1.00) and (2.00, 2.00). Statistical analysis shows that the calculated

regression line does not differ significantly from the expected line in respect to slope ($t = 0.62$; $p \sim 0.5$), nor does the mean of the observed values differ from the mean of the expected ($t = 0.48$; $p > 0.6$).

It is interesting to note that the expected values are probably better estimates of the extent of DNA duplication than the observed dye contents, even though both are based on the same photometric readings. In the first place, values of $1 + \frac{r}{2-r}$ cannot under any circumstances be less than 1 or greater than 2; such is not the case for the observed photometric data, which may vary outside these limits. Secondly, the value of r may be closely estimated from inaccurate data if the experimental errors are of certain systematic types. Imagine, for instance, a group of nuclei in all of



TEXT-FIG. 4. Comparison of the DNA (Feulgen) contents of the anterior and posterior duplicated zones in fifteen macronuclei undergoing duplication. The regression of anterior on posterior is shown as the dashed line; equivalence of dye content in the two zones would give points along the solid line. The two lines do not differ significantly in slope ($t = 1.37$; $p = 0.2$), but the anterior values are perhaps slightly higher than the posterior ($t = 2.34$; $0.02 < p < 0.05$).

which a certain fraction of DNA has duplicated, but in which the total dye contents vary because of differential dye binding (or to actual differences in individual g_0 values). The *ratio* of the dye contents in the duplicated and unduplicated parts of the nucleus might, nevertheless, be unaffected and the function $1 + \frac{r}{2-r}$ be a good measure of the extent of DNA duplication. The data for fast green binding of *Euplotes* nuclei, which will be presented shortly, suggest just this situation. Or suppose that the nuclei have been squashed to different degrees. The photometric measurements, for obvious practical reasons, cannot be taken too near the edge of the nucleus. Therefore, in the more poorly squashed cylindrical nuclei the measured extinction values will be too high. However, they will be high in both the duplicated and unduplicated parts of the nucleus, resulting in a better estimate of r than of the total dye content. Similar considerations apply to inhomogeneous, but well flattened nuclei, in which the extinctions are low as a result of distributional errors. In short, the expected values are based upon a ratio, and systematic errors tend to cancel out in the calculation of ratios.

It is interesting to compare the dye content in the two distal regions of the same nucleus, as is done in Text-fig. 4. In these fifteen nuclei the waves of DNA synthesis are proceeding at almost the same rate from the ends of the nucleus, so that the amount of duplicated material is nearly the same in the two distal zones. This fact is rather striking in view of the clear morphological differences between the ends. As shown in Text-fig. 1, the typical nucleus of *Euplotes eurytostomus* is actually a shallow W, one end being rounded, the other shaped somewhat like an arrowhead. An asymmetry is also indicated by the position of the micronucleus, which is regularly imbedded in the bend of the W next to the pointed end. Since the preceding measurements were made, some individuals have appeared which have only a single duplication band proceeding from the pointed end of the nucleus. It is not yet clear whether these traverse the whole nucleus or whether a second band starts belatedly from the other end.

The discussion so far has concerned only the amount of DNA in the duplicated and unduplicated parts of the nucleus and not the concentrations within these areas. However, the observation prompting this study was that regions distal to

TABLE II

Average extinction in the two distal duplicated zones	Extinction in central non-duplicated zone	Ratio of extinctions
0.382	0.158	2.42
0.210	0.117	1.79
0.322	0.140	2.30
0.433	0.162	2.67
0.292	0.177	1.65
0.335	0.152	2.20
0.295	0.179	1.65
0.292	0.152	1.92
0.342	0.154	2.22
0.246	0.097	2.54
0.279	0.151	1.85
0.279	0.146	1.91
0.281	0.119	2.36
0.612	0.309	1.98
0.598	0.286	2.09
Average.....		2.10

Comparison between the Feulgen dye extinctions in the duplicated and non-duplicated zones of 15 nuclei. Arranged in order of increasing total dye content. If these flattened nuclei are of constant thickness along their length, the extinctions are proportional to dye concentration. The data suggest a rough doubling in DNA concentration during duplication.

the duplication bands stain more intensely than the rest of the nucleus, suggesting that DNA synthesis might be accompanied by a doubling of DNA concentration, or at least by an increase in concentration not completely counterbalanced by a volume increase. That such a situation exists, at least to a first approximation, is suggested by the data in Table II. For each individual the average extinction of the two distal zones has been compared with the extinction of the central area. The ratios fall between 1.65 and 2.67 with an average of 2.10. If we make the reasonable assumption that these squashed nuclei are of uniform thickness along their length, we can say that the extinctions are proportional to dye concentration. The data suggest, therefore, that DNA duplication is accompanied by a rough doubling of the DNA concentration.

The over-all impression which one gains, both from the extinction data and from simple observation of the nuclei, is that the nucleus is a roughly uniform ribbon in which gross form changes do not occur until late in the process of duplication (Figs. 1 to 7). It should be possible, therefore, to predict the extent of DNA duplication simply

from the relative distance travelled by the bands or the relative areas of the distal zones. We will assume that x in equation (1), the fraction of DNA which has duplicated, can be estimated as follows:

$$x = \frac{l_1}{l_1 + l_2}$$

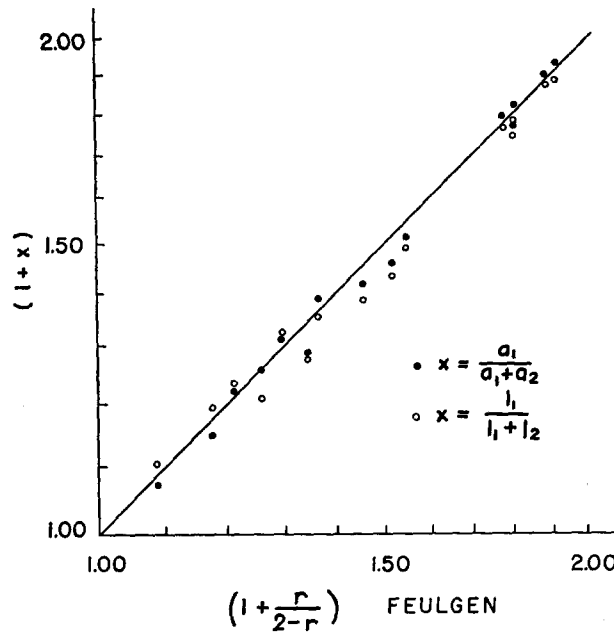
or

$$x = \frac{a_1}{a_1 + a_2}$$

in which l_1 and l_2 are the lengths of the duplicated and non-duplicated zones respectively and a_1 and a_2 their areas. Sample calculations are shown in Table I, and the values obtained from area and length measurements are plotted in Text-fig. 5 against the expected values previously determined from r . It is clear from the graph that the extent of DNA duplication can be reasonably well predicted from the morphological features of the nucleus alone. This fact will be of some value in the discussion of the histone content of the nucleus.

Alkaline Fast Green (Histone) Photometry.—Let us next consider the basic proteins associated with the DNA of the macronucleus. Numerous studies in the past have shown that DNA isolated from natural sources is combined with basic proteins, the histones, or in certain cases with the simpler protamines. We know very little about the association of these substances in the intact cell, although considerable progress has been made recently in elucidating the structure of isolated nucleoprotamine and nucleohistones (*e.g.* 12, 36). Staining procedures offer certain possibilities for studying the relationship between nucleic acid and protein at the cellular level. The staining technique used here to demonstrate basic nuclear proteins is that developed by Alfert and Geschwind (3). In this technique an acid dye, fast green, is used at pH 8.1. Most proteins do not bind appreciable quantities of acid dye at such an elevated pH, but histones do so. The factors which influence the degree of dye binding are numerous and in most cases obscure, so that extreme caution must be used in interpreting the staining data quantitatively.

Preliminary experiments showed that fast green staining of *Euplotes* macronuclei resembles Feulgen staining in that the regions distal to the duplication bands stain more intensely than the central zone (Fig. 3). It seemed likely that some



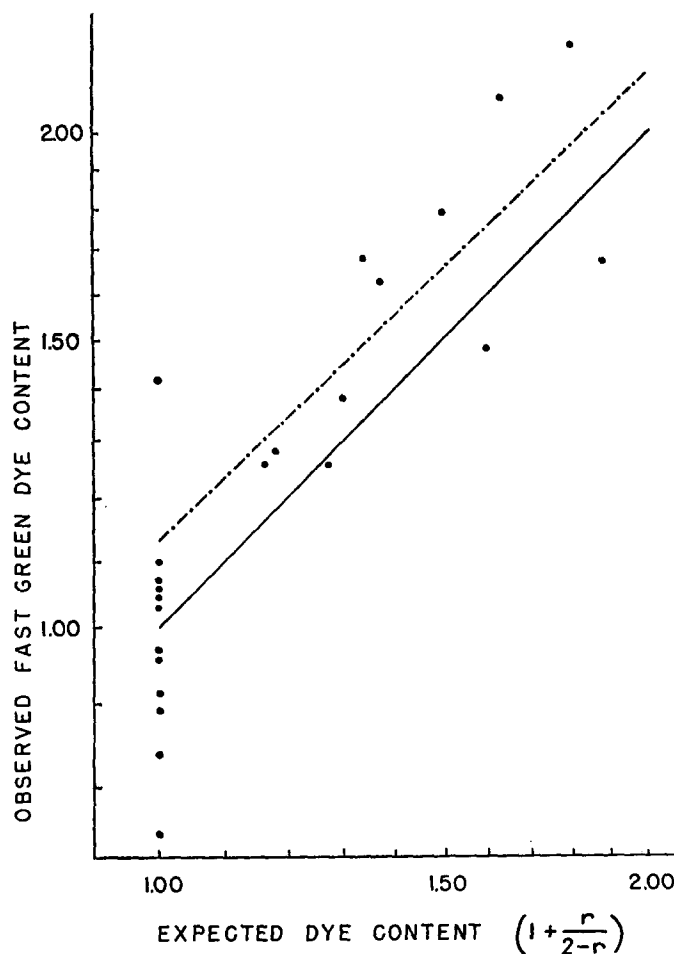
TEXT-FIG. 5. Plot of $\left(1 + \frac{a_1}{a_1 + a_2}\right)$ and $\left(1 + \frac{l_1}{l_1 + l_2}\right)$ against $\left(1 + \frac{r}{2 - r}\right)$ for the fifteen macronuclei undergoing duplication. These functions express the extent of DNA duplication predicted from (a) a_1 and a_2 , the areas of the duplicated and non-duplicated zones, (b) l_1 and l_2 , the lengths of these same zones, and (c) r , the fraction of Feulgen dye in the duplicated zone.

insight into the synthesis of the basic nuclear proteins might be gained by estimating the dye content photometrically. For convenience, we shall refer to "histone" in these nuclei, implying only that we are dealing with basic nuclear proteins stained by alkaline fast green.

A series of twelve nuclei, none of which possessed duplication bands, had a mean fast green dye content in arbitrary units of 41.0 ± 1.8 (mean \pm standard error of the mean). As seen in Text-fig. 6, the spread of values was about twice that for similar Feulgen measurements, the standard deviation amounting to about 15 per cent. There is no obvious reason that the fast green nuclei should be subject to more experimental error in photometry than the Feulgen nuclei, since the measuring technique was similar for the two sets of animals. It seems likely, therefore, that the actual fast green content varies somewhat more than the Feulgen content in a series of similar nuclei. How much of the additional variability is due to experimental factors and how much to real differences in histone content are not known. Differential dye extraction probably accounts for some spread of the data, since the fast green dye

can be removed rather readily from these nuclei. Distilled water and buffer at pH 8.1, both of which are used briefly after the specimens have been stained, extract some of the dye.

No individuals could be found in the stage immediately preceding macronuclear division, so that an estimate of fast green binding in completely duplicated nuclei was not obtained. However, it was soon found that individuals with duplication bands have a higher fast green content than those without. The fraction of dye in the distal regions was once again used in equation (2) to give an expected total dye content. In Text-fig. 6 the observed values $\left(\frac{\text{arbitrary units}}{41.0}\right)$ are plotted against the expected $1 + \frac{r}{2 - r}$ for eleven individuals and it can be seen that the points fall roughly along the theoretical line. The regression of observed on expected was calculated for the duplicating nuclei and is shown in the same figure. Statistical analysis shows that the slope of this regression line does not differ significantly from 1.00, the slope of the theoretical line ($t = 0.06$; $p > 0.8$). The regression line as a whole is some-

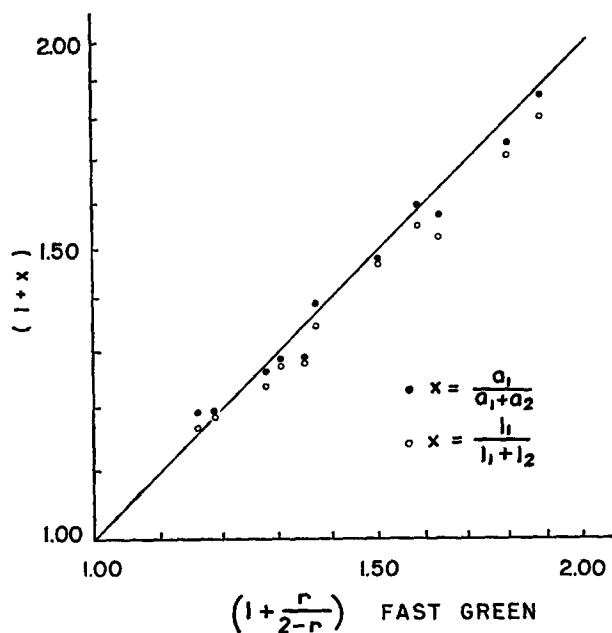


TEXT-FIG. 6. Plot of observed fast green dye contents against expected contents $\left(1 + \frac{r}{2-r}\right)$ where the expected contents are calculated from the fraction of dye in the duplicated zones according to Text-fig. 2. The dashed line is the regression of observed on expected for the eleven nuclei undergoing duplication. It does not differ significantly from the expected (solid) line. Measured preduplication dye contents are included at the expected value of 1.0.

what elevated but not significantly so ($t = 0.76$; $p > 0.4$ that the mean of the observed values is a random variant of the mean of the expected values).

We have already seen in the case of the Feulgen nuclei that the areas and lengths of the duplicated and non-duplicated nuclear zones can be used to predict $1 + \frac{r}{2-r}$ with a fair degree of accuracy. What, then, do measurements of the fast green nuclei show? In Text-fig. 7 the fast green data are plotted as before for the Feulgen data, that is, $1 + \frac{r}{2-r}$ has been plotted against

$1 + \frac{a_1}{a_1 + a_2}$ and $1 + \frac{l_1}{l_1 + l_2}$ in which r is the fraction of dye in the duplicated zones, a_1 and a_2 the areas, and l_1 and l_2 the lengths of the duplicated and non-duplicated parts of the nucleus respectively. The fit, as with the Feulgen data, is good. It seems likely that r is rather well estimated despite the variability of the fast green data. This would follow, as already discussed, if the factors which cause variability among individuals affect the two parts of a particular nucleus to the same extent. These factors are probably experimental, *e.g.* differential dye extraction, but similar results would be expected if each individual had a char-



TEXT-FIG. 7. plot of $\left(1 + \frac{a_1}{a_1 + a_2}\right)$ and $\left(1 + \frac{l_1}{l_1 + l_2}\right)$ against $\left(1 + \frac{r}{2 - r}\right)$ for the eleven macronuclei undergoing duplication. These functions express the extent of histone duplication predicted from (a) a_1 and a_2 , the areas of the duplicated and non-duplicated zones, (b) l_1 and l_2 , the lengths of these same zones, and (c) r , the fraction of fast green dye in the duplicated zone.

acteristic preduplication histone content different from that of others.

Taken together the data suggest that histone undergoes a duplication similar to that of the DNA, synthesis beginning at the ends of the nucleus and proceeding toward the middle. As yet, however, we do not have confirmatory evidence based upon the incorporation of labelled amino acids.

DISCUSSION

Apparently only one author has clearly stated the significance of reorganization among the hypotrichs. Sonneborn (29), writing in 1947, suggests that, "one may at least raise the question as to whether the 'progression' of the reorganization band may not represent a wave of mitoses passing through successive component subnuclei along the length of the macronucleus." It is now clear that during the process both DNA (Feulgen) and histone (alkaline fast green) undergo a doubling in amount. This doubling begins at the tips of the elongated macronucleus and proceeds sequentially toward the middle, so that at any instant only a small region at or near the duplication band is

actually synthesizing new material. The quantitative data, moreover, are consistent with a scheme whereby the nucleus is thought to contain a large number of units, each of which duplicates once and only once. This latter conclusion is quite understandable, if we assume that chromosomes retain their identity within the macronucleus. The results presented here are in agreement with those of McDonald (18), who recently showed that a doubling of DNA content occurs prior to macronuclear division in *Tetrahymena*.

It is generally believed that the protozoan macronucleus arises by some type of endomitotic process from a diploid micronucleus, and there is furthermore some reason to suppose that entire genomes remain intact during its formation. The evidence, though by no means conclusive, is based upon a variety of genetic and cytological observations. In the first place, the gigantic size of the macronucleus and its abnormally high DNA content bespeak a polyploid condition. The macronucleus of *Paramecium caudatum*, for instance, contains about 40 times the DNA of the micronucleus (19); and measurements made during the course of this study show that the ratio in

Euplotes eurystomus is about 200:1. As expected, the formation of the macronucleus is accompanied by a progressive increase in DNA content (28). In some cases, such as the suctorian, *Ephelota*, bundles of chromosomes are distinctly visible during the endomitotic replications (16). But perhaps the most spectacular case is the primary nucleus in the radiolarian *Aulacantha*, which contains over a thousand chromosomes readily visible in the living condition (16). An excellent summary of macronuclear cytology is to be found in Grell's recent volume, *Protozoologie* (16).

The genetic evidence for intact genomes or subnuclei within the macronucleus comes largely from the phenomenon of macronuclear regeneration in *Paramecium* (29). Here macronuclei with an apparently normal complement of genes arise from small fragments of a pre-existing macronucleus. Interestingly about $\frac{1}{20}$ to $\frac{1}{40}$ of a macronucleus in *P. aurelia* is sufficient for macronuclear regeneration, and we have already mentioned that the macronucleus of *P. caudatum* contains some 40 times as much DNA as the micronucleus. In many other cases macronuclei are normally formed by a process of budding, as for instance in the suctorian *Ephelota* (16). Presumably such buds contain at least one complete genome, although direct genetic evidence is not available to prove this assertion.

We will assume, therefore, that the macronucleus of *Euplotes* contains multiple genomes, or at least a large number of chromosomes. If we knew the spatial arrangement of these chromosomes, then presumably we could interpret the sequential character of macronuclear duplication in terms of chromosomal duplication. Lacking this information, we must turn to other cases for suggestions. Quite recently Taylor (34) has shown that DNA duplication in the plant *Crepis capillaris* begins at the ends of each chromosome and proceeds to the centromere region. The evidence consists of a gradient of isotope concentration in labelling experiments, in some instances labelling being entirely restricted to the centromere regions. Unfortunately, generalizations from this one case are unwarranted, as the same phenomenon apparently does not occur in *Bellevalia*. Nevertheless, it is tempting to see more than an accidental similarity between *Euplotes* and *Crepis*, even though details are difficult to picture. From Turner's drawings (35) it appears that the haploid set of *Euplotes* contains eight telocentric chromo-

somes of nearly equal length. We might imagine the chromosomes to be oriented longitudinally in two groups, and very much elongated so that each has its end in one of the two ends of the macronucleus and its centromere in the middle. Simultaneous duplication of all the chromosomes from their ends would then give the over-all pattern actually observed. Unfortunately, such a scheme cannot be reconciled easily with the phenomenon of macronuclear regeneration, which demands that complete genomes form relatively small packets within the nucleus. Alternatively, we might suppose that the chromosomes are arranged in numerous small groups, in each of which the centromeres are oriented toward the middle of the macronucleus. In this case we need the subsidiary hypothesis that duplication of a distal packet triggers the duplication of the next packet in line.

Evidence for oriented material was sought in polarized light observations, but there were no signs of birefringence in the macronucleus. Nor is there any striking orientation of materials at the electron microscopical level, as shown by Roth's (27) recent study of *Euplotes patella*. Neither the polarization nor electron microscopical observations necessarily rule out the possibility of chromosomal orientation in the macronucleus, since the finest strands of chromosomes are complexly folded in most cases (26), and chromosomes as such are only faintly birefringent (17). Clearly additional evidence is needed to make further speculation profitable.

The present data bear directly on the question of the relationship between DNA and the histones. Several years ago Alfert and Bern (2) showed that the Feulgen/alkaline fast green ratio remains nearly constant during the period of DNA synthesis in onion root tip nuclei. Bloch and Godman (6) demonstrated essentially the same fact in rat liver nuclei, although in the latter case there appears to be a population of nuclei with an abnormal ratio (7). In other cases the Feulgen/alkaline fast green ratio seems to be fairly constant in nuclei of one type but to differ from that in other nuclei of the same organism. For instance, the macronucleus of *Tetrahymena* takes up less fast green relative to the Feulgen stain than does the micronucleus (4). Again in the centipede, *Scutigera*, and in the pentatomid bug, *Loxa*, the nuclei which show asynapsis during meiosis bind a relatively greater amount of fast green than the

normal nuclei (5). In none of these cases is there independent evidence to indicate whether the variability in staining ratio is due to differences in the DNA/histone ratio, to variability in types of histone or their association with the DNA, or to other factors. In one case, that of sperm formation in the salmon, we know that changes in the fast green binding capacity of the nuclei are correlated with the change from histone to protamine as the basic material associated with the DNA (1). These examples indicate that considerable caution is necessary in interpreting data based on fast green staining.

In *Euplotes* the staining data suggest a simultaneous duplication of DNA and histone, or to speak more precisely, of DNA and a basic nuclear protein fraction which stains with alkaline fast green. The same is true for onion root tip and rat liver nuclei. In the latter cases we can only infer that the syntheses are spatially coincident, whereas in *Euplotes* we have seen that new material is formed only in a narrowly defined region close to the duplication bands. This observation is consistent with the idea that nucleoprotein synthesis is a unitary process involving simultaneous duplication of the associated nucleic acid and protein molecules.

Euplotes is a favorable organism in which to study certain other aspects of nucleic acid and protein synthesis. What of the non-histone proteins? Are they duplicated during DNA-histone synthesis or do they behave independently? A few preliminary experiments using tritium-labelled cytidine show that this compound is incorporated into RNA in the central zone between the approaching duplication bands. Presumably, therefore, RNA formation is independent of DNA and histone synthesis in this part of the nucleus. Cytidine incorporation also occurs distal to the bands, but as yet an experimental distinction between RNA and DNA radioactivity has not been made. It will be particularly interesting to discover if both RNA and DNA synthesis take place at the duplication bands. If DNA is directly involved in RNA synthesis, as has been suggested in the past, then perhaps RNA synthesis cannot occur during DNA duplication. These questions are now being studied by autoradiographic techniques.

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EXPLANATION OF PLATES

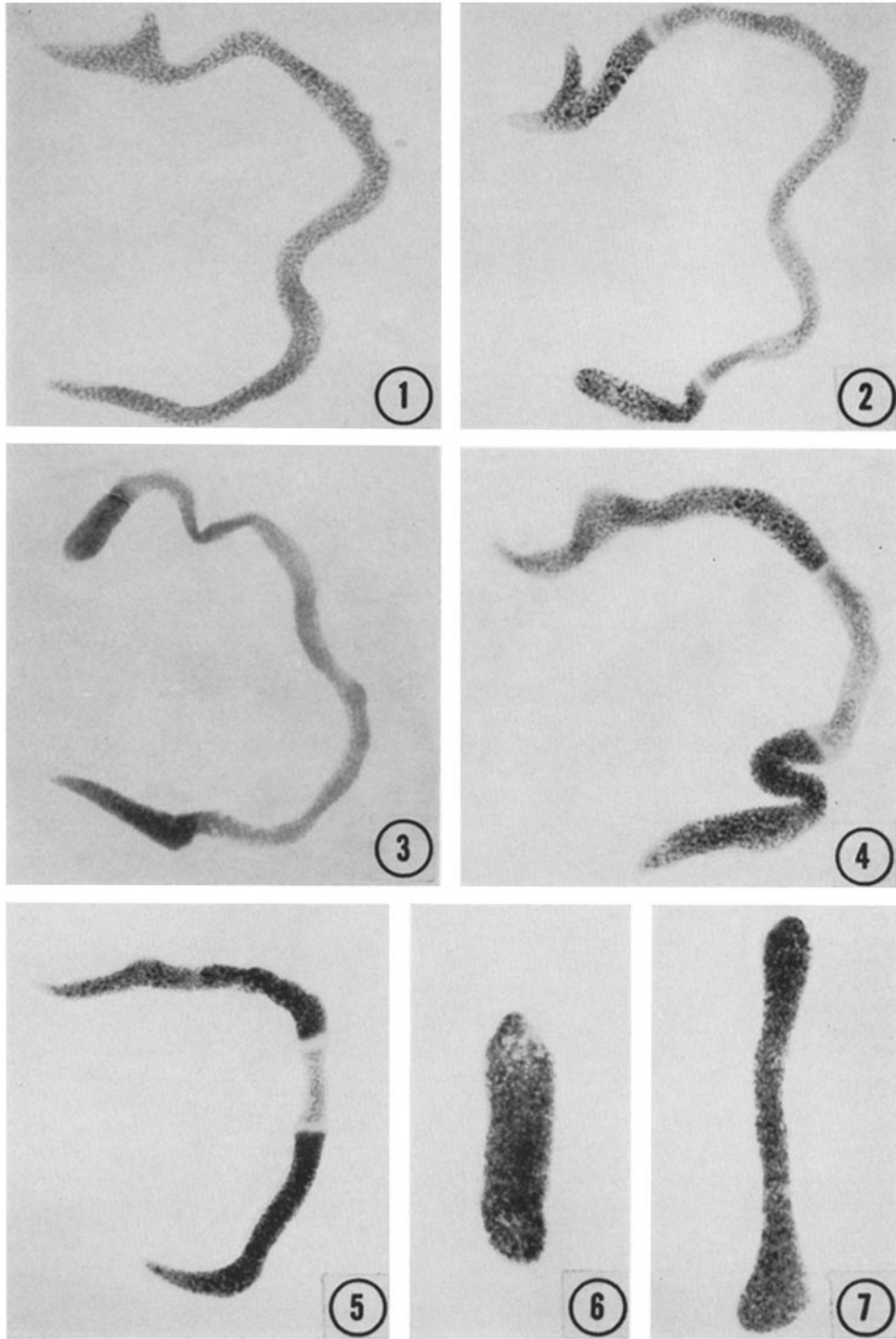
PLATE 124

FIG. 1. Typical macronucleus of *Euplotes eurytomus* before the onset of DNA-histone duplication. Feulgen stain.

FIGS. 2 to 5. Various stages in the progression of the duplication bands ("reorganization bands") from the ends to the middle of the nucleus. Distal to the bands in each nucleus new DNA and histone have been synthesized, but no synthesis has occurred in the central zone. As shown by the intensity of the Feulgen stain (Figs. 2, 4, 5) or alkaline fast green (Fig. 3) the concentration of DNA and histone is greater in the distal regions.

FIGS. 6, 7. Nuclei in which the duplication bands have met near the middle and disappeared. Such nuclei contain twice as much DNA (Feulgen) as those prior to the appearance of the duplication bands. The animal will soon undergo transverse fission with an amitotic splitting of the macronucleus into two equal fragments. Feulgen stain.

FIGS. 1 to 7. Approximately $\times 380$.



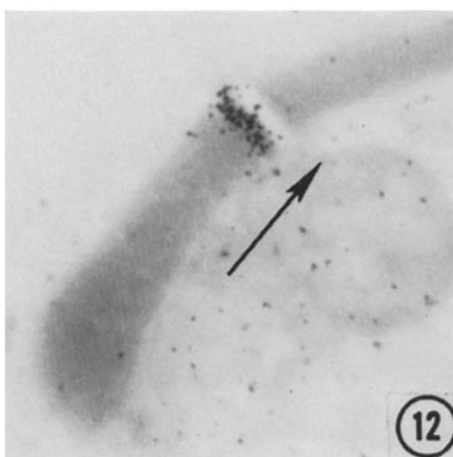
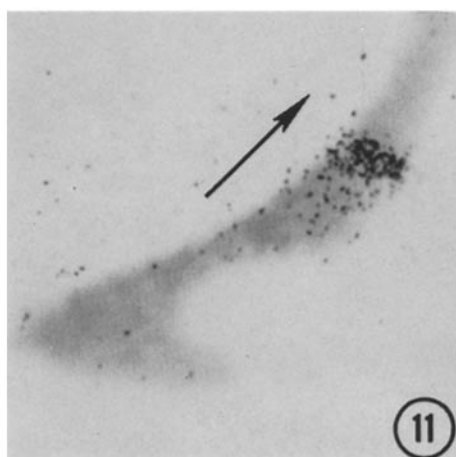
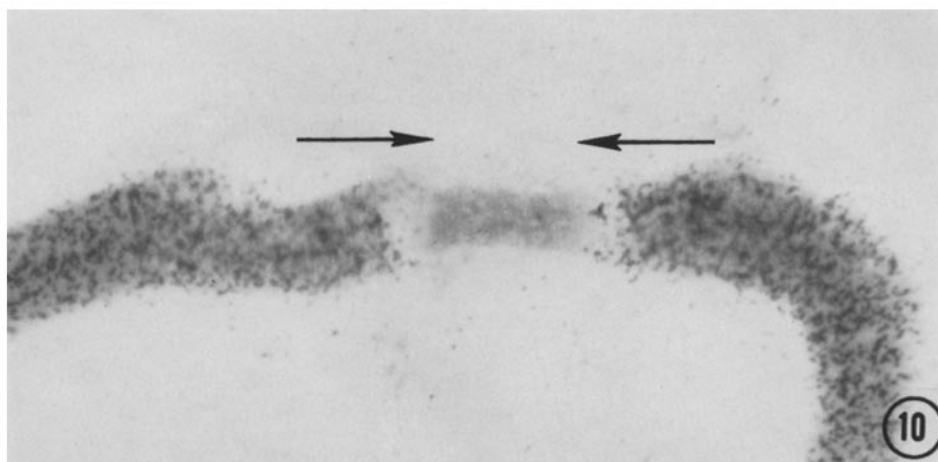
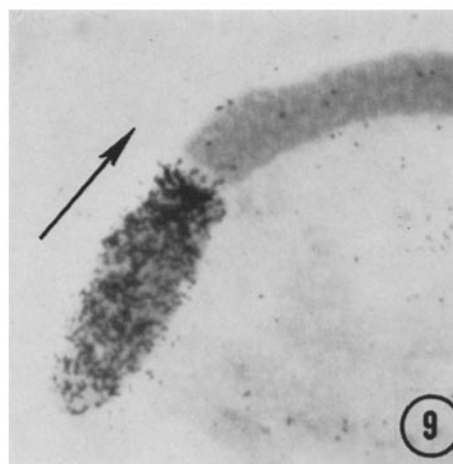
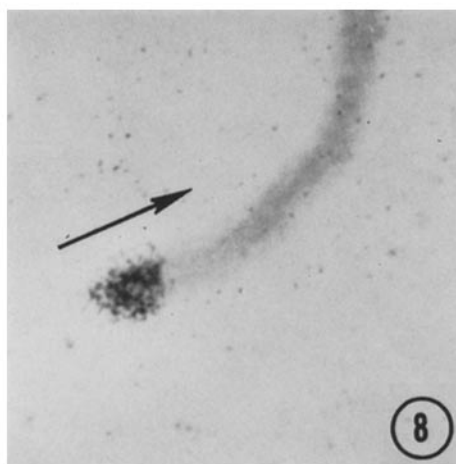
(Gall: Macronuclear duplication in *Euplotes*)

PLATE 125

FIGS. 8 to 12. Autoradiographs showing incorporation of H^3 -thymidine in the macronucleus of *Euploes eury-stomus*. Stained by the Feulgen reaction (Figs. 8, 11) or azure B at pH 4.0 (Figs. 9, 10, 12). The duplication bands are shown most clearly in Figs. 9 and 12. The direction of movement of the bands is indicated in each case by an arrow. $\times 1300$.

FIGS. 8 to 10. Animals left 8 hours in the isotope solution, showing label throughout the zone distal to the duplication bands. In Figs. 8 and 9 the duplication bands have progressed only a short distance from the ends of the nucleus, in Fig. 10 they will soon meet near the middle.

FIGS. 11 and 12. Animals left 3 hours in the isotope solution. Here the label is present only in a short region distal to the duplication bands. This fact indicates that synthesis of DNA takes place in or next to the band, but does not persist in regions through which the band has already passed.



(Gall: Macronuclear duplication in *Euplotes*)