QUANTITATIVE STUDIES ON THE MACRONUCLEI OF *PARAMECIUM AURELIA* DOUBLETS: DETERMINATION, DNA CONTENT AND DISTRIBUTION OF DNA TO DAUGHTER MACRONUCLEI

by

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ABSTRACT

This study examined the nuclear events in Paramecium aurelia doublets following sexual reorganization to: 1) Determine how loss in number of macronuclei in older clones occurred; 2) Quantitatively analyse the DNA content of Feulgen-stained doublet macronuclei and the distribution of DNA to daughter cells at division; and 3) Quantitatively compare singlet and doublet P. aurelia with respect to DNA content, total protein content, and length of the cell cycle.

If the situation was analogous to that in singlets, four macronuclear anlagen would be expected in doublets following conjugation or autogamy. Results, however, showed that the number of macronuclear anlagen in doublets varied considerably. Also, a number of "abnormal" anlagen were observed in doublets which appeared to be undergoing macronuclear regeneration. Genetic evidence indicated that the cause of variability in the number of anlagen was due to abnormal patterns of determination of the products of the second post-zygotic division of the synkarya, presumably as a consequence of the abnormal morphology of doublet cells. Distribution of macronuclear anlagen at the first cell division was also irregular; only 34.3% of the daughter cells showed the expected 2:2 distribution of anlagen. Loss of the second macronucleus occurred by the fourth to fifth cell cycle. Misdivision (failure to divide) and missegregation of macronuclei, mechanisms which could account for macronuclear loss, were observed to be occurring approximately 15-20%

and five-eight percent of the time. Genetic evidence showed that fusion of macronuclei, a third mechanism which could account for loss of macronuclei, could not take place more than about 15% of the time, if it in fact occurred at all.

The DNA content of doublets varied slightly between stocks, as well as between cells within stocks. DNA content in doublets was not strictly proportional to the number of macronuclei per cell; additional macronuclei increased the DNA content, but not in proportion to the number of macronuclei. Therefore, mechanisms were present which regulated the amount of DNA per cell to a more nearly constant amount than would be expected from the number of macronuclei alone. When daughter cells contained equal numbers of macronuclei, the mean difference in DNA content between sister cells was about five percent of the G_2 DNA content and the intraclass correlation coefficient between sister cell DNA was high (+0.7 or more). In contrast, when unequal numbers of macronuclei were present in daughter cells, the DNA contents were quite unequal; however, this inequality was not strictly proportional to the number of macronuclei per cell. No net regulation of DNA content occurred during the DNA synthesis period of the cell cycle as evidenced by the fact that there was no significant difference in variation between doublet cells having the G_1 *versus* the G_2 amount of DNA.

A comparison of DNA content in *P. aurelia* singlets and doublets showed that the mean G_1 DNA content of doublets was approximately 192% that of singlets. Further, the mean DNA content of micronuclei in singlet and doublet cells was the same. A similar comparison of total protein

content showed that doublets had approximately twice the macromolecular dry mass of singlets. As reported by Kimball (1967), it was found that the ratio between dry mass and DNA content in doublets is constant when both the cell and the macronucleus are doubling in each cell cycle.

Finally, it was shown that doublet P. aurelia have a vegetative cell cycle lasting approximately 164% of the length of singlets. It was suggested that the G_1 period in doublets may be shorter with increased cell size as was observed by Killander and Zetterberg (1965a,b) in mammalian cells, thereby allowing more time for growth in the smaller than in the larger cells before DNA synthesis begins.

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DEDICATION

To

My Parents

and

Phyllida

INTRODUCTION

The doublet biotype has been observed in a variety of Ciliates including Paramecium aurelia. This study examines the consequences of the doublet biotype on the number of macronuclei, their DNA content and the distribution of macronuclear DNA to the daughter cells at division.

The Surface Morphology of Paramecium aurelia Singlets

Paramecium aurelia is a holotrichous Ciliate which contains in its cortex approximately 5,000 cilia and basal bodies in about 4,000 unit territories arranged in some 75 rows or kineties which run, for the most part, from pole to pole. This pattern is interrupted on the ventral surface of the cell by the mouth or buccal cavity around which the anterior and posterior ends of the kineties bend inward to form anterior and posterior sutures (Figure 1). The ingestatory apparatus of P. aurelia consists of a funnel shaped gullet, or cytopharynx, located at the base of an oval-shaped depression of the cell surface, the vestibule. The entire ingestatory apparatus is a continuation of the cortex and appears to be composed of modified kineties. Other surface landmarks include two contractile vacuole pores on a meridian about 180° away from the mouth (35-40 kineties to the right), and a cytoproct which lies posterior to the mouth at the junction between the right and left posterior fields of kineties (Sonneborn, 1963, 1970b for review).

Surface Structure of P. aurelia Doublets

Doublet paramecia occasionally arise as a consequence of delayed separation and subsequent complete fusion of two conjugating normal singlet cells. Thus each doublet contains two complete sets of cortical structures with vestibules and gullets 180° apart on the surface of the cell (Figure 2). The two sets of cortical structures are effectively fused with the right ventral surface of one of the original cells adjoining the left ventral surface of the other and vice versa (Figure 3). Following doublet formation the cortical phenotype is stable; events which occur at division in the oral segment of singlets happen in both oral segments of doublets. (See Sonneborn, 1963, for a detailed review.)

Sonneborn (1960, 1963, 1970b) further demonstrated in an elegant series of experiments that the hereditary difference between singlets and doublets is not due to differences in nuclear genes, macronuclear differentiation, or freely flowing cytoplasm; the pattern of cortical inheritance is semi-autonomous in a genetic sense.

The Structure and Development of Nuclei

As in nearly all Ciliates, two types of nuclei of strikingly different structure and organization are present in P. aurelia: micronuclei and macronuclei (Raikov, 1972, for review). The diploid micronucleus is a compact structure which can be as small as 1.5 to 2.0 μm in diameter, and which divides mitotically during the latter part of each vegetative cell cycle by means of an intranuclear spindle (Stevenson and Lloyd, 1971). The DNA in micronuclei is very tightly packed; almost

nothing is known of its physical configuration. However, its pattern of replication has been studied.

The restriction of DNA synthesis to a certain part of the cell cycle forms the basis for subdivision of the cell cycle into four segments. The interval between the end of nuclear division and the start of DNA synthesis is called G_1, the period of DNA synthesis, S, the period extending from the end of DNA synthesis to the beginning of nuclear division, G_2, and the period of cell division, D.

Woodard, Gelber and Swift (1961) reported that micronuclear DNA synthesis began midway through the cell cycle and terminated after only thirty to forty minutes. Their conclusions were based on microspectrophotometric measurements of Feulgen stained cells of known age. Pasternak (1967), however, using a more sensitive autoradiographic analysis found that micronuclear DNA synthesis was initiated about halfway through the cell cycle and lasted for approximately 80 minutes. Synthesis was completed by the end of the first 2/3 of the cycle. Pulse labeling experiments using ^{3}H-Uridine and ^{3}H-Leucine further indicate that micronuclei are competent in RNA and protein synthesis which precedes the onset of (micronuclear) DNA synthesis and continues concurrently during the midportion of the cell cycle (Pasternak, 1967).

The macronucleus is much larger (approximately 15 x 40 μm in size) and has a G_1 DNA content of approximately 860 times the haploid DNA content (Woodard et al., 1961; Allen and Gibson, 1972; Berger, 1973). The exact arrangement of the DNA in the macronucleus is unknown; however, ultrastructural examination reveals two distinct types of bodies within

it: Large bodies (Jurand, Beale and Young, 1962; Dippell and Sinton, 1963; Stevenson and Lloyd, 1971b) up to 1.2 μm in diameter, irregular in outline, and fibrogranular in appearance and small bodies (Jurand, Beale and Young, 1962; Dippell and Sinton, 1963; Stevenson and Lloyd, 1971b) about 0.1-0.2 μm in diameter, also irregular in shape, and fibrillar in ultrastructure. The large bodies are nucleoli and the small bodies are centres of condensed chromatin (Nanney and Rudzinska, 1960; Dippell and Sinton, 1963; Wolfe, 1967; Jurand and Jacob, 1969). Wolfe (1967) observed unsectioned chromatin granules and suggested that they are interconnected by fibrils of about 100 Å in diameter, forming a continuous network. Biochemical observations by Allen and Gibson (1972) suggest that macronuclear DNA is organized in full genomes and that the macronuclear DNA molecules are double stranded and of a high molecular weight.

Studies on the time course of macronuclear DNA synthesis using photometric techniques (Walker and Mitchison, 1957; Kimball and Barka, 1959; Kimball et al., 1960; Woodard et al., 1961) report that DNA synthesis begins about 50% of the way through the cell cycle and continues up to division. Autoradiographic analysis of the pattern of macronuclear DNA replication, however, shows that macronuclear DNA synthesis begins abruptly about 25% of the way through the cell cycle and continues until the following division with little or no G_2 period (Berger and Kimball, 1964; Berger, 1971). The sensitivity of autoradiographic methods in detecting small amounts of DNA synthesis may explain why the onset of DNA synthesis was observed earlier in the cell cycle than in previous photometric studies.

Conjugation and Development of the Macronucleus in P. aurelia Singlets

Since P. aurelia has two micronuclei and both undergo meiosis, there are eight haploid meiotic products. One located near the boundary between the two mates enters a newly formed protrusion, the "paroral cone" (Diller, 1936) and undergoes a modified mitotic division while the other seven disintegrate and are resorbed. One of these haploid nuclei becomes a stationary ("female") pronucleus, while the other becomes a migratory ("male") pronucleus. In the case of conjugation, male pronuclei are exchanged near the region where the paroral cones of the two mates overlap, producing a heterozygous diploid synkaryon in each mate. In autogamy, a similar process occurs in single cells, the male pronucleus fuses with its "sister" female pronucleus to produce a homozygous diploid synkaryon (Sonneborn, 1937, 1947). The ultrastructural details of meiosis in the micronuclei of P. aurelia during conjugation and autogamy have been described by Stevenson (1972).

At the beginning of each sexual generation new macronuclei develop from the diploid division products of the synkaryon formed at fertilization. The nuclear events occurring during conjugation or autogamy were first described by Hertwig (1889); Maupas (1889); and Diller (1936). The normal macronuclear condition is restored after mating by the passage of one of the two macronuclear anlagen or new macronuclei, without division to each of the two daughter cells formed at the first fission (Figure 5a). The new macronuclei divide at the second and subsequent fissions. Approximately 95% of the DNA in macronuclear anlagen is synthesized during the second cell cycle (Berger, 1973).

During conjugation the old macronucleus breaks down to form approximately 35 macronuclear fragments which do not divide, and are segregated among the cells of the exconjugant clone. Fragments may persist as long as 13 cell cycles before they are completely autolysed; however, autolysis of some fragments begins as early as the fourth cell in well-fed exconjugants (Berger, 1973). The rate of DNA synthesis in fragments is much lower than that in normal macronuclei, and decreases exponentially with time. RNA synthesis in macronuclear fragments continues at a rate similar to that in macronuclei for at least as long as nine cell cycles after conjugation (Berger, 1973). Gene activity in fragments has been detected as late as the eleventh cell cycle (Berger, in press).

Nuclear Events in *P. aurelia* Doublets

In newly formed doublets, four macronuclear anlagen would be expected if the situation was analogous to that in singlets (Figure 5b). At the first fission, two anlagen would segregate to each daughter cell, giving rise to two macronuclei. After a relatively short time, the two macronuclei give way to a single larger macronucleus in the later progeny (Sonneborn, 1963). The current study investigates the kinetics of this phenomenon which, until now, has not been examined in detail. Normally, the macronucleus of a singlet occupies a position close to the gullet with its major axis extending longitudinally; in doublets the macronucleus usually lies close to both gullets, with its major axis transverse (Figure 4c) (Sonneborn, 1963). Four micronuclei remain in the

later progeny of the doublet clone (two on each side) and function during conjugation or autogamy (Figure 5b).

Doublets can mate bilaterally with two singlet mates, one on each of their two ventral surfaces. Mating may also occur between two doublets, each of which can accept another doublet. If only one mate is present, conjugation occurs on its mated side and autogamy occurs simultaneously on its unmated side (Chun, 1969).

Variations in the normal distribution of macronuclear anlagen have been reported in Stock 51, syngen 4 of P. aurelia doublets (Butzel, 1970) and in a tandem doublet clone (Can 18) of P. bursaria (Chen, 1949). In conjugating cells which became "bridged-pairs" (Figure 3a) and failed to separate before the first fission, the number of macronuclear fragments decreased while the fragments increased in size (Butzel, 1970). Abnormal distribution of anlagen resulted in only 1/3 of the doublets showing two anlagen in each half of the fused pair. Later experiments (Butzel, 1973) revealed that 84% of cytoplasmically-bridged pairs (CBPs) contained four anlagen (each control singlet contained two), and only about 1/2 of the CBPs had a 2:2 distribution of the macronuclei between their halves. Micronuclear numbers and positions were also irregular. In all, only 12% of the CBPs were found to have a normal distribution of two macronuclei and two micronuclei in each half of the doublet while the remainder possessed some type of abnormal distribution.

The kinetics of loss of the second macronucleus in doublets has been examined by Samoiloff (personal communication), who observed an approximately exponential decrease in the number of macronuclei following

a sexual cycle, the later progeny containing only a single large macronucleus with variable numbers of micronuclei.

Goals of the Present Study

The major goals of the study are: 1) To give an account of the nuclear events following conjugation in doublets; how many macronuclei are determined, their distribution to cells at the first fission after reorganization and to examine the mechanism(s) by which a single macronucleus remains in older clones; 2) To quantitatively analyse the DNA content of doublet cells and the distribution of DNA to daughter cells at fission; and 3) To quantitatively compare singlets and doublets with respect to DNA content, macromolecular dry mass and length of the cell cycle.

Figure 1. Surface morphology of P. aurelia singlets.
a. Ventral or oral surface. a, anterior pole; al, anterior left kinety field; ar, anterior right kinety field; c, circumoral or vestibular kinety field; cvp, contractile vacuole pore; m, mouth; p. posterior pole; pl, posterior left kinety field; pr, posterior right kinety field; py, cytoproct; s, preoral suture.
b. Dorsal or aboral surface.

Figure 2. Surface morphology of P. aurelia doublets.
a. Surface at upper focal level. a, anterior pole; al, anterior left kinety field; ar, anterior right kinety field, cvp, contractile vacuole pore; g, gullet; m, mouth; p, posterior pole; pr, posterior right kinety field, pl, posterior left kinety field; py, cytoproct; s, preoral suture; c, circumoral or vestibular kinety field.

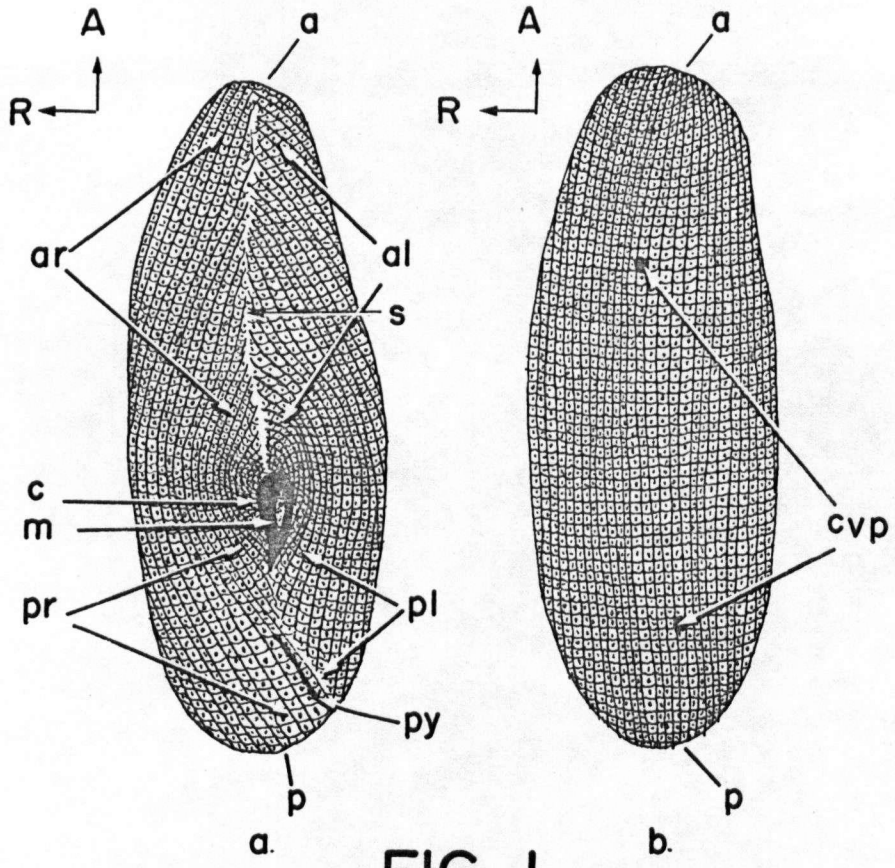


FIG. 1

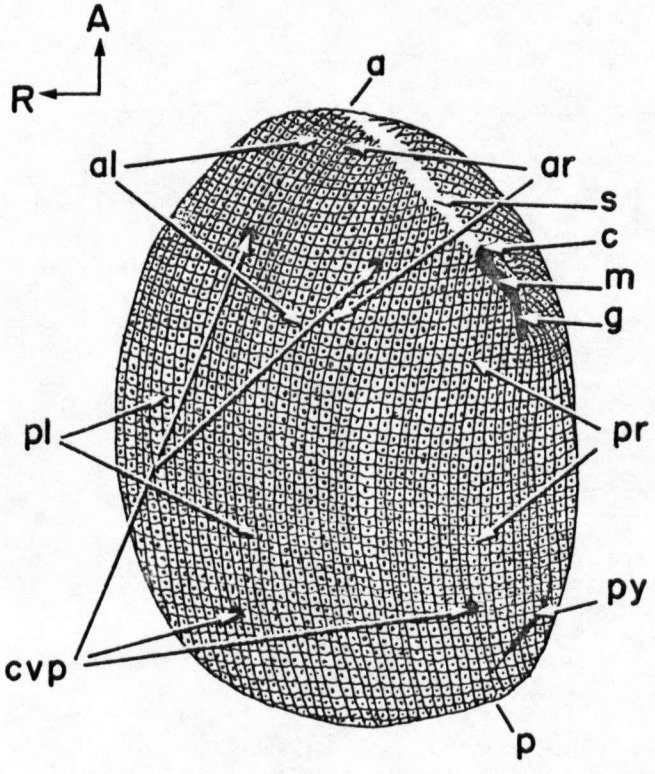


FIG. 2

Figure 3. Doublet formation resulting from delayed separation of two conjugating singlet cells.
a. Two single cells joined as a "bridged-pair" at the point where ("male") gametes are exchanged.
b. A later doublet resulting from the "bridged-pair" showing an enlarged area of cortical fusion.
c. A completely formed doublet showing the cortices of the two mates joined from pole to pole. a, anterior pole; m, mouth; p, posterior pole.

Figure 4. Position of nuclei in singlet and doublet P. aurelia.
a. Position of the macronucleus in P. aurelia singlets.
b. Typical position of macronuclei in P. aurelia doublets in the second cell cycle following a sexual generation.
c. Typical position of the macronucleus in P. aurelia doublets during vegetative growth. a, anterior pole; mac, macronucleus; m, mouth; g, gullet; mic, micronucleus; p, posterior pole.

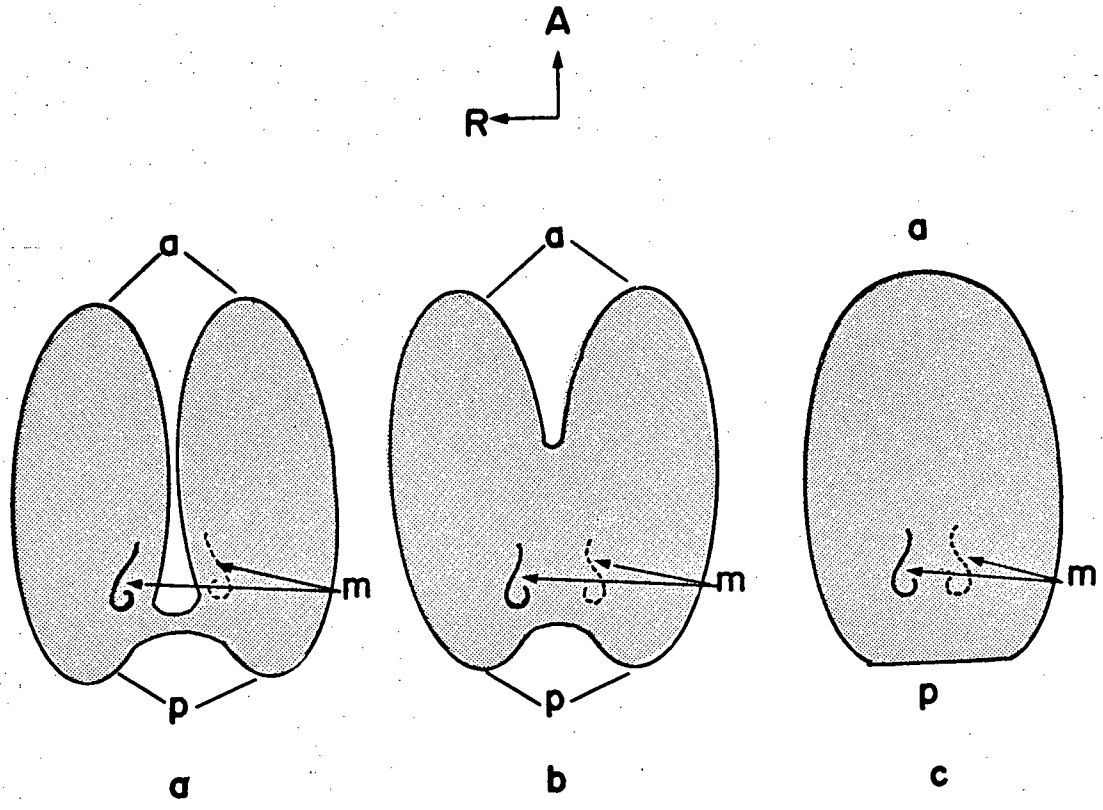


FIG. 3

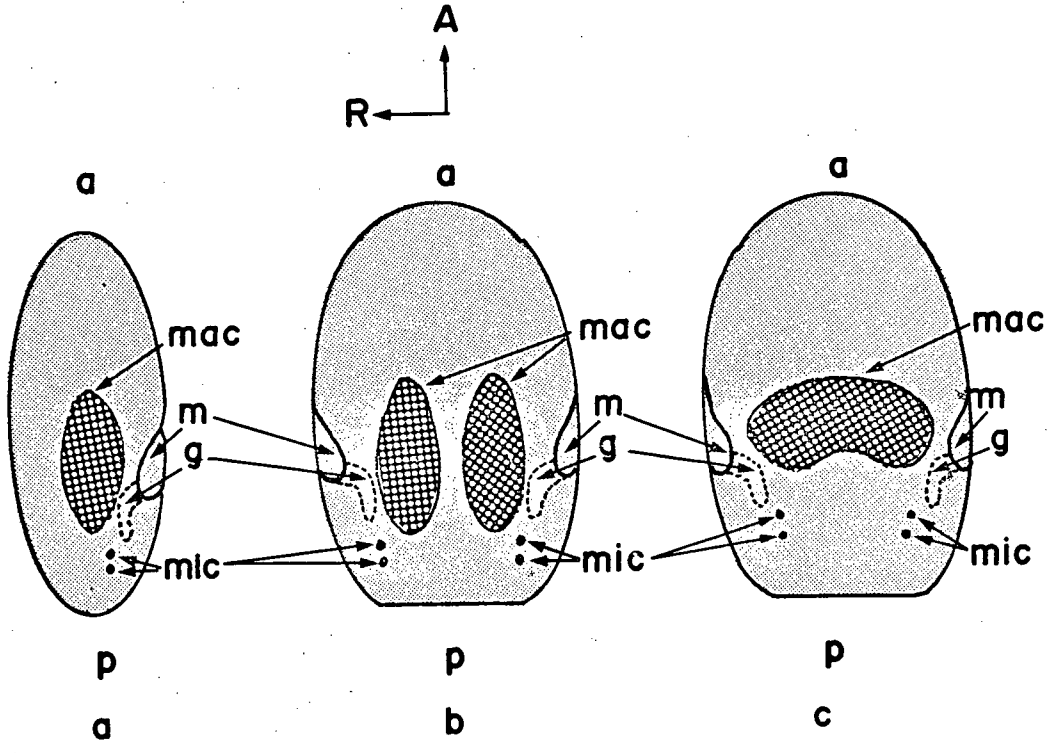


FIG. 4

Figure 5. Diagram of nuclear behavior during autogamy in Paramecium aurelia singlets Figure 5a and in doublets if the situation were analogous to that in singlets Figure 5b. I, II, III - respective maturation divisions of the micronuclei, pn - pronuclei, sk - synkaryon, 1, 2 - synkaryon divisions, a - first exautogamous cell division. In the exautogamont and its offspring, hatched circles are macronuclear anlagen, small black circles are new micronuclei. Crosses mark degenerating nuclei. The dotted lines in Figure 5b shown at the first exautogamous cell division denote that two of the four macronuclear anlagen in the parent exautogamont have an equal probability of going to either one of the two daughter cells.

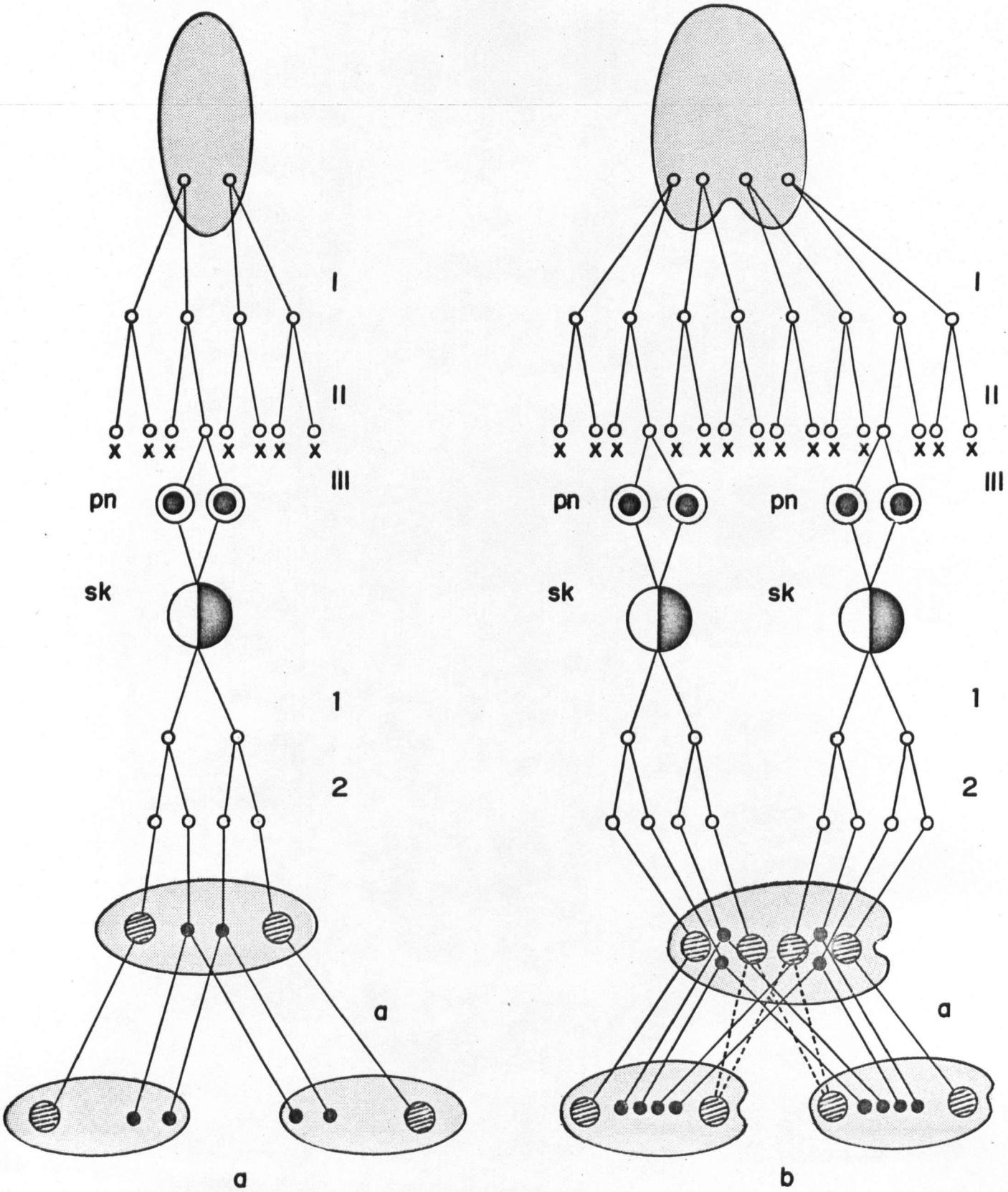

Singlet P. aurelia
Doublet P. aurelia
I
II
III
pn
pn
sk
sk
1
2
a
a
a
b

FIG. 5

MATERIALS AND METHODS

Culture of Paramecia

Various strains of doublet Paramecium aurelia, syngen 4, stock 51-S (wild-type) were grown at 27°C on Cerophyl rye grass medium (Cerophyl Laboratories, Kansas City, Mo., U.S.A.) with Aerobacter aerogenes as the food organism (Sonneborn, 1970a). The pH of the culture fluid was adjusted to 6.8-7.0 before use. All stocks were maintained in previously autoclaved depression slides and stored in plastic moist chambers.

Stocks

Doublet strains $3A_2$SplA, $3A_2$SplB, H9W, KVD4, 3A, and 2B were obtained from the Sonneborn Laboratories, Indiana University, Bloomington. They are derived from stock 51; the parental genotypes are unknown. Stock GM_1 was obtained from a cross of wild-type cells using antiserum treatment (Appendix I). Stocks GM_2 and GM_3 were obtained similarly; stock GM_2, however, contains the mutant gene amac (am) in homozygous condition. This gene causes mis-segregation of macronuclei at division in a variable fraction of dividing cells (Sonneborn, 1954) and therefore was used only in investigations concerning the cytoplasmic content of doublets.

Cytological Techniques

Cells were prepared for staining by isolating single cells in a micro-drop of culture fluid and allowing them to dry in rows on an albumenized microscope slide. Following fixation in ethanol-acetic acid (3:1) for 20 minutes and hydrolysis in 1 N HCL (60°C) for 11 minutes, the nuclei were stained with 0.5% Azure A or Leucobasic fuchsin (Appendix I), dehydrated by drying and mounted in Cargill's type 'A' index of refraction immersion oil. Relative cytoplasmicccontent of singlet and doublet cells was determined by drying the cells in micro-drops of culture fluid on albumenized slides. After fixation, cells were stained with Acid Fast-Green or Napthol yellow-S (Appendix I). Dippell's stain (Appendix I) (Dippell, 1955) was used to identify macronuclear fragments. This was a test for nuclear reorganization which occurs during conjugation or autogamy.

Values of relative DNA and cytoplasmic content for singlet and doublet cells were obtained by scanning photographic negatives of stained cells with a scanning integrating microdensitometer (Appendix I).

RESULTS AND DISCUSSION

SECTION I

DETERMINATION, DEVELOPMENT AND DISTRIBUTION OF MACRONUCLEI IN P. aurelia DOUBLETS

A. The Number and Distribution of Macronuclear Anlagen in Doublet Paramecium aurelia Following Sexual Reorganization

The purpose of these experiments was to examine the number and distribution of macronuclear anlagen in doublets following conjugation using cytological and genetic means.

1. Initial Number of Anlagen Per Cell

The initial number of macronuclear anlagen per cell in stock GM_3 doublets was examined after bilateral conjugation with singlet mates. Both doublet and singlet exconjugants were dried on albumenized microscope slides and stained with Azure-A during the first cell cycle. A small number of abnormal exconjugant doublets which failed to divide within 120 hours after conjugation were also examined.

While four anlagen would theoretically be expected in doublet exconjugants, the number of anlagen observed in doublets varied (Table I). Four macronuclear anlagen were found in the largest number (71/133) of exconjugants in which the number of anlagen could be unambiguously determined. However, nearly as many cells (60/133) contained only two anlagen.

TABLE I

NUMBERS OF MACRONUCLEAR ANLAGEN IN DOUBLETS IN THE FIRST CELL CYCLE FOLLOWING CONJUGATION

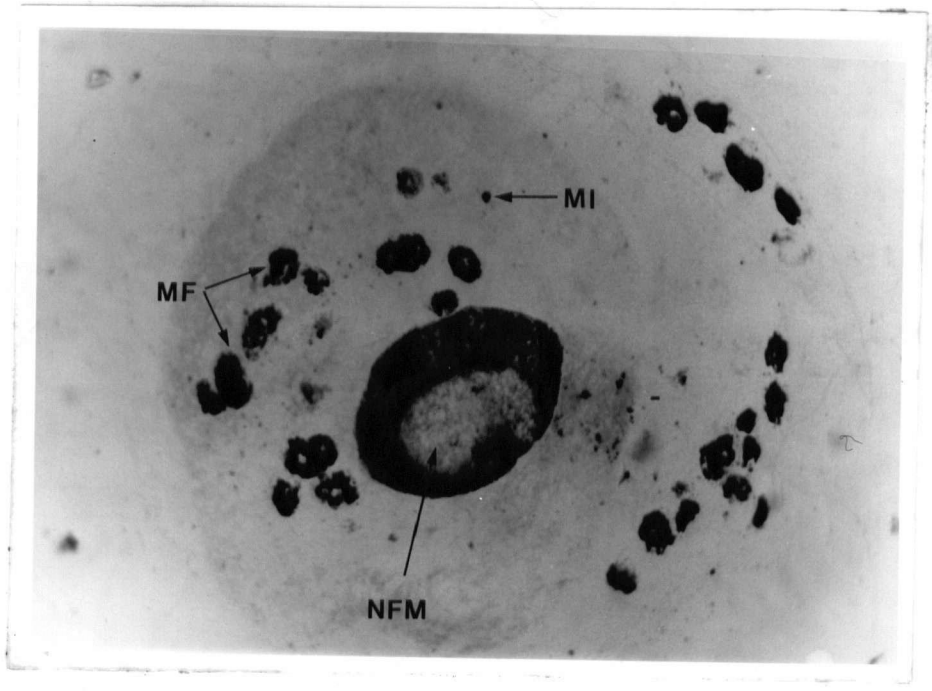
No. of Anlagen/Cell	No.	%
0	1	0.7
1	4	3.0
2	60	45.1
3	2	1.5
4	71	53.3
Total	133	

These results imply that, at least in this stock, there is substantial departure from the number of anlagen (4) expected in doublets.

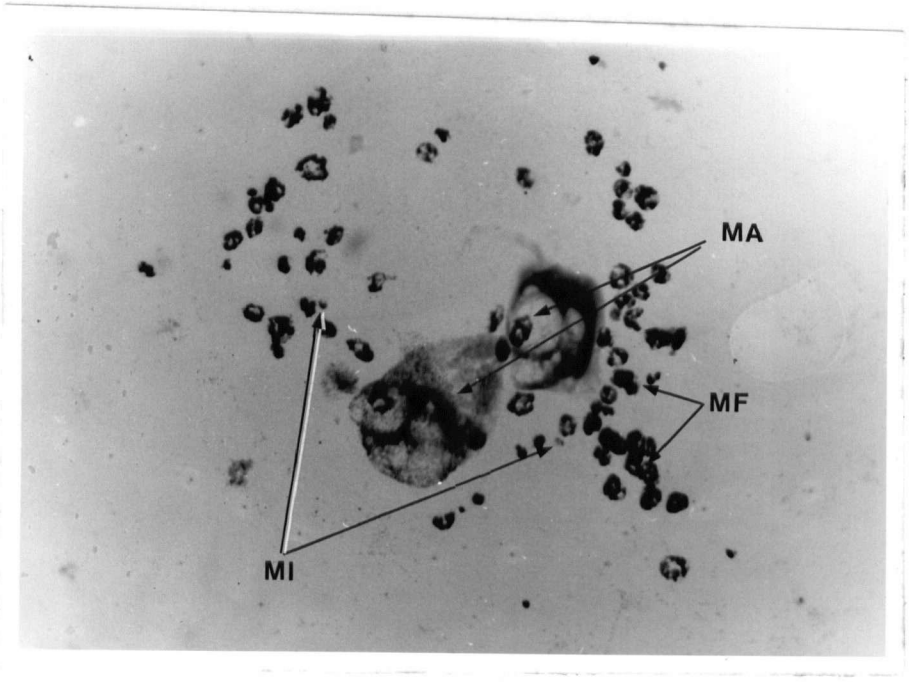
In most experiments there were some cells which did not divide after reorganization. A sample of 42 of these abnormal cells were also stained 120 hours after conjugation and examined. These cells, which occurred in several experiments with a variety of doublet stocks, grew very large and changed progressively from the stubby "torpedo" shape of a normal doublet to a nearly spherical form. Thus they were nicknamed "basketballs." The "basketball syndrome" was seen in varying degrees in every experiment following macronuclear reorganization, particularly in exautogamonts. The basis of this change is unknown. Nuclear morphology in "basketballs" varies, although most cells usually have one or two large faintly staining anlagen with fragments of the former macronucleus in late stages of autolysis (Figure 6a). Large nucleolar fusion masses which are normally seen in developing anlagen in singlets (Berger, 1973) were also seen in the large macronuclear anlagen of basketballs (Figure 6b). Earlier workers have not reported such abnormal cells although perhaps cells showing this phenotype have been discarded and their details omitted from the data. The number of anlagen in the sample of 42 "basketballs" is observed in Table II. The numbers of micronuclei and abnormal anlagen for a selected sample of "basketballs" is given in Table III.

The data indicate that deviations from the normal pattern of nuclear events during sexual reorganization (Figure 5) occur frequently in doublet P. aurelia. It is apparent from the data in Table I that

Figure 6 (a and b). Photographs of doublet cells which did not divide for a period of 120 hours after conjugation and were referred to as "basketballs."
ma - macronuclear anlagen, mf - macronuclear fragments, nfm - nucleolar fusion mass, mi - micronucleus. x 890.



(a)



(b)

FIGURE 6

TABLE II

DISTRIBUTION OF MACRONUCLEAR ANLAGEN IN DOUBLETS WHICH DO NOT DIVIDE FOLLOWING NUCLEAR REORGANIZATION

No. of Anlagen/Cell	No.	%
0	1	2.3
1	25	59.5
2	13	30.9
3	1	2.3
4	2	4.7
Total	42	

TABLE III

NUMBERS OF NORMAL ANLAGEN, ABNORMAL ANLAGEN, AND MICRONUCLEI OBSERVED IN A SELECTED SAMPLE OF NON-DIVIDING DOUBLETS FOLLOWING NUCLEAR REORGANIZATION

No. of Normal Anlagen/Cell	No. of Abnormal Anlagen/Cell	No. of Micronuclei	Total Number of Derivatives of the Two Synkaryons
2	0	6	8
1	0	11	12
1	0	6	7
2	5	4	11
2	4	2	8
4	0	4	8
1	7	0	8
0	8	0	8
2	0	7	9
3	1	4	8

only slightly more than half of the cells examined during the first cell cycle had four anlagen, as would be expected from the pattern of events in normal singlets. I speculate that either one of two events must be occurring:

a) that doublets containing two anlagen resulted from a unilateral mating with only one of the singlet cells while presumably the other pair of micronuclei failed to undergo or complete meiosis and degenerated (Sonneborn, 1951); or

b) that every doublet starts with four diploid division products of each of the two synkarya of which variable numbers are determined to become macronuclei.

First, genetic evidence shows that cells with only two anlagen are present in which the doublet indeed mated on both sides. Wild-type doublets (stock GM_3) were mated on both sides to pawn (pw/pw) singlets. After conjugation, the three cells were isolated and the phenotype of the singlet cells determined. Both singlet and doublet cells were then stained with Azure-A during the first exconjugant cell cycle to determine the number of macronuclear anlagen. A schematic diagram of the experiment is shown in Figure 7.

Of 42 mating triplets in which the behavioral phenotype of the singlet exconjugants was scored, 18 singlets continued to display the pawn phenotype indicating they had not mated. This means that their doublet partners had only mated on one side. Every singlet contained two macronuclear anlagen and numerous macronuclear fragments showing that all had undergone nuclear reorganization, either through cytogamy

Figure 7. Diagram of experiment in which wild-type doublet cells were bilaterally mated with singlets bearing the behavioral mutant gene pawn (pw/pw). Exconjugant singlets were scored for avoidance behavior in Dryl's solution (Appendix I) after conjugation to determine if mating had occurred. In both Case 1 and Case 2, exconjugant doublet cells were found to contain variable numbers of macronuclear anlagen. (See explanation in text.) +/+ - wild-type doublet conjugant, pw/pw - pawn singlet conjugants.

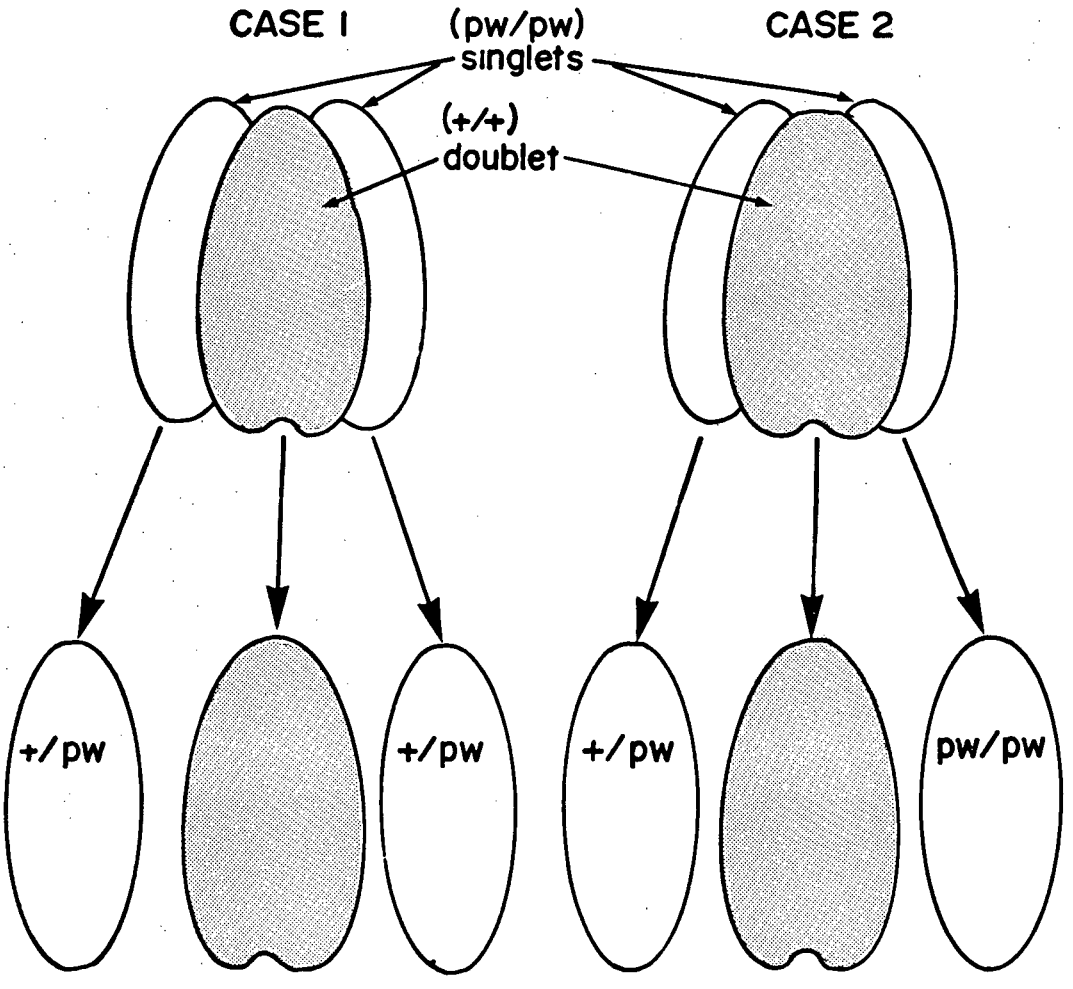


FIGURE 7

or conjugation. Furthermore, nine of the 18 doublets which mated on only one side had four anlagen, while five were found to have two anlagen and one cell contained only one anlage. The other three were unscoreable because one cell burst while being transferred and identification of the anlagen in the other two was impossible because the numerous darkly-staining macronuclear fragments obscured the anlagen. Of the 24 remaining doublets which mated on both sides, seven had four anlagen, six had two, two had one, and one cell had three. The remaining eight cells could not be scored because macronuclear fragments obscured the anlagen. These results rule out the possibility that doublets containing two anlagen in the first cell cycle arise only following unilateral fertilization.

Further, the occurrence of unilateral fertilization in doublets seems unlikely as judged from Chen's observations of tandem doublets (Chen, 1949), where he reported that in Paramecium bursaria, chains of two cells frequently occurred when a cell divided and the two daughter cells failed to separate; both later growing to adult size but remaining connected by a slender protoplasmic bridge. In an experiment where a singlet cell was mated to the anterior component of the tandem pair, the micronucleus in the posterior component underwent the usual meiotic changes. This indicates that the influence of the contact between the anterior component and the single animal must have passed to the posterior component by means of the protoplasmic bridge. After the onset of conjugation the differentiation of the two pronuclei takes place in both individuals of the pair, even though the posterior half of the doublet does not mate with any animal. Exchange of the haploid pronuclei takes

place only between the anterior half of the doublet animal and the single animal but the migratory pronucleus in the posterior half of the tandem doublet moves to the vicinity of the posterior mouth region as if a conjugant were attached to that part of its body. No case was observed in which the migratory pronucleus in the posterior half of the doublet breaks through the cell membrane; rather it fuses with the stationary pronucleus in the same half to form a synkaryon. Autogamy thus occurs in the posterior half of the doublet animal. When the conjugants separate, the exconjugant double animal usually contains eight nuclei, while the single animal contains four nuclei. The number of nuclei that become macronuclear anlagen is variable. In most of the exconjugant single animals, two (sometimes three) of the four nuclei become anlagen. In the exconjugant tandem doublet animals, two or four or five of the eight nuclei developed into anlagen.

Although tandem doublets differ in morphology and origin from the homopolar doublets used in my own experiments, examining the data in Table III on "basketballs" it becomes apparent that the type of nuclear behavior seen in these cells is similar to that observed by Chen in P. bursaria. Six out of 10 of these cells had eight synkaryon division products which would have arisen from two synkaryons, although it is notable that for the most part, the variations occurred in the number of micronuclei or abnormal anlagen. The important question to be considered at this point is how do doublet cells starting with eight diploid division products of the two zygote nuclei following sexual reorganization end up with varied numbers of anlagen, micronuclei, and anlagen which appear abnormal.

The discovery that displacement of synkaryon derivatives by centrifugation in Paramecium (Sonneborn, 1953) and Tetrahymena (Nanney, 1953) is followed by deviations from the normal pattern of differentiation of macronuclear anlagen may, however, shed some light on this question. These and other experiments demonstrate that the different fates of the derivatives of the last synkaryon division depend on their different positions in the cytoplasm. The spindles of the last synkaryon division are usually parallel to each other and parallel to the longitudinal axis of the exconjugant's body, so that derivatives of these spindles lie in two groups, the anterior and the posterior. The nuclei of the anterior group usually develop into macronuclear anlagen, and nuclei of the posterior group into micronuclei. The synkaryon derivatives seem to be "sensitive" to the regional cytoplasmic influences during a short time only; oonce differentiation has set in, it becomes irreversible (Sonneborn, 1953; Nanney, 1953). Maupas (1889) reported that differentiation of the anterior synkaryon derivatives into macronuclear anlagen occurs in P. aurelia as well as P. caudatum, Colpidium colpoda, and Tetrahymena patula. Further, Egelhaaf (1955) demonstrated that cytoplasmic gradients are responsible for the determination of macronuclear anlagen and micronuclei, by cutting P. bursaria exconjugants transversely immediately after the last synkaryon division.

Chen (1951) also showed that in exconjugants resulting from crosses between a young and an old clone of P. bursaria that the ratio of macronuclear anlagen to micronuclei may change towards an increase of the number of micronuclei at the cost of the anlagen, as well as in

the opposite direction. However, the total number of synkaryon derivatives remained normal.

The most reasonable explanation for the occurrence of doublets with various distributions of macronuclear anlagen, micronuclei, and abnormal anlagen may be due to the grossly abnormal makeup of the cell. Following the second post-zygotic division of the two synkarya, when determination normally occurs, most of the eight derivatives of the two synkarya must have been dislocated from their proper orientation. In the case of the cells having 11-12 derivatives, a third post-zygotic division of the products of one of the synkarya must have occurred. Additional synkaryon divisions are frequently followed by formation of supernumerary macronuclear anlagen; abnormal supernumerary macronuclear anlagen arising from a third division of the synkaryon have been reported in P. aurelia exconjugants (Hertwig, 1889; Maupas, 1889). An abnormal fourth synkaryon division also may occur in ciliates usually having three divisions, i.e. in Bursaria truncatella (Poljansky, 1934), and Paramecium caudatum (Diller, 1940; Maupas, 1889; Ossipov and Skoblo, 1968) where instead of the usual four, five to nine macronuclear anlagen may appear in the latter species following conjugation, and one to 10 anlagen, following induced autogamy (Raikov, 1972, for review). It is not unlikely that doublet P. aurelia with variable numbers of macronuclear anlagen and anlagen which are abnormal in morphology arise due to similar events.

It should be obvious, but commonly is not recognized as such, that the nuclei alone cannot direct their fate when they are maintained

in a cell having an abnormal cortex and cytoplasm. This is not merely because the rest of the cell provides a limiting membrane with the function of regulating ingress and egress of materials, for there are regionally diverse decisive areas in the interior of a specific cell. Nuclear events within a cell therefore depend on a unique overall organization. The various developmental and genetic events which are regionally localized in different parts of the cell interior may thus be dependent upon specific molecular combinations between newly formed molecules deriving from the genes and pre-existing molecular patterns already present in the cortex.

The cause of the abnormal phenotype and lack of division in the "basketball" cells is unknown. Perhaps events which could give rise to variable numbers of macronuclear anlagen as mentioned previously, might also inhibit development of nuclei or other critical events following reorganization.

2. Distribution of Macronuclear Anlagen at the First Cell Division

The pattern of distribution of macronuclear anlagen at the first exconjugant cell division in doublets was examined. Stock GM_3 doublet cells were mated on both sides with singlets. After separation, the exconjugant doublet cells were maintained in individual depression slide cultures. At the first exconjugant cell division both daughter doublets were dried on albumenized microscope slides and stained with Azure-A.

The distribution of anlagen between daughter cells at the first cell division varied (Table IV). While nearly 50% of the parent doublets

TABLE IV

DISTRIBUTION OF MACRONUCLEAR ANLAGEN BETWEEN EACH SISTER CELL AT THE FIRST POST-CONJUGATIONAL DIVISION

Type	No.	%	Approximate Mean No. of Micronuclei Per Parent Cell
4:4	1	3.1	0
4:2	1	3.1	2
4:0	1	3.1	4
3:1	4	12.5	4
2:2	11	34.3	4
2:1	5	15.6	5
2:0	5	15.6	6
1:1	4	12.5	6

contained four anlagen (each control singlet exconjugant contained two), only 34.3% of the daughter cells had the normal 2:2 distribution of anlagen.

Further, five out of 32 cells examined in the second cell cycle had three anlagen present in the parent exconjugant doublet. The two cases in which doublets were found to have a 4:2 and a 4:4 distribution of anlagen in the second cell cycle, suggest that the parent cells must have contained six and eight anlagen respectively. Here, as was observed previously (Table III), there was a total of eight derivatives of the two synkaryons (including micronuclei) for each pair of daughter cells.

Cells examined in the second cell cycle also support the hypothesis of abnormalities of nuclear determination occurring in the first cell cycle. Some cells had several large dark-staining fragments and one or two very small abnormal looking anlagen, and appeared to be in the early stages of macronuclear regeneration or "MR" (Sonneborn, 1947) (Figure 8). If this is true it suggests that DNA synthesis in the macronuclear fragments was not repressed to the normal extent by the abnormal anlagen and were undergoing MR as if no anlagen were present at all.

The wide variation in thennumber of macronuclear anlagen between sister cells (Table IV) arose as a result of missegregation of anlagen at the first exconjugant cell division (Figure 9). My data for distribution ratios of anlagen are comparable with the results reported by Butzel (1973) who used incompletely fused doublets or cytoplasmically-bridged pairs (CBPs). The fact that my data concern events occurring in fully formed doublets emerging from conjugation, while Butzel was describing nuclear phenomena in CBPs, may account for the small discrepancies.

Figure 8. Photograph of a doublet emerging from sexual reorganization illustrating abnormal nuclear development. aa- abnormal macronuclear anlagen, rf - regenerating macronuclear fragments. x 2240.

Figure 9. Examples of the variation in distribution due to missegregation of macronuclear anlagen at the first post-conjugational cell division between daughter cells derived from a parent cell containing two or four anlagen. a. Normal 2:2 distribution of anlagen to each daughter cell from a parent doublet containing four anlagen. b. Missegregation of one anlage at the first post-conjugational cell division producing a 3:1 distribution in the daughter cells. c. Missegregation of two anlagen at the first post-conjugational cell division producing a 4:0 distribution in the daughter cells. d. Normal 1:1 distribution of anlagen to each daughter cell from a parent doublet containing two anlagen. 3. Missegregation of both anlagen at the first post-conjugational cell division producing a 2:0 distribution in the daughter cells.

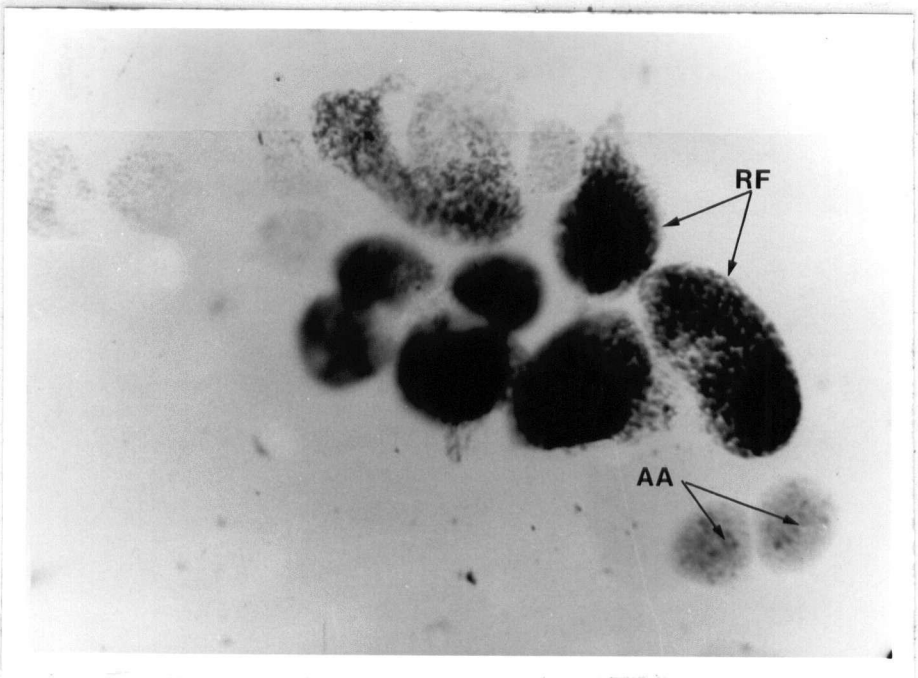


FIGURE 8

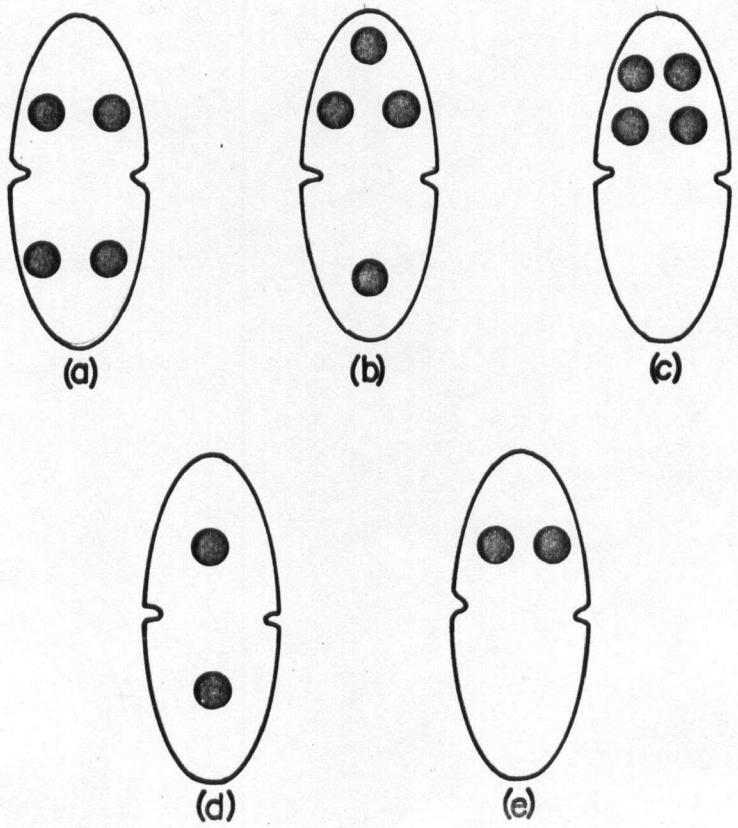


FIGURE 9

3. Change in Number of Macronuclei in Exconjugant and Exautogamous Doublet Clones

The presence of a single large macronucleus lying transversely between the two gullets in doublet P. aurelia (Sonneborn, 1963) invited a further series of experiments to examine the kinetics of macronuclear loss in doublets following sexual reorganization. Exconjugant and exautogamous lines were maintained as single cell isolates. Each time a cell divided, one of the two daughter cells was dried on an albumenized slide. A record was kept indicating that the cell had divided (to keep track of the number of fissions that each line had undergone), and a list of the line number and its appropriate place on the slide map was maintained. The object was to fix one daughter cell at each cell cycle for each of the lines through a period of 10 cell cycles. When all of the cells had been fixed, the slides were stained with Azure-A or Leucobasic fuchsin (Appendix I) and the number of macronuclei in each cell were scored. From the line numbers on the slide map and a table of the number of fissions undergone by each line, the history of each line could be reconstructed.

This experiment was done a number of times using both exconjugant and exautogamous doublets from a number of stocks. Exconjugants produced fewer "basketballs" resulting in higher numbers of examinable lines than did exautogamonts.

The first successful experiment of this type was done using exautogamous cells of Stock KVD4. Of 46 viable lines examined at the second cell cycle, 89% of the cells had two nuclei, while 11% had one nucleus (Figure 10a). Loss of the second nucleus in cells containing

two in the second cell cycle was roughly exponential (Figure 10b), although it was not until the ninth cell cycle that 100% of the cells of the 12 remaining lines had only one macronucleus. The reliability of the graphs in Figures 10a and 10b for the later cell cycles is not as great as that for the earlier, because of the declining sample size in the later cell cycles. Occasionally, loss of the second macronucleus was more rapid as in experiments 1 and 3 shown in Table V, in which complete loss of the second macronucleus occurred by the fifth to sixth cell cycles. In experiment 3 of Table V, a large fraction of cells (48/82) had only a single macronucleus during the second cell cycle.

The data show that macronuclear loss in doublets emerging from sexual reorganization follows a definite pattern. By the time most doublets have reached the fifth to sixth cell cycle they contain only one macronucleus; the fraction of cells having two macronuclei decreased exponentially from the second cell cycle. These results are similar to those obtained by Samoiloff (personal communication) who also observed an approximate exponential decrease in the number of cells containing two macronuclei in exautogamous doublets.

The occurrence of abnormalities in nuclear determination in doublets leads to the conclusion that the reasons for these abnormalities lie in the nature of the balance between cortex and cytoplasm. Further, the reoccurrence of abnormal nuclear determination in exconjugants after periods of vegetative reproduction indicates that the frequency of abnormal nuclear determination does not decrease with clonal age.

Figure 10a. Percent of doublets with two macronuclei in exconjugant and exautogamous doublet clones.

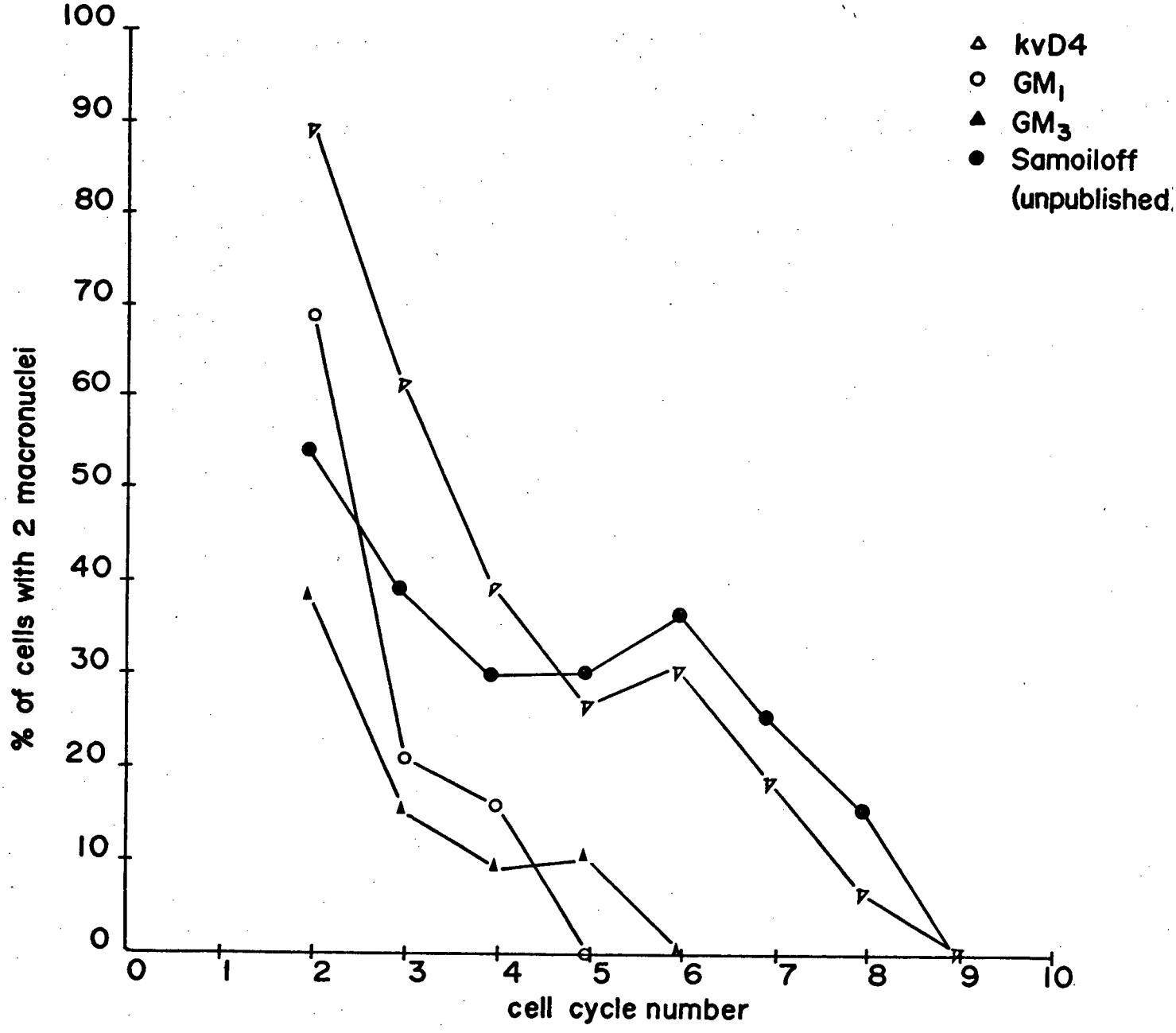

100
90
80
70
60
50
40
30
20
10
0
% of cells with 2 macronuclei
0
1
2
3
4
5
6
7
8
9
10
cell cycle number
kvD4
GM1
GM3
Samoiloff
(unpublished)

FIGURE 10a

Figure 10b. Log_2 percent of doublets with two macronuclei in exconjugant and exautogamous doublet clones.

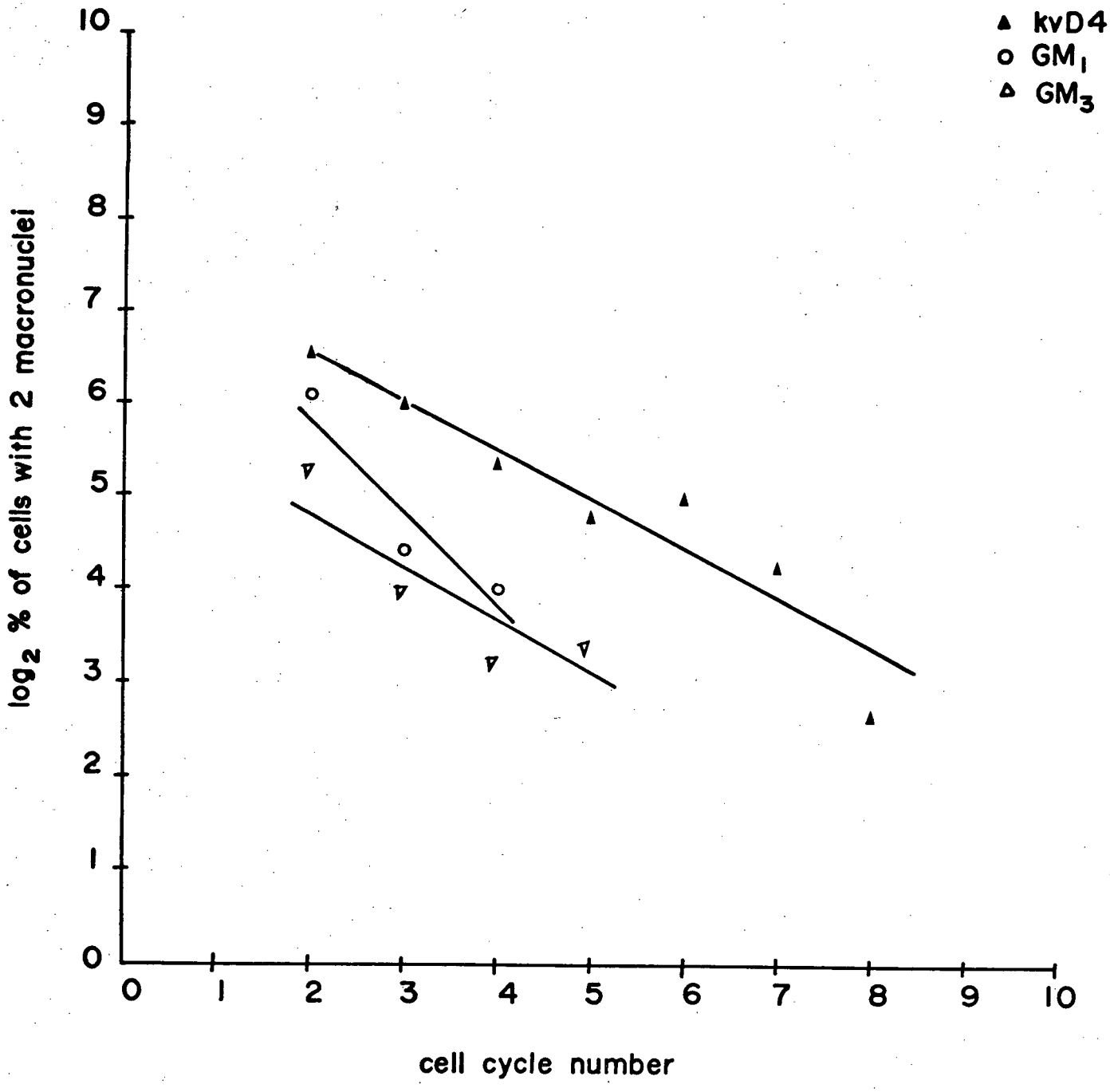

▲ kvD4
o GM1
△ GM3
log2 % of cells with 2 macronuclei
cell cycle number
0
1
2
3
4
5
6
7
8
9
10

FIGURE 10b

TABLE V

FRACTION OF OBSERVED DOUBLETS WITH TWO OR MORE MACRONUCLEI

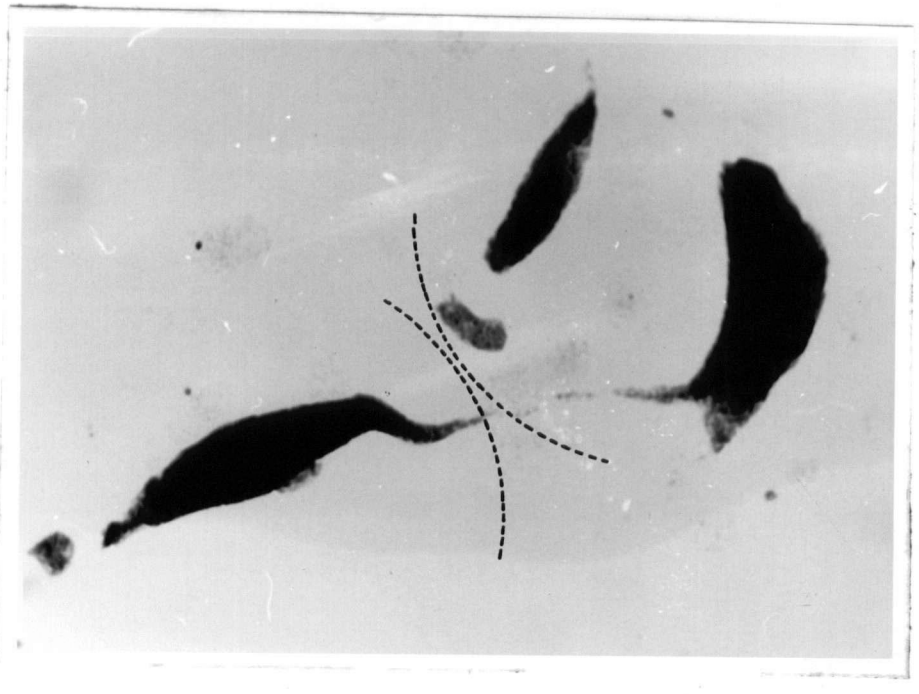
Expt. No.	Doublet Stock	Cell Cycle 2	3	4	5	6	7	8	9	10
1*	GM_1	90/133	7/34	4/25	0/24	0/20	0/19	0/18	0/11	0/7
2*	KVD4	41/46	22/36	11/28	6/23	7/23	4/22	1/16	0/12	0/4
3*†	GM_3	31/82	7/48	2/23	2/19	0/13	0/13	0/12	0/11	0/9

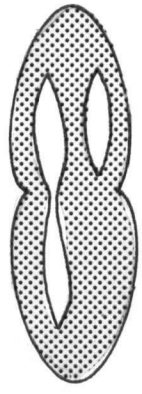
*Exautogamonts

†Exconjugants

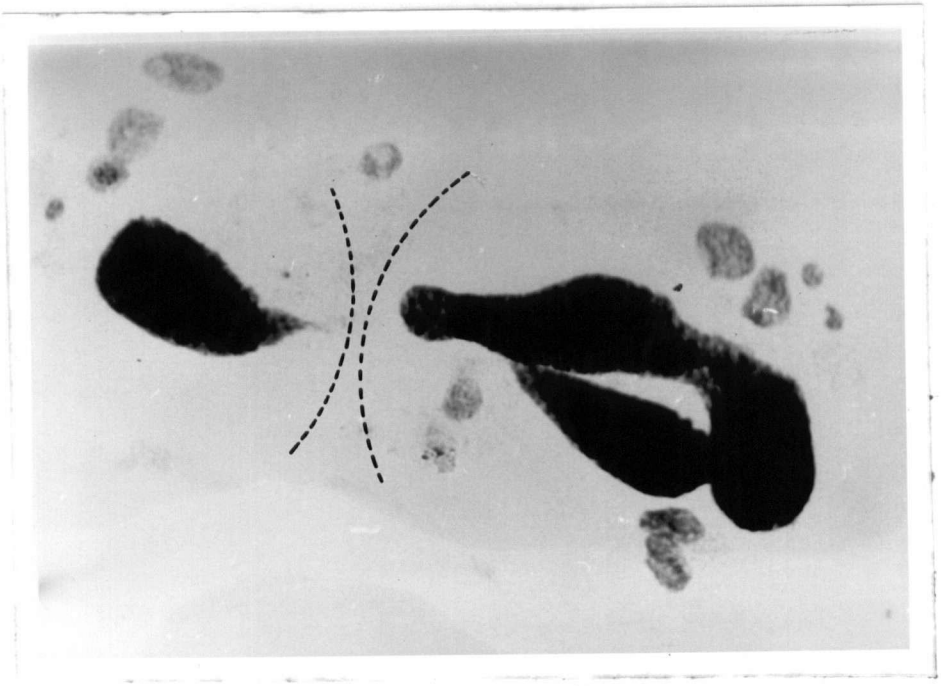
In the foregoing experiments, deviations from the "theoretical" pattern of nuclear behavior in P. aurelia doublets (Figure 5b) were frequently noted. Further, loss of the second macronucleus occurred at or near the fourth exconjugant cell cycle. In considering these observations, a number of possible macronuclear events at cell division which could give rise to doublet cells with one macronucleus were apparent; these are a) misdivision, b) missegregation, and c) fusion of macronuclei as shown below.

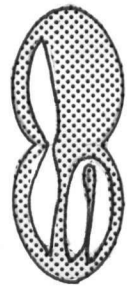
Misdivision



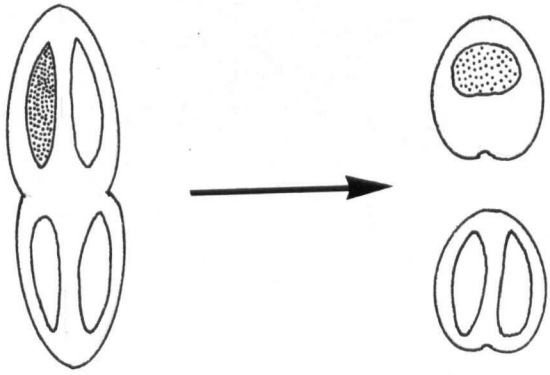


Missegregation





Fusion



A reduction in thennumber of macronuclei in daughter cells could be achieved by misdivision and missegregation (or both) of macronuclei in the parent cell. Further, both misdivision and missegregation of macronuclei were observed at low frequency in the population. However, the third possibility of fusion of macronuclei causing a reduction in macronuclear number has not yet been examined. Therefore a genetic experiment (Part B, Section I) was devised to test whether fusion of macronuclei is the usual method causing a decrease in macronuclear number.

B. Does Fusion of Macronuclei Play a Significant Role in Reducing the Number of Macronuclei Per Cell?

1. Genetic Evidence to Test the Hypothesis of Regular Macronuclear Fusion

The hypothesis that reduction in macronuclear number resulted from a regular fusion of macronuclei following a sexual cycle was tested genetically.

Single cells marked with the behavioral mutant pawn (pw/pw) (Kung, 1971) were mated on one side to wild-type (+/+) doublet paramecia (Figure 11a). After pair separation the exconjugant singlets were tested for avoidance in Dryl's solution (Appendix I) to be sure that mating had occurred (Figure 11b). At the first cell division after conjugation, one of the daughter doublet cells of each line that had mated was stained with Azure-A. If the stained cell was found to contain two anlagen, its sister cell, also presumably containing two anlagen, was isolated and its progeny were grown vegetatively for approximately 30 fissions.

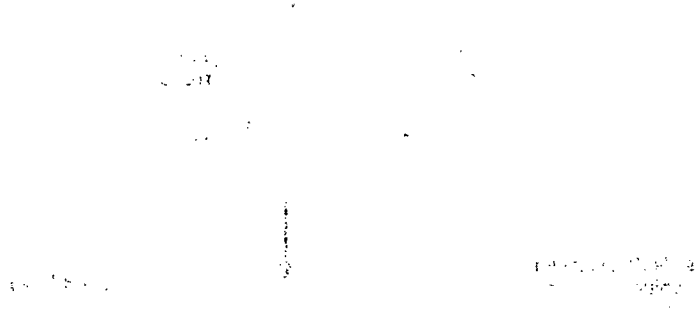


Figure 11. Diagram of the genetic experiment to test the hypothesis of regular macronuclear fusion.

GENETIC EXPERIMENT TO TEST THE HYPOTHESIS
OF REGULAR MACRONUCLEAR FUSION

EXPERIMENTAL DESIGN

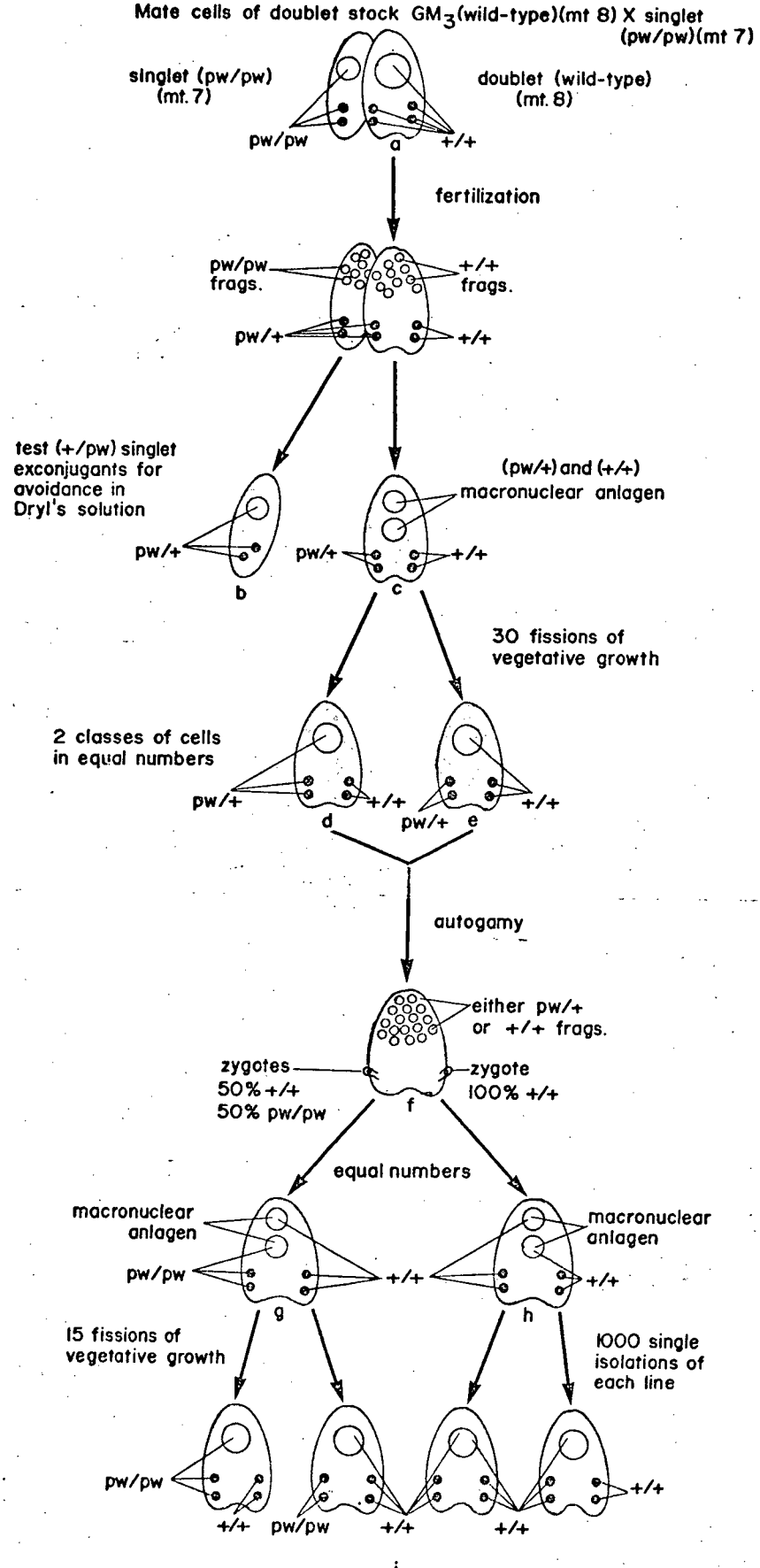


FIGURE 11

If the original doublet that was mated to a (pw/pw) singlet contained four anlagen in the first exconjugant cell cycle, one would expect that the daughter cells at the first cell division would contain two anlagen of different genotypes (i.e. one anlage would be (pw/+) being derived from the heterozygous synkaryon produced by conjugation, the other anlage would be (+/+) having been derived from a homozygous wild-type synkaryon produced by autogamy on the side of the doublet which did not mate) (Figure 11c). If segregation of these two genetically different anlagen occurs at the second post-zygotic cell division one would expect two classes of cells in equal numbers but of different genotypes to result: one class would contain a heterozygous (pw/+) macronucleus, two heterozygous (pw/+) micronuclei, and two homozygous (+/+) micronuclei (Figure 11d). The other class would contain a homozygous (+/+) macronucleus, two heterozygous (pw/+) micronuclei, and two homozygous (+/+) micronuclei (Figure 11e).

After the vegetative growth period of 30 fissions, both classes of doublets were starved, inducing them to undergo bilateral autogamy (Figure 11f) (as was checked by the presence of two anlagen in the sister exautogamont in the second cell cycle), from which a further two classes of progeny in equal numbers resulted. One class of cells contained two anlagen, one of which would be homozygous (pw/pw) and the other homozygous (+/+), with one pair of homozygous (pw/pw) micronuclei and one pair of homozygous (+/+) micronuclei (Figure 11g). The other class would contain two anlagen and four micronuclei, all of which would be genetically homozygous (+/+) (Figure 11h).

One thousand single cell isolations of each of the two clones which survived at this point were made and grown vegetatively for 15 fissions. Individual cell lines were then scored for avoiding behavior in Dryl's solution. If segregation of macronuclear anlagen following autogamy occurred, one would expect to see a ratio of three wild-type (+/+) to one pawn (pw/pw) (Figure 11i). The results of this experiment are given in Table VI.

Correcting the values in Table VIa after taking into account the frequency of macronuclear regeneration (MR), which was observed to be approximately eight percent in the doublet stock used, the data in Table VIb were obtained. These data are reasonably consistent with the expected ratio.

Had fusion of non-sister pronuclei occurred after the third maturation division, two classes of doublets in equal numbers but of different genotypes would result; one class would be heterozygous (pw/+) for both macro- and micronuclei, the second class would be homozygous (+/+) for both macro- and micronuclei. Both classes would be scored as having wild-type avoidance behavior in Dryl's solution. Therefore, the presence of the pawn phenotype observed in the final scoring, rules out the possibility of synkaryon formation resulting from the fusion of non-sister pronuclei in this experiment.

Further, less than 15% fusion of homozygous pawn (pw/pw) macronuclei with wild-type (+/+) macronuclei (Figure 11g) would be required to produce the observed wild-type to pawn ratio (Table VIb). Therefore, although it has not been proven that macronuclear fusion does not occur,

TABLE VI

GENETIC EXPERIMENT TO TEST THE HYPOTHESIS OF REGULAR MACRONUCLEAR FUSION EXIT RATIO (WILD-TYPE/PAWN)

	Wild-type	Pawn	Ratio (wild-type/pawn)
Line No. 19-1	365	62	5.88:1
Line No. 15-1	119	20	5.95:1
	(a)		
Line No. 19-1	335.8	91.2	3.68:1
Line No.115-1	109.4	29.5	3.72:1
	(b)		

it has been shown that observed reduction in number of macronuclei per cell cannot be the result of macronuclear fusion in the majority of cases.

The high ratio of wild-type (+/+) to pawn (pw/pw) (3.68:1 and 3.72:1) (Table VIb) instead of the 3 (+/+):1 (pw/pw) ratio expected, may have also resulted from a lower viability of cells homozygous for the pawn gene (personal observations) or from errors in scoring avoidance behavior (Appendix I).

In conclusion, these experiments have shown that the number of macronuclear anlagen varied considerably from the number expected (four) if the situation in doublets was analogous to that in singlet P. aurelia. Genetic evidence indicates that the most plausible explanation for variability in numbers of anlagen was abnormal patterns of macronuclear determination of the products of the second post-zygotic division of the two synkarya, presumably occurring because of the somewhat abnormal morphology of doublet cells. Further, the distribution of macronuclear anlagen at the first cell division was also irregular (only 34.3% of the daughter cells had the expected 2:2 distribution of anlagen). As well, a number of "abnormal" anlagen were observed in cells which appeared to be undergoing macronuclear regeneration. Most doublets that initially contained two macronuclei in the second cell cycle lost the second macronucleus by the fourth to fifth cell cycle. The pattern of loss is approximately exponential. Misdivision and missegregation of macronuclei, mechanisms which could account for macronuclear loss, were observed to be occurring at low frequency. Genetic evidence showed that fusion of macronuclei, a third mechanism for change in macronuclear number, if it occurs, could not take place more than about 15% of the time.

Section II will now examine quantitatively by microdensitometry (Appendix I) the DNA content of doublets with various numbers of macronuclei.

SECTION II

QUANTITATIVE ANALYSIS OF DNA CONTENT AND ITS DISTRIBUTION TO DAUGHTER CELLS AT FISSION

The consequences of variation in the number of macronuclei on the DNA content of individual macronuclei and total DNA content per cell were examined with the following fact in mind: In P. aurelia doublets there are two types of divisions; those at which there is no change in the number of macronuclei per cell, and those at which the number of macronuclei is changed. The latter events lead to a reduction of the number of macronuclei as shown in section I. These events correspond to misdivision and missegregation. Analysis of these division events in comparison with "normal" division events in which there is no change in macronuclear number make it possible to: 1) acquire some idea about how the change in macronuclear DNA content which accompanies the transition from two macronuclei to one occurs, 2) learn something about how the DNA content is regulated, and 3) examine the relative frequencies of misdivision and missegregation.

The relative DNA content of Feulgen-stained doublet macronuclei was estimated by microdensitometry (Appendix I). Dividing cells were

selected from log-phase cultures and were isolated into drops of culture fluid. After division, each daughter cell was fixed and prepared as described in the methods section. The G_2 DNA content of the original divider was assumed to be the sum of the DNA contents of the G_1 daughter macronuclei. In analysing the data, several questions were examined:

A. DNA Content of Macronuclei in Doublets

1. Is the DNA content of all doublet stocks the same?

Three different doublet stocks were used in the course of these experiments. It was desirable to pool data from the three stocks to increase sample sizes. Data from the three stocks were tested for homogeneity by one-way analysis of variance (Table VII). While the G_2 DNA contents of stocks 2B and 3A were similar, the mean DNA content of stock H9W was about 17% larger. This difference was not significant at the five percent level. Variances, however, were highly heterogeneous. Therefore, to reduce some of the heterogeneity of variance the data were reclassified. Cells were grouped according to the number of macronuclei per cell and the homogeneity of the data was re-examined. Data from stocks 2B and 3A were homogeneous throughout, while the mean DNA content of stock H9W was significantly greater in both uni- and bi-macronucleate cells (Table VIII). Data for DNA content of cells with three macronuclei per cell from all three stocks were homogeneous. In subsequent analyses data from stocks 2B and 3A were pooled (unless otherwise indicated).

TABLE VII

G_2 DNA CONTENT OF DOUBLETS

Stock	N	$E._{tot} \pm$ S.E.	Coefficient of Variation (%)
2B	44	222.7±12.7	64.2
3A	42	213.2±11.3	34.5
2B & 3A (pooled)	86	218.0±8.9	49.3
H9W	50	255.5±13.6	37.6

F (pooled data from 3 doublet stocks) = 1.98

Probability of greater F = 0.1

Probability of homogeneity of variances = 0.00

TABLE VIII

MEAN G_2 DNA CONTENT OF ALL DOUBLETS WITH ONE, TWO OR MORE THAN TWO MACRONUCLEI

Stock	No. of Macronuclei	N	$E._{tot}$ ±S.E.	Coefficient of Variation (%)	F	P_α	P_v
A.							
*2B, 3A	1	80	170.2±8.9	31.2	0.21	>0.10	0.42
H9W	1	58	229.1±14.7	34.6	7.34 (3 stocks)	0.00	0.03
B.							
*2B, 3A	2	102	214.7±8.1	27.0	0.86	>0.10	0.93
H9W	2	38	258.2±21.0	35.5	3.13 (3 stocks)	0.05	0.04
C.							
*2B, 3A, H9W	>2	32	260.6±24.9	36.4	0.43	>0.10	0.15

F (among stocks and numbers of macronuclei) = 6.61. Probability of greater F = 0.00.
Probability of homogeneity of variances = 0.06. Data are not homogeneous by Dunnett's procedure.
*Homogeneous data as judged by one-way Anova and Bartlett's test.
F (among cells with one and two macronuclei for stocks 2B and 3A) = 15.57). Probability of greater F = 0.00. Probability of homogeneity of variances = 0.40.

Unimacronucleate cells of stock H9W had approximately 35% more DNA per cell than unimacronucleate cells of stocks 2B or 3A. Bimacronucleate cells of stock H9W contained approximately 20% more DNA per cell than bimacronucleate cells of stocks 2B or 3A. Subsequent analysis (section III) indicated that the mean cytoplasmic content (macromolecular dry mass) of stock H9W was also substantially (27%) higher than that of the other stocks. The implications of those observations will be discussed further in section III.

2. Is DNA content a function of the number of macronuclei per cell?

The ciliate macronucleus is a compound nucleus containing a large number of subnuclei or units (Nanney and Rudzinska, 1960; Wolfe, 1967; Preer, 1968; Stevenson and Lloyd, 1971b), each of which consists of an entire genome. Thus, variation in DNA content can occur without genic imbalance. Doublets have been observed with up to six macronuclei (section I). It there was no mechanism to regulate the amount of DNA per cell, the DNA content per cell might be expected to be proportional to the number of macronuclei per cell. A glance at Table VIII, however, shows that this is only partly true. The mean DNA content per cell increases only slightly as the number of macronuclei increases. In stocks 2B and 3A, cells with two macronuclei have an average of 126% of the DNA content of unimacronucleate cells. This difference is significant ($P_{\alpha} > 0.05$). When the number of macronuclei per cell was increased to three the mean DNA content increased a further 27% to 153% of the mean value for unimacronucleate cells. This difference was also significant

($P_\alpha > 0.05$). Trimacronucleate cells had an average of 121% of the DNA content of the bimacronucleates. This last difference was also significant ($P_\alpha > 0.05$). In stock H9W which had a higher initial DNA content, there was only a 13% increase in the mean DNA content of bimacronucleate cells and no further increase in trimacronucleates. Further, the observed mean DNA content per cell was significantly higher ($P_\alpha > 0.05$) in unimacronucleate cells and lower ($P_\alpha > 0.05$) in trimacronucleates than the expected value if the DNA content per cell depended totally on the number of macronuclei each cell contained (Table VIII).

A graph of the values of observed mean DNA content for doublets from stocks 2B and 3A with one, two and three macronuclei (Table VIII) along with the expected values of mean DNA content if DNA content was totally dependent on the number of macronuclei per cell, is shown in Figure 12. The slope of the line connecting the points for observed mean DNAccontent is 0.27; the slope of the line connecting the points for the 'expected' mean DNA content is 1.00. Cells with two macronuclei have a mean DNA content which is approximately 63% of the 'expected' value. Cells with three macronuclei similarly have a mean DNA content which is approximately 51% of the 'expected' value if DNA content was completely ɔdependent on the number of macronuclei per cell.

These data suggest that the DNA content of macronuclei is regulated in such a way that the number of macronuclei per cell has relatively little effect on the total DNA content. The G_2 cell DNA content is largely determined by other factors which presumably include the nucleocytoplasmic ratio. This will be further discussed in section III.

Figure 12. Observed vs. expected mean DNA content for doublets with one, two and more than two macronuclei. Vertical bars are 95% confidence limits of the mean.

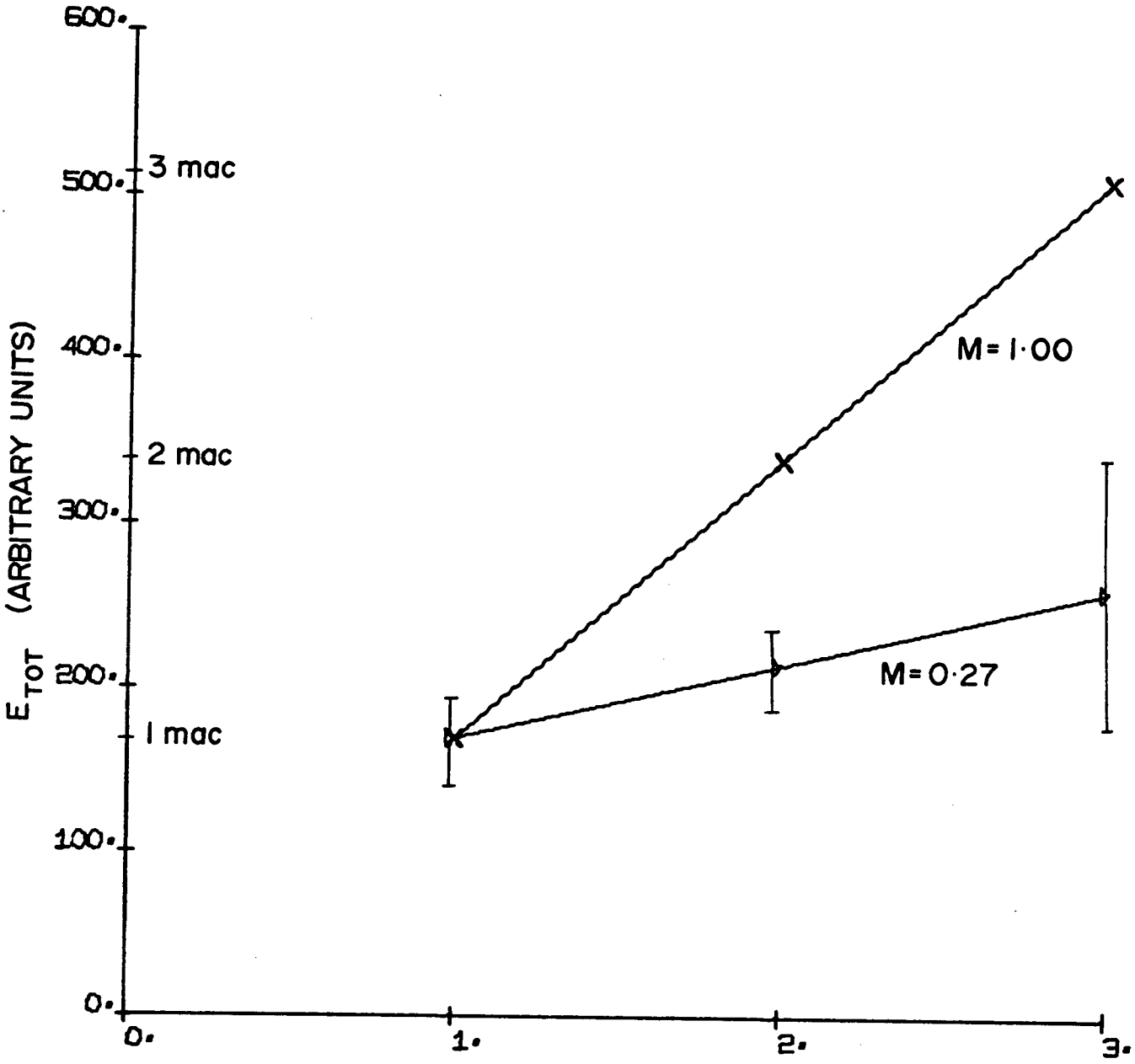


FIGURE 12

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B. Is the Distribution of DNA to Daughter Macronuclei and to Daughter Cells Equal?

As shown above (Table VIII), the DNA content of doublets is quite variable, even when only cells from a single stock containing the same number of macronuclei are considered. In this sub-section the distribution of DNA to daughter cells at division will be considered as a source of part of the variance in DNA content observed in doublet stocks. This variance (CV = 45%) is substantially greater than that observed in singlets (9.8% to 11.0%) (Kimball and Barka, 1959; Kimball, 1967; Berger, 1973).

1. Cases in which the number of macronuclei in daughter cells is equal

a) Distribution of DNA per cell

The variance in G_1 DNA content was separated into components attributable to variation between pairs of daughter cells and variation within pairs of daughter cells by application of one-way analysis of variance. The intraclass correlation coefficient was then calculated. This statistic is the ratio of the between-pair variance to the sum of the between- and within-pair variances. The greater this coefficient, which ranges from -1/(n-1), where 'n' is the sample size, to unity, the more significant are the differences between pairs. The significance of the intraclass correlation coefficient can be tested by calculating the F statistic for the intraclass correlation coefficient (Ostle, 1963). The variance attributable to differences within pairs includes the variance due to measuring error (Appendix II) as well as that due to true

differences in the DNA content between pairs of daughter cells. The variance attributable to differences between pairs should be a measure of the true difference between pairs, free of measuring error. The mean difference in DNA content of daughter cells was also calculated as a percentage of the parental G_2 DNA content.

The data (Table IX) show that in stocks 2B and 3A, daughter cells were quite similar in DNA content as judged both by the intraclass correlation coefficient and the low mean difference in DNA content between sister cells. On the other hand, in stock H9W, the similarity of daughter cells was low as judged by the lack of significant intraclass correlation and the higher mean difference in DNA content between sister cells indicating a greater inequality of division of the G_2 macronucleus. The variance of the mean DNA content between sister cells was also substantially greater than that observed in the other stocks.

When two macronuclei were present, both the intraclass correlation coefficient and the mean difference between DNA content of sister cells were similar to those observed in unimacronucleate cells. The variance of the mean difference in DNA content of sister cells with two macronuclei was, however, somewhat higher. As only one pair of daughter cells from each stock contained an equal number of more than two macronuclei in each cell, the sample sizes were too low to draw any definite conclusions. In these cases, the intraclass correlation coefficient was not calculated.

TABLE IX

DISTRIBUTION OF G_1 DNA TO DAUGHTER CELLS CONTAINING EQUAL NUMBERS OF MACRONUCLEI

Stock	No. of Macronuclei	N	Average Diff. Between Cells (% of G_2 Mac)	Coefficient of Variation (%)	Intraclass Correlation Coefficient(r_i)	P_α
*2B, 3A	1	64	5.3±0.4	68.7	+0.81**	<0.01
H9W	1	45	10.2±2.2	103.7	+0.16	>0.05
*2B, 3A, H9W	2	118	5.8±0.6	104.5	+0.72**	0.05-0.01
2B	>2	6	4.8±1.3	39.7		
3A	>2	6	0.0±0.0	00.0		
H9W	>2	8	7.3±0.0	00.0		

*Homogeneous data as judged by one-way Anova and Bartlett's test.

** Variance in G_1 DNA content among pairs of sister cells is significantly greater than variance within pairs.

Pooled data for average differences between cells bearing various numbers of macronuclei was judged homogeneous by one-way Anova and Dunnett's procedure. F = 0.68. Probability of greater F = 0.1. Probability of homogeneity of variances = 0.23.

b) Distribution of DNA per macronucleus

A study of the degree of similarity of G_1 DNA content among macronuclei in pairs of daughter cells with two or more macronuclei in each cell was done to determine whether macronuclei of similar DNA content tended to segregate together. By comparing the variance within pairs or groups of macronuclei to the variance between pairs of daughter cells it was possible to determine if macronuclei with similar DNA contents segregated together or independently. To show selective segregation, macronuclei within a single daughter cell would have to be more similar in DNA content (variance within reduced) than macronuclei among the pairs of daughter cells (variance between increased). A diagrammatic representation of selective vs. nonselective segregation of macronuclei in doublets is shown in Figure 13.

The values for average differences between macronuclei for the various stocks as well as the intraclass correlation coefficients are given in Table X.

The data in Table Xa show that variation in G_1 DNA content among pairs of macronuclei significantly exceeded variation within pairs for the three doublet stocks. However, the intraclass correlation coefficient within pairs varied from +0.36 to +0.60 between stocks indicating that the degree of similarity of macronuclei within cells varied from stock to stock. Further, when comparing the pooled value for coefficient of variation for average differences between macronuclei (102.4%) (Table Xa), with the value for the coefficient of variation for average differences between daughter cells with an equal number of two macronuclei

Figure 13. Selective vs. non-selective segregation of macronuclei from a parent to the daughter cells.

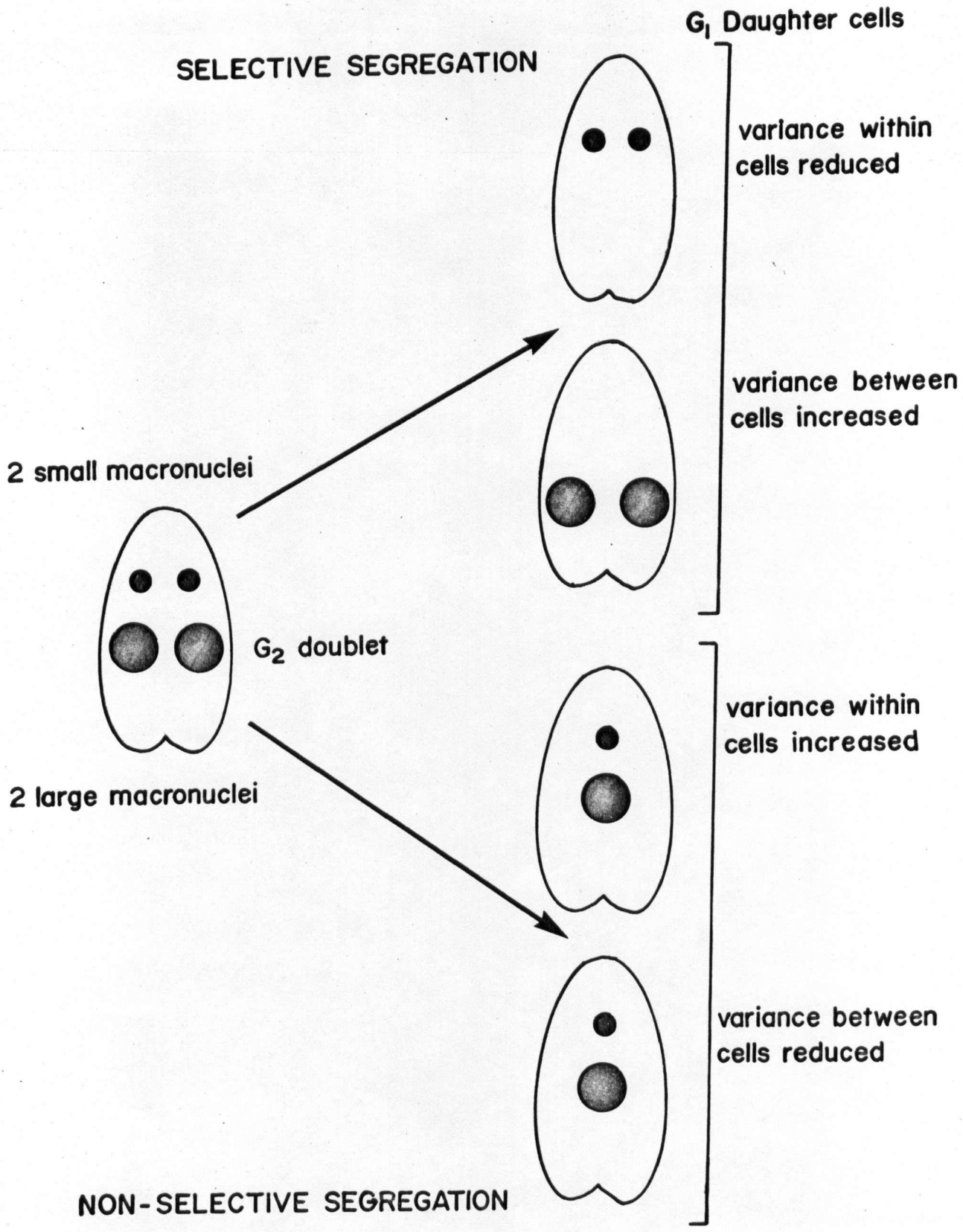
G1 Daughter cells
SELECTIVE SEGREGATION
variance within cells reduced
variance between cells increased
2 small macronuclei
G2 doublet
variance within cells increased
2 large macronuclei
variance between cells reduced
NON-SELECTIVE SEGREGATION

FIGURE 13

TABLE X

DISTRIBUTION OF G_1 DNA BETWEEN MACRONUCLEI WHEN EQUAL NUMBERS OF MACRONUCLEI ARE PRESENT IN EACH DAUGHTER CELL

	Stock	No. of Macronuclei	N	E. tot ±S.E. %G_1 Macronucleus	C.V.(%)	r_i	P_α	Average Diff. Between Macs (% of G_2 Mac)	C.V.(%)
	A.								
	2B	2	99	51.6±2.9	57.7	+0.41**	0.05-0.01	4.7±0.6	94.8
*	3A	2	76	53.1±2.2	36.2	+0.60**	<0.01	3.1±0.5	104.4
	H9W	2	82	61.7±3.8	56.9	+0.36**	0.05-0.01	5.7±0.8	99.2
	Pooled	2	257	55.2±1.7	50.2	+0.45		4.5±0.4	102.4
	B.								
	2B	3	6	44.3±7.4	50.0	0.00	>0.05	7.0±1.8	37.1
*	3A	3	6	25.0±2.3	22.6	0.00	>0.05	1.1±0.1	12.8
	H9W	4	8	23.7±2.4	29.4	+0.42**	0.05-0.01	0.7±0.2	40.9
	Pooled	3-4	14	24.2±1.3	26.0	0.21		0.9±0.1	26.8

*Homogeneous data as judged by one-way Anova and Bartlett's test.

** Variance in G_1 DNA content among groups of macronuclei is significantly greater than variance within groups.

which was 104.5% (Table IX), it was found that the variances in both cases were statistically homogeneous at the one percent level. Also, a tally was made of the number of cases in which non-selective segregation of a single larger and a single smaller macronucleus to each daughter cell in a pair occurred. In this instance, four macronuclei in the parent cell gave rise to two larger and two smaller macronuclei in the daughter cells (Figure 13). Further, presuming that the larger macronuclei in the daughter cells were derived from the two larger macronuclei in the parent, 76% of the cases showed that the two larger macronuclei segregated to different daughter cells; and that the two smaller macronuclei similarly segregated to different daughter cells. Therefore, daughter cells in a 2:2 distribution of macronuclei tend to contain one large and one small macronucleus and are consequently more nearly equal in DNA content than by chance alone.

By non-selective segregation (Figure 13), the two daughter cells would receive an approximately equal share of parental DNA. Thus, an equal distribution of DNA to each daughter cell appears to be directly related to the non-selective manner of macronuclear segregation in the majority of cases. A similar pattern of non-selective segregation of macronuclei for cells with more than two macronuclei (Table Xb) was shown by comparing the variation in G_1 DNA content between macronuclei within a daughter cell to the variation in G_1 DNA content between pairs of daughters (Table IX). Further, the mean G_1 DNA content ($E._{tot} \pm S.E.$) of each macronucleus in sister cells containing two macronuclei was exactly half of the mean G_1 DNA content of macronuclei in daughter cells in which each cell contains one macronucleus.

As would be expected, the DNA content of each macronucleus in cells with more than two macronuclei is proportionately lower (Table Xb). Further, in the cases of stocks 3A and H9W, the mean G_1 DNA content per macronucleus in cells with three and four macronuclei was approximately equal. However, a higher degree of similarity (r_i = +0.42) existed among the four macronuclei in each daughter cell of stock H9W.

2. Cases in which the number of macronuclei in daughter cells is not equal

a) Distribution of DNA per cell

It was shown in the preceding sub-section that the mean difference in G_1 DNA content between sister doublets was about five percent of the G_2 macronucleus for both uni- and bimacronucleate cells (with the exception of unimacronucleate doublets of stock H9W). This result raises the question of whether daughter cells which receive unequal numbers of macronuclei will show a comparable equality of DNA distribution, or whether the daughter cells would have a DNA content proportional to the number of macronuclei per cell. Difference in DNA content of daughter cells receiving different numbers of macronuclei is shown in Table XI.

In those cases in which one daughter cell received two macronuclei and the other daughter cell received only one macronucleus, the distribution of G_1 DNA differed markedly between cells as evidenced by the low within-pair correlation coefficient (+0.24). A similar result was obtained in cases in which one of the daughter cells received three macronuclei and the other daughter received one macronucleus. When one daughter cell received two macronuclei and its sister cell received three

TABLE XI

DISTRIBUTION OF G_1 TO DAUGHTER CELLS CONTAINING UNEQUAL NUMBERS OF MACRONUCLEI

Stock	No. of Macronuclei	N	$E._{tot}$±S.E.	C.V.(%)	r_{ri}	P_α	Average Diff. Between Cells (% of G_2 Mac)	C.V.(%)
A. 1:2 Distribution								
*2B, 3A, H9W	1	21	116.0±9.3	36.9	+0.24	>0.05	9.4±1.8	89.3
			**					
*2B, 3A, H9W	2	21	143.0±9.9	31.7				
B. 1.3 Distribution								
*2B, 3A, H9W	1	9	71.8±10.3	43.3	0.00	>0.05	16.5±3.4	62.7
*2B, 3A, H9W	3	9	150.0±26.6	53.3				
C. 2:3 Distribution								
*2B, 3A, H9W	2	5	73.4±21.4	65.2	+0.51	<0.01	9.8±3.2	73.7
			**					
*2B, 3A, H9W	3	5	88.6±21.9	55.3				

*Homogeneous data as judged by one-way Anova and Bartlett's test.

**Homogeneous means as judged by one-way Anova.

Average differences between cells judged homogeneous for 1:2, 1:3, and 2:3 distributions of macronuclei by one-way Anova and Dunnett's procedure. F = 2.16. Probability of greater F = 0.1. Probability of homogeneity of variances = 0.68.

macronuclei, the distribution of parental G_2 DNA was more nearly equal as judged by the higher intraclass correlation coefficient (r_i = +0.51). Further, the average differences in mean G_1 DNA content between sister cells with 1:2 and 2:3 distributions of macronuclei were nearly the same. The average difference in mean G_1 between daughter cells in a 1:3 distribution was nearly twice as great.

These results show that the G_1 DNA content per daughter cell is not strictly proportional to the number of maconuclei per cell. Further, the mean DNA content of daughter cells with two macronuclei in a 1:2 distribution was only 23% higher than the DNA content of their unimacronucleate sisters. Similarly, cells with three macronuclei in a 1:3 distribution contained only 211% of the mean DNA content of their sister cells with one macronucleus. In a 2:3 distribution, cells with three macronuclei contained only 20% more DNA than their sister cells with two macronuclei.

b) Distribution of DNA among macronuclei in cells with more than one macronucleus

The distribution of parental DNA among macronuclei in cases where the number of macronuclei in daughter cells was not equal was investigated by comparing the individual values of mean G_1 DNA content per macronucleus in cells receiving more than one macronucleus. For example, in the case of a 1:2 distribution, the question was asked whether a parent cell may have had one large and two small macronuclei and passed the larger macronucleus on to one daughter cell and the two smaller macronuclei on to the other daughter cell (Figure 14a). Similarly in a 1:3

Figure 14. Variations in unequal distribution of macronuclei to daughter cells.

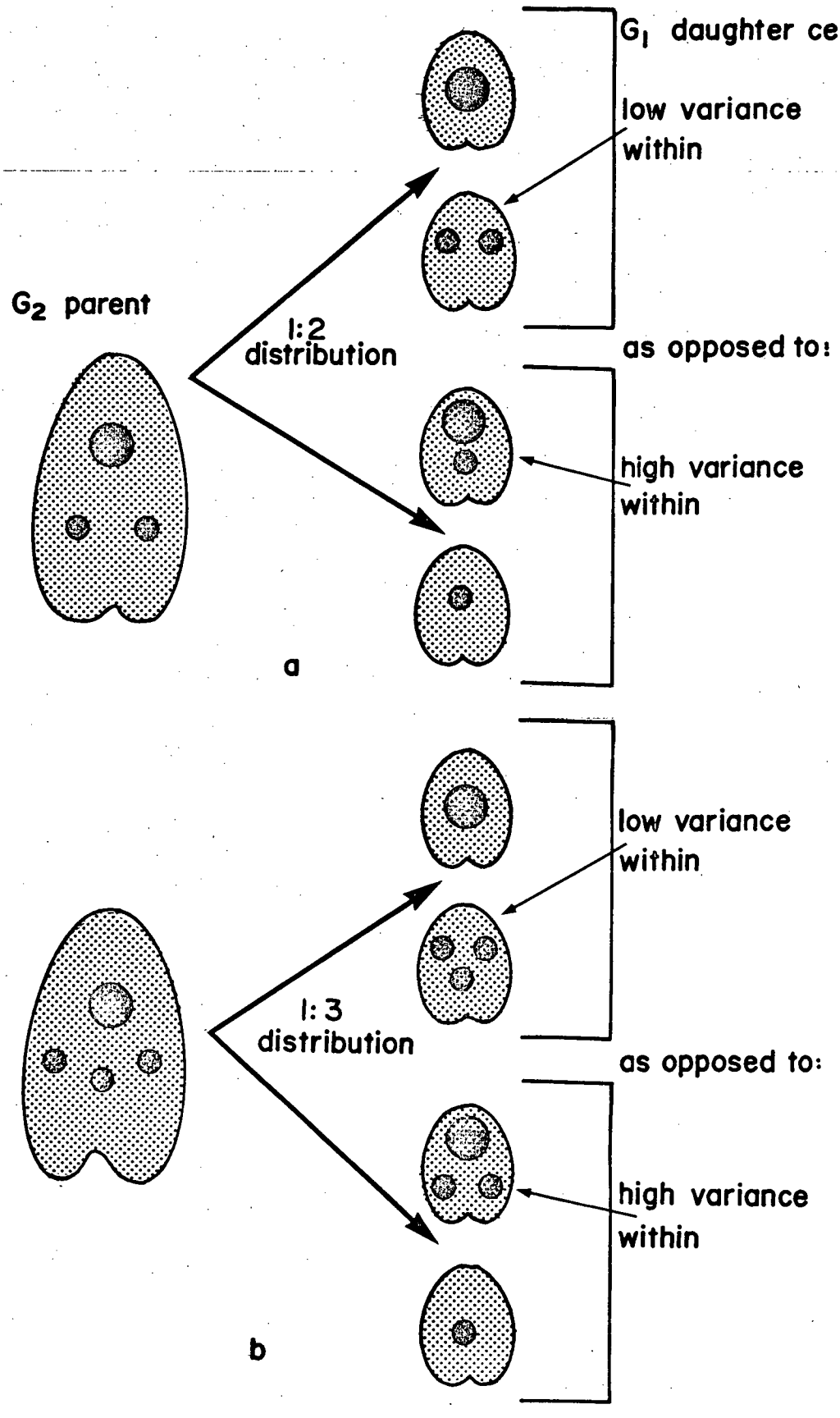
G_1 daughter cells
low variance within
G_2 parent
1:2 distribution
as opposed to:
high variance within
a
low variance within
1:3 distribution
as opposed to:
high variance within
b

FIGURE 14

distribution, the parent cell may have had one large macronucleus and three smaller macronuclei and passed the larger macronucleus on to one daughter cell and the three smaller macronuclei to the other daughter cell (Figure 14b). If the situation is as described in the examples above, one would expect that the two or three smaller macronuclei within a daughter cell would be more similar (variance within reduced) than macronuclei between pairs of daughter cells (variance between increased). Further, it should be possible to determine if the DNA content per cell and per macronucleus in a 1:2 distribution and in a 1:3 distribution resulted from misdivision or missegregation of the parent macronuclei by counting the number of cases in which the largest macronucleus ends up in a cell with a single macronucleus. The data in Table XII show that as the number of macronuclei in daughter cells increases, the DNA content per macronucleus ($E._{tot} \pm S.E.$) decreases. This decrease is not strictly proportional to the number of macronuclei per cell. Cells with three macronuclei in a 1:3 distribution have a mean DNA content of 170% of the mean DNA content of the three macronuclei in a 2:3 distribution. The values of intraclass correlation within pairs or groups of macronuclei varied from +0.36 for the two macronuclei in a 1:2 distribution to greater than +0.60 for macronuclei in 1:3 and 2:3 distributions. The correlation between the three macronuclei in a 2:3 distribution was very high (+0.91). The average difference as a percent of the G_2 macronucleus was greatest in the case of the three macronuclei in a 1:3 distribution and least in the case of the three macronuclei in a 2:3 distribution. Coefficients of variation for average differences were also higher in most cases than comparable coefficients of variation between daughter cells (Table XI).

TABLE XII

DISTRIBUTION OF G_1 DNA BETWEEN MACRONUCLEI WHENE THE NUMBER OF MACRONUCLEI IN DAUGHTER CELLS IS NOT EQUAL

Stock	No. of Macronuclei	N	$E._{tot} \pm$S.E.	C.V.(%)	r_i	P_α	Average Diff. Between Macs (% of G_2 Mac)	C.V.(%)
A. Between two macronuclei in a 1:2 distribution								
*2B, 3A, H9W	2	44	69.0±3.5	48.0	+0.36	>0.05	4.2±0.7	116.1
B. Between three macronuclei in a 1:3 distribution								
*2B, 3A, H9W	3	27	49.7±4.3	64.6	+0.62	<0.05	6.0±1.7	114.4
C. Between two and three macronuclei in a 2:3 distribution								
*2B, 3A, H9W	2	12	40.9±6.6	79.1	+0.67	<0.05	5.8±2.0	80.3
*2B, 3A, H9W	3	18	29.4±2.5	46.2	+0.91	<0.01	1.8±0.4	61.6

*Homogeneous data as judged by one-way Anova and Bartlett's test.

In cells with a 1:2 distribution, the largest macronucleus ended up in the cell with a single macronucleus in 18/22 cases. As all cells were in G_1, these 18 cases presumably resulted from the misdivision (failure to divide) of one macronucleus with the undivided macronucleus going to one of the daughter cells, and both daughter macronuclei segregating away from the non-divided macronucleus to the other sister cell. The other 4/22 pairs might have been cases of misdivision of a macronucleus plus missegregation of one of the resulting smaller macronuclei to the cell receiving only one macronucleus.

In the case of 1:3 distributions, the macronucleus with the highest DNA content was present in the cell containing a single macronucleus in seven out of nine cases. In eight of the nine cases, a single large macronucleus was present in each daughter cell of the pair, with two smaller macronuclei of approximately equal DNA content in one cell along with one of the larger macronuclei. The largest macronucleus was found in the cell with two macronuclei in 5/6 cases in a 2:3 distribution. Thus, it would appear that the largest macronucleus tends to segregate away from other (smaller) macronuclei resulting in greater equality of DNA content in daughter cells than would be expected by chance alone.

Loss of macronuclei in exconjugant or exautogamous doublets is achieved through misdivision or missegregation of macronuclei until each daughter cell is left with only a single macronucleus by the fourth to fifth cell cycle as shown in section I. Further, upon scoring the frequency or misdivision versus missegregation using the criteria above, it was found that misdivision occurs approximately 15-20% of the time depending on the stock, and missegregation occurs approximately five to eight percent of the time.

C. Does Regulation of DNA Content Occur During the S Phase?

The question of regulation of DNA content in doublets during the DNA synthesis period was examined by comparing the coefficients of variation of G_1 and G_2 DNA in daughter cells. If regulation of DNA content had occurred during S, one would expect to find a reduction in the coefficient of variation for parental G_2 DNA as compared to the corresponding coefficient of variation for G_1 DNA in the daughter cells. Pooled data for mean G_1 and G_2 DNA contents for cells containing various numbers of macronuclei plus their coefficients of variation were tabulated and the results are summarized in Table XIII.

Dunnett's multiple range test was used to compare G_1 and G_2 DNA content of the pooled data with the result that there was no significant difference at the one percent level in mean G_1 or G_2 DNA regardless of the number of macronuclei per cell. Further, there was no significant difference in variation in G_1 DNA content in daughter cells compared to the corresponding variation in G_2 DNA content in the parent cell as judged at the one percent level by one-way Anova and Bartlett's test. Therefore, it was concluded that no net regulation of DNA content occurred during the S period of the cell cycle.

SUMMARY

The data presented in this section show that the DNA content of doublets varies slightly between stocks, as well as between cells within stocks. The DNA content of doublet cells is not strictly proportional

TABLE XIII

MEAN G_1 AND G_2 DNA CONTENT OF DOUBLETS

Stock	No. of Macronuclei/Cell	N	G_1 DNA E.$_{tot}$ ±S.E.	C.V.(%)	G_2 DNA E.$_{tot}$ ±S.E.	C.V.(%)
2B, 3A, H9W	1	110	104.0±6.0	49.1	208.0±15.1	43.7
2B, 3A, H9W	2	118	109.1±5.4	42.1	218.3±14.4	39.5
2G, 3A, H9W	1, 2 & >2	272	115.2±3.8	44.7	230.2±8.8	45.4

Pooled means and variances for G_1 and G_2 DNA content respectively for cells with one, two and more than two macronuclei were judged homogeneous by Dunnett's procedure.

to the number of macronuclei per cell. Additional macronuclei increase the DNA content, but not in proportion to the number of macronuclei. Cells with two macronuclei contained only about 26% more DNA than did unimacronucleate cells. Thus, mechanisms exist which regulate the amount of DNA per cell to a more nearly constant total than would be expected from the number of macronuclei alone.

The distribution of macronuclei and DNA was examined in two types of fission events: those that produce daughter cells with equal numbers of macronuclei, and thus do not cause a net change in the number of macronuclei per cell; and those which produce daughter cells with unequal numbers of macronuclei, and bring about changes in the number of macronuclei. In the former case, the mean difference in DNA content between sister cells was about five percent of the G_2 DNA content and the intraclass correlation coefficient between sister cell DNA was high (+0.7 or more).

In contrast, when unequal numbers of macronuclei occurred in daughter cells the DNA contents were quite unequal. However, the mean DNA contents were not strictly proportional to the number of macronuclei per cell. The DNA content of sister cells was in all cases more similar than would be expected on the basis of the number of macronuclei alone.

Finally, it was shown that there was no net regulation of DNA content during the S phase of the cell cycle as evidenced by the fact that there was no significant difference in variation between cells having the G_1 *versus* the G_2 amount of DNA.

SECTION III

QUANTITATIVE COMPARISON OF DOUBLET *P. aurelia* WITH SINGLETS

The previous section (Section II) described the results of a study to determine the DNA content in *P. aurelia* doublets containing various numbers of macronuclei. The relative DNA content of doublets *versus* singlet cells, however, has not yet been discussed. In this section DNA content, cytoplasmic protein (macromolecular dry mass), and length of the cell cycle in singlets and doublets are quantitatively compared. DNA content and cytoplasmic protein were examined by microdensitometry (Appendix I). The G_2 values for macromolecular dry mass are the sum of the G_1 values for pairs of daughter cells fixed after division.

A. G_1 DNA Content of Doublet vs Singlet Cells

The mean extinction values and the corresponding DNA contents (c) of G_2 macronuclei in singlet and doublet cells are shown in Table XIV. The data allow the following observations and conclusions:

1. The mean G_1 DNA content of *P. aurelia* doublets is approximately twice (192%) that of singlets.

2. The value calculated for the macronuclear DNA content of singlets is in agreement with the previous estimates (Woodard *et al.*, 1961; Allen and Gibson, 1972; Berger, 1973).

TABLE XIV

G_1 DNA CONTENT OF SINGLET AND DOUBLET
P. aurelia

Type	No. of Macronuclei	N	$E._{tot}$ ±S.E.	Coefficient of Variation (%)	DNA Content (c)
Singlet	1	36	55.7±4.5	36.8	844.0±69
*Doublet	1 & 2	228	106.5±8.0	45.6	1614.5±151
Micronuclei from Doublets		56	0.13±0.00	44.7	2.0**

* Pooled data from 1 mac and 2 mac doublets from Table XIII.

** Micronuclei assumed to have a 2c DNA content.

3. The DNA content of micronuclei in singlet and doublet cells is the same.

4. A slightly higher variation in G_1 DNA content as judged from the coefficients of variation in Table XIV is present in doublet as compared to singlet cells.

A similar comparison of the cytoplasmic content or macromolecular dry mass of singlet and doublet P. aurelia is presented in Table XV in the following sub-section.

B. Macromolecular Dry Mass of Doublet and Singlet P. aurelia

Dividing cells were isolated from vegetative cultures of four doublet stocks and one wild-type singlet stock. Immediately after separation, G_1 sister cells were dried on albumenized microscope slides and stained with acidified Napthol yellow S which stains basic and non-basic proteins (Appendix I). Stained cells were photographed and the amount of dye was estimated by the microdensitometric technique (Appendix I).

The results (Table XV) indicate that doublet P. aurelia have roughly twice the protein content (macromolecular dry mass) of singlets. As the cells were fed on solid food (Aerobacter aerogenes), estimates of total protein content would include undigested food as well as Paramecium protein.

Intraclass correlation analysis showed that variation in macromolecular dry mass among pairs of sister cells did not exceed variation

TABLE XV

MACROMOLECULAR DRY MASS OF SINGLET AND DOUBLET *P. aurelia*

Stock	N	$E._{tot} \pm$ S.E. G_1 cells	C.V.(%)	N	$E._{tot} \pm$ S.E. G_2 cells	C.V.(%)	r_i	P_α
51s (singlet)	38	3.5±0.1	23.3	19	7.1±0.3	17.4	+0.12	>0.05
*KVD4, GM_1, GM_2 (doublet)	58	7.5±0.3	29.4	29	15.1±0.6	22.5	+0.28	>0.05
H9W (doublet)	36	9.5±0.2	17.5	18	19.0±0.6	15.0	+0.44	0.05-0.01

*Homogeneous data as judged by one-way Anova and Bartlett's test.

within pairs for stock 51s (singlets), and stocks KVD4, GM_1 and GM_2 (doublets), therefore implying an unequal division of cytoplasm to the daughter cells in these stocks. However, variation among pairs exceeded variation within pairs of sister cells in stock H9W. Coefficients of variation between G_1 and G_2 cells from the same stock were not significantly different at the five percent level. Further, it was noted that the mean cytoplasmiccontent of cells from stock H9W was approximately 20% higher than in the other three doublet stocks examined. As mentioned in Section II, part A1, cells from stock H9W had an approximately 27% higher DNA content than cells from other doublet stocks. Thus, as was reported by Kimball (1967), it appears that the ratio between dry mass and DNA content is constant under equilibrium conditions, i.e. when both the cell and the macronucleus are doubling in each cell cycle.

Since stock H9W doublets have been reported to have an increased number of ciliary rows (M. Schneller, personal communication), it was expected that these cells would be larger. Also, since the pattern of cortical inheritance in P. aurelia was shown to be independent of the genetic constitution of the macronucleus (Sonneborn 1960, 1963, 1970b), one might interpret the correlation between macromolecular dry mass, DNA content and the number of ciliary rows to mean that the row variations are primary and that cell size and DNA content are adjusted to the number of rows. In support of this idea, Tartar (1961) has shown that in Stentor the size of the macronucleus is not the cause of, but a response to, the size of the cell.

Among the factors that could limit the growth rate in doublets are the amount of cytoplasm, the number of ribosomes and the amount of

ribosomal RNA, and the amount of messenger RNA. Since Kimball (1967) found little or no positive correlation between dry mass and division rate in singlet P. aurelia, one might expect to find a similar lack of correlation in doublets. For this reason division rates in singlet and doublet cells are compared in the following sub-section.

C. Length of the Cell Cycle in Doublet and Singlet P. aurelia

A number of dividing cells from two doublet stocks and a wild-type singlet stock were isolated into individual depression slide wells. Immediately after separation, each sister cell was reisolated into a separate depression and the elapsed time from separation to the following division was recorded in minutes. A 28°C walk-in incubator was used to ensure uniformity of temperature throughout the course of the experiment.

The results (Table XVI) show that doublet P. aurelia have a vegetative cell cycle lasting approximately 164% of the length of singlets. Intraclass correlation analysis showed that variation in cell cycle length among pairs exceeded variation within pairs of daughter cells for both doublet and singlet stocks. Thus, related daughter cells showed a high degree of similarity in the length of cell cycle. Further, there was no significant difference in the coefficients of variation for mean cell cycle lengths between doublet and singlet cells at the five percent level.

The reasons why doublet P. aurelia have a longer vegetative cell cycle than singlets is unknown. From the data already discussed it is known that doublets have roughly twice the DNA content and macromolecular

TABLE XVI

CELL CYCLE LENGTH IN DOUBLET AND SINGLET
P. aurelia

Stock	N	Mean Length of Cell Cycle (min.)±S.E.	C.V.(%)	r_i	P
*GM_1, GM_2 (doublet)	98	486.4±5.4	10.9	+0.43	0.05-0.01
51s (singlet)	61	296.0±2.7	7.1	+0.42	0.05-0.01

* Homogeneous data as judged by one-way Anova and Bartlett's test.

dry mass of singlets. Further, doublets have two mouths and therefore could potentially feed twice as quickly. Therefore, one might expect that they should grow as quickly as singlets but they obviously do not. Results similar to these have also been observed by other workers for P. aurelia doublets (personal communication from M. Samoiloff). Kimball (1941) noted, however, that the rate of division in singlet and doublet Euplotes patella was approximately equal. Moreover, as was noted by Kimball (1967) for singlets, the situation in P. aurelia differs from that reported by Prescott (1956) for Amoeba proteus. Unequal division of the cytoplasm resulted in unequal generation times, so that essentially the normal cell size was restored before the next division. However, cells from wild-type singlet as well as several doublet stocks were found to have an unequal division of cytoplasm to daughter cells, but the degree of similarity of cell cycle lengths within pairs of daughter cells was significantly high. Thus it appears that generation time is less readily influenced by cell size in P. aurelia than in A. proteus.

In some respects a similar variation was found within populations of mammalian cells (Killander and Zetterberg, 1965a,b). The macromolecular dry mass and, to some extent, DNA content at division varied appreciably in non-neoplastic and even more so in neoplastic cell lines. In the latter, there was a high correlation between the dry mass and DNA content at the time of division. Unequal division, variations in growth rate, and possible genetic differences were suggested as sources of this variation. The generation time also varied somewhat but was not clearly related to cell size. However, the G_1 period was shorter with increased

cell size, and the size at the start of S tended to be less variable than at other stages. It was suggested, as could also be the case in P. aurelia doublets, that these relations tend to restore the normal size by allowing more time for growth in the smaller than in the larger cells before DNA synthesis begins.

CONCLUDING STATEMENT

The occurrence of Paramecium doublets has made it possible to examine the nature of regulation of DNA content in a cell type not commonly found in nature. In paramecium doublets an artificial situation is created in which a normal singlet genome develops into a single macronucleus in a cytoplasmic unit twice the normal size. The results show that the size and DNA content of the macronucleus is determined by two primary factors, the size of the cell and the number of macronuclei. When more than one macronucleus is present, the macronuclei interact competitively so that the total macronuclear DNA content per cell is approximately constant. When the cytoplasmic unit is twice as large, as in doublets, the mean macronuclear DNA is twice as great as in normal singlets, even when only a single macronucleus is present in both.

These studies have shown that regulation of DNA content per cell as a result of macronuclear loss by the fourth to fifth post-autogamous cell division, occurs chiefly in two ways: 1) by missegregation of a dividing macronucleus at fission, and 2) by misdivision of one or more macronuclei at fission.

Regulation of DNA content in doublets is further refined in such a way that DNA content, in cases of unequal distributions of macronuclei, is not strictly dependent upon the number of macronuclei per cell. Generally, the largest macronuclei tend to segregate away from smaller

macronuclei. Once a single macronucleus arrives in a doublet cytoplasm, the DNA content in the macronucleus increases rapidly, resumably partially in response to the decreased nucleo-cytoplasmic ratio. Because of the increased length of the cell cycle in doublets, the G_1 period may be longer in singlets than in doublets, allowing more time for growth in smaller rather than in larger cells.

NOTES

1. The Feulgen nuclear reaction for DNA is the cytochemical procedure that has been most used in cytophotometric studies. Quantitative use of the Feulgen reaction has helped to establish that the average amount of DNA per chromosome set is constant for animal and plant species, and that replication of DNA is restricted to the interphase stage of the cell life cycle. Feulgen cytophotometry has been used to determine the extent of polyploidy in mammalian liver tissue (Alfert and Geschwind, 1958) and Paramecium aurelia macro- and micronuclei (Kimball and Barka, 1959; Berger, 1973) among other things.

The Feulgen reaction depends upon a prior acid hydrolysis of DNA which preferentially removes purines, unmasking the aldehyde group of the deoxyribofuranose sugars to which they were bound. The aldehydes thus formed react with a decolorized Schiff reagent, which is converted into its colored form and is bound in situ to the DNA. Proof that an aldehyde group is necessary for the positive nuclear reaction comes from the observation that aldehyde coupling reagents block the Feulgen reaction if applied after acid hydrolysis (Lessler, 1956).

2. The cytoplasmic content of acecell may be determined cytophotometrically using an acid dye such as Napthol yellow S (NYS). The predominant mechanism of staining proteins by acid dyes is electrostatic bonding of the dye anions to the available cationic groups of proteins.

The latter are the ε-amino groups of lysine and hydroxylysine, the guanidyl groups of arginine, the imidazole groups of histidine, and the free terminal α-amino groups. The extent to which an anionic dye will bind to these cationic groups is limited by a number of factors, including the pH of the staining solution, the concentration of the dye and its affinity for protein, the effect of the fixative, the availability of the protein basic groups to the dye, the nature of the solvent ions, and the ionic strength of the staining solution (Deitch, 1966).

Dye binding with NYS was found to reach a maximum largely independent of dye concentration and staining time, and I have found it to be an ideal stain for doing microdensitometric measurements on the cytoplasmic content (macromolecular dry mass) of Paramecium aurelia. Hydrolysis in 1 N HCl for the Feulgen reaction causes about a 10% decrease of NYS staining, probably as a result of extraction of some histone during the acid hydrolysis, while DNase or hot trichloroacetic acid pretreatment on the other hand causes a 20-30% increase in dye binding, indicating that DNA may mask some of the protein basic groups and prevent them from staining.

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APPENDIX I

DETAILS OF CYTOLOGICAL AND MICRODENSITOMETRIC TECHNIQUES USED.

A. Culturing Methods

1. Media

a) Cerophyl - Prepared according to the methods described by Sonneborn (1970a).

b) Baked lettuce medium - Prepared according to the methods described by Sonneborn (1950).

c) Dryl's solution (Dryl, 1959)

i) Stock solutions

Soln. A - a. 0.1 M $NaH_2PO_4 \cdot H_2O$ (1.38g/100 ml)

b. 0.1 M $NAH_2PO_4 \cdot 7H_2O$ (2.68g/100 ml)

c. 0.1 M Na citrate (2.94g/100 ml)

Soln. B. a. 0.1 M CaCl (1.11g/100 ml)

ii) Dryl's soln. pH 7.0 (1000 ml)

a. 40 mls Soln. A

b. 945 mls distilled water

c. 15 mls Soln. B added last

B. Selection of Doublet Dividers in Vegetative Growth

Cultures of exautogamous exponentially dividing doublet cells were grown in depression slides for a period of one-two days. Dividing

cells were collected with a micropipette and each daughter cell was isolated into individual "A" and "B" depressions immediately after separation which occurred approximately 10-15 minutes after the collection of the divider (Kimball and Barka, 1959).

C. Preparation of Antiserum

1. Antiserum was prepared as described by Sonneborn (1970a).

D. Generation of Doublet Stocks GM_1, GM_2, and GM_3.

Mating reactive cells of mating type VII (Stock 51) and mating type VIII (Stock 51) were mixed according to standard methods (Sonneborn, 1970a). Conjugating pairs were later individually isolated into depression slides containing sterile Dryl's solution with a 1/800 concentration of Stock 51 antiserum (serotype A). After a period of two hours in the antiserum solution, conjugating pairs were then transferred to individual depressions of Cerophyl medium and left overnight. Pairs which failed to separate were isolated as prospective doublet cells and were transferred daily to fresh culture media.

E. Cytological Techniques

1. Fixation

a) Clark's ethanol-acetic acid

3 parts absolute ethanol

1 part glacial acetic acid

2. Albumenized slides

a) Meyer's albumen fixative was spread in a thin layer applied to one end of a clean microscope slide with the finger. The slide was then heated over a burner flame until the albumen film was dry but not scorched.

3. Staining Procedure

a) Feulgen reaction

i) Reagents

a. Leucobasic fuchsin (Schiff's reagent)

- 100 mls distilled water
- 0.5 g potassium meta-bisulfite
- 0.5 g basic fuchsin
- 10 mls 1 N HCl

The dye (basic fuchin) was added to water which was brought to a boil. After the mixture had cooled to room temperature the potassium meta-bisulfite and hydrochloric acid were added. After the mixture had set for several hours in a stoppered bottle, approximately one gm of Norit-A 'decolorizing charcoal' (Fisher Chemicals) was added and removed by filtration once all traces of color had been removed from the solution. The filtered reagent was stored at four degrees C in a foil-wrapped bottle.

b. Acid bisulfite wash

- 0.5 g potassium meta-bisulfite
- 5 mls 1 N HCl
- 95 mls distilled water

ii) Method

Individual cells were dried in rows on previously albumenized microscope slides (Fisher M6155) and fixed in Clark's fixative for 20 minutes followed by a rinse in distilled water and hydrolysis in 1 N HCl at 60° C for 11 minutes. Following hydrolysis, the cells were again rinsed in distilled water and placed in a covered Coplin jar containing leucobasic fuchsin for approximately 45 minutes where staining took place. After staining the slides were again rinsed in distilled water and placed into another Coplin jar containing acid bisulphite for one minute. The slides were then removed and washed in running tapwater for 1/2 hour and air dried.

In preparing slides for microdensitometry, the stain was made fresh for each use. Special care was taken to ensure that the temperature of the slides and the acid-bath were correct during hydrolysis as this is the most critical step of the procedure. Slides were stained approximately 45 minutes (as described below), rinsed in distilled water, and put through two changes of acid-bisulphite wash to remove any non-specifically bound dye. Slides were then washed in running tap water for 1/2 hour and air dried.

b) Azure A technique for DNA (modified from DaLamater, 1951, by S. W. Perdue, unpublished)

i) Reagents

a. Stock solution

- 0.5% Azure A in distilled water

b. Staining solution

- 30 mls 0.5% Azure A

- 0.45 g potassium metabisulphite

- 1.5 mls 1 N HCl

ii) Method

Cells were dried individually in rows on previously albumenized microscope slides and fixed 20 minutes in Clark's fixative. Following fixation, slides were rinsed in distilled water and hydrolysed in 1 N HCl for 11 minutes at 60° C. Slides were then rinsed in distilled water and placed in a covered Coplin jar containing the staining solution for 20-30 minutes. After staining, slides were put through multiple rinsings of distilled water and then washed at least 20 minutes in running tap-water and allowed to air dry. After drying, the slides were mounted in Cargill's type 'A' refractive index immersion oil (index of refraction 1.530) to reduce the loss of light by scatter which would normally occur at the air-glass interface between the specimen and the coverslip. Coverslips were sealed with clear fingernail polish.

c) Acid Fast-Green (for staining non-histone protein) after Kaye and McMaster-Kaye (1966)

i) Reagents

a. 5% trichloroacetic acid (TCA) 2.5 g TCA/50 mls distilled water

b. 1 mg/ml Fast Green stain

c. 1 N HCl

ii) Method

Individual cells were dried on albumenized microscope slides. When the drop had dried enough to make the cells adhere to the surface of the glass, the slides were immersed in hot trichloroacetic

acid (five percent for 15 minutes at 90°C), washed in distilled water, and stained in covered Coplin jars for 60 minutes in a one mg/ml aqueous solution of Fast Green which had been adjusted to an acid pH with 1 N HCl. Following staining the slides were transferred directly to 95% ethanol for several minutes, run up to xylene, and mounted in immersion oil or permount sealing the border of the coverslip with clear nail polish.

d) Napthol yellow S (for basic and non-basic cytoplasmic proteins after Deitch (1955, 1966)

i) Reagents

a. 50 mls 0.1% Napthol yellow S dissolved in 1% glacial acetic acid

b. 50 mls 1% glacial acetic acid

c. 90 mls tert-butyl alcohol

ii) Method

Individual cells were dried in rows on previously albumenized microscope slides, fixed for 20 minutes in Clark'sffixative, and rinsed in distilled water. The slides were then transferred to covered Coplin jars of 0.1% Napthol yellow S in one percent glacial acetic acid and stained for 30 minutes at room temperature. Following staining, slides were rinsed in a one percent solution of acetic acid for one minute, and dehydrated in three changes of tert-butyl alcohol and two changes of xylene. Coverslips were mounted in Cargill's index of refraction immersion oil and sealed with clear nail polish.

e) Dippell Stain (Dippell, 1955)

i) Reagents

a. 200 mls 45% glacial acetic acid

b. 1 1/2-2 g carmine

c. 4.5 parts 45% glacial acetic acid

d. 2 parts 1 N HCl

e. 1 part 1% Fast-Green in 95% methyl alcohol

ii) Method

The acetocarmine was prepared by refluxing 200 mls of 45% acetic acid with 1 1/2-2 gms carmine for 1 1/2 hours. The Dippell stain was prepared by mixing together 10.5 parts acetocarmine, 4.5 parts 45% glacial acetic acid, two parts 1 N HCl, and one part one percent Fast Green in 95% methyl alcohol. This stain, in prepared form, simultaneously fixes and stains the macronuclei of paramecia an orange-brown colour while staining the cytoplasm light green. It is generally applied with a dropper to a small culture of cells either in a depression or in a droplet on a microscope slide. After a coverslip has been applied the nuclear morphology of the cells is easily seen. This technique provides a quick, easy method for observing the status of the macronucleus and was used almost exclusively to check for cells in autogamy. In this case the fragments of the old macronucleus were easily recognized as such.

F. Microdensitometry

1. Preparation of Slides

Feulgen stained macro- and micronuclei of dividing Paramecium aurelia (Stocks 3A, 2B, and H9W) following sexual reorganization were photographed using an Olympus microscope and overhead attached camera with a Zeiss 40x water immersion objective and 10x ocular. Kodak high

contrast copy film #5069 was used throughout. The negatives of the nuclei were cut out so that they could be mounted in refractive index immersion oil on a glass microscope slide and a coverslip was applied and sealed with clear nail polish.

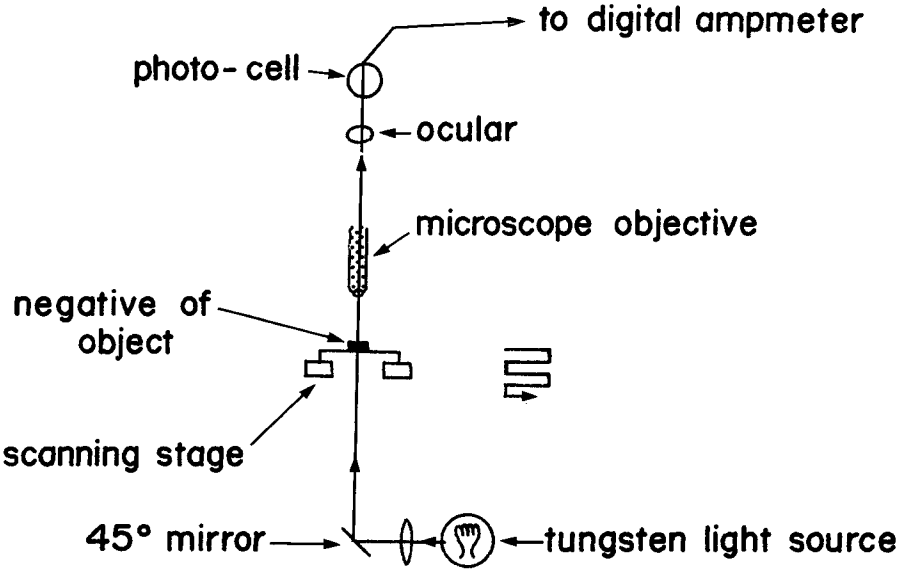
2. Equipment

A Zeiss microscope with a tungsten light source was used. The light, after passing through the condenser, passed through a slide containing the photographic negative of a nucleus mounted on the microscope stage. The cone of light, after passing through the negative as a small spot, passed through the optical system and struck a photocell located at the image focal plane. A current proportional to light intensity was generated by the photocell, and the current values were recorded on punched paper tape.

The digital stage control unit operated servo-motors which controlled the movements of the microscope stage. Each nuclear image was scanned in a preprogrammed rectangular array of points at even intervals so that after completing a scan of the image along a given line, the stage would index and begin another scan along an adjacent parallel line. After completing a whole set of scans of the entire object image, the instrument automatically shut off. Minute variations in the optical density of each negative were thus obtained.

The scanning data recorded on paper tape was analysed on an IBM 1130 computer using a special program (DENSI) written by Dr. J. D. Berger which plots extinction values to give a numerical density "map" of each scanned object. The cumulative extinction of light into the negative of each

scanned nucleus provided a relative basis for comparison of the DNA content in macronuclei. Cytoplasmic content (macromolecular dry mass) was estimated in a similar manner employing the stain Napthol yellow S for cytoplasmic proteins. A diagram of the optical system of the scanning device is shown below.



3. Theoretical Aspects of Microdensitometry

Microdensitometry provides a quantitative estimate of the amount of light transmitted or absorbed by serially scanning a photographic negative of the object which has been specifically stained, and measuring the total cumulative extinction (absorbence) of light into the silver grains of the scanned array. Quantitative measures of the amount of absorbing material are obtained by making measurements of optical density defined as the logarithm of the ratio of light input intensity to output intensity through an absorbing material (Bloom, 1966). The problem of non-uniform distribution of material in the object (i.e. DNA in a cell)

make it desirable to break up the specimen into smaller pieces of uniform optical density and to then add up all the information obtained in this manner. This process is known as integration and for this reason the instrument is known as an integrating microdensitometer.

The relation between transparency, opacity (1/T), and density (log 1/T) was first described by Hurter and Driffield (1890) who found that the density of a film should be proportional to the amount of silver per unit area and to the logarithm of the exposure time. On the basis of their study Hurter and Driffield constructed a D-log E curve. The H- and D-curve for Kodak high contrast copy film #5069 is shown in Figure 15.

The slope, γ, of the curve is characteristic of the emulsion under specified conditions of processing. It is used as a measure of contrast. In any application of photographic photometry, the correct exposure range for the film must not extend beyond the limits of the straight-line portion of the appropriate H- and D-curve.

The total absorbence of an object on a negative by microdensitometry may be determined by the argument:

$$A = \log (I/I_o) \tag{1}$$

$$A_{bkg} = \log (I_{btg}/I_{bkg}) \tag{2}$$

$$A_{image} = \log (I_{btg}/I_{image}) \tag{3}$$

$$A_{object} = A_{bkg} - A_{image} \tag{4}$$

Substituting (2) and (3) into (4) and simplifying:

$$A_{object} = \log I_{image} - \log I_{bkg} \tag{5}$$

Figure 15. Plot of optical density vs. log exposure time for Kodak high contrast copy film #5069.

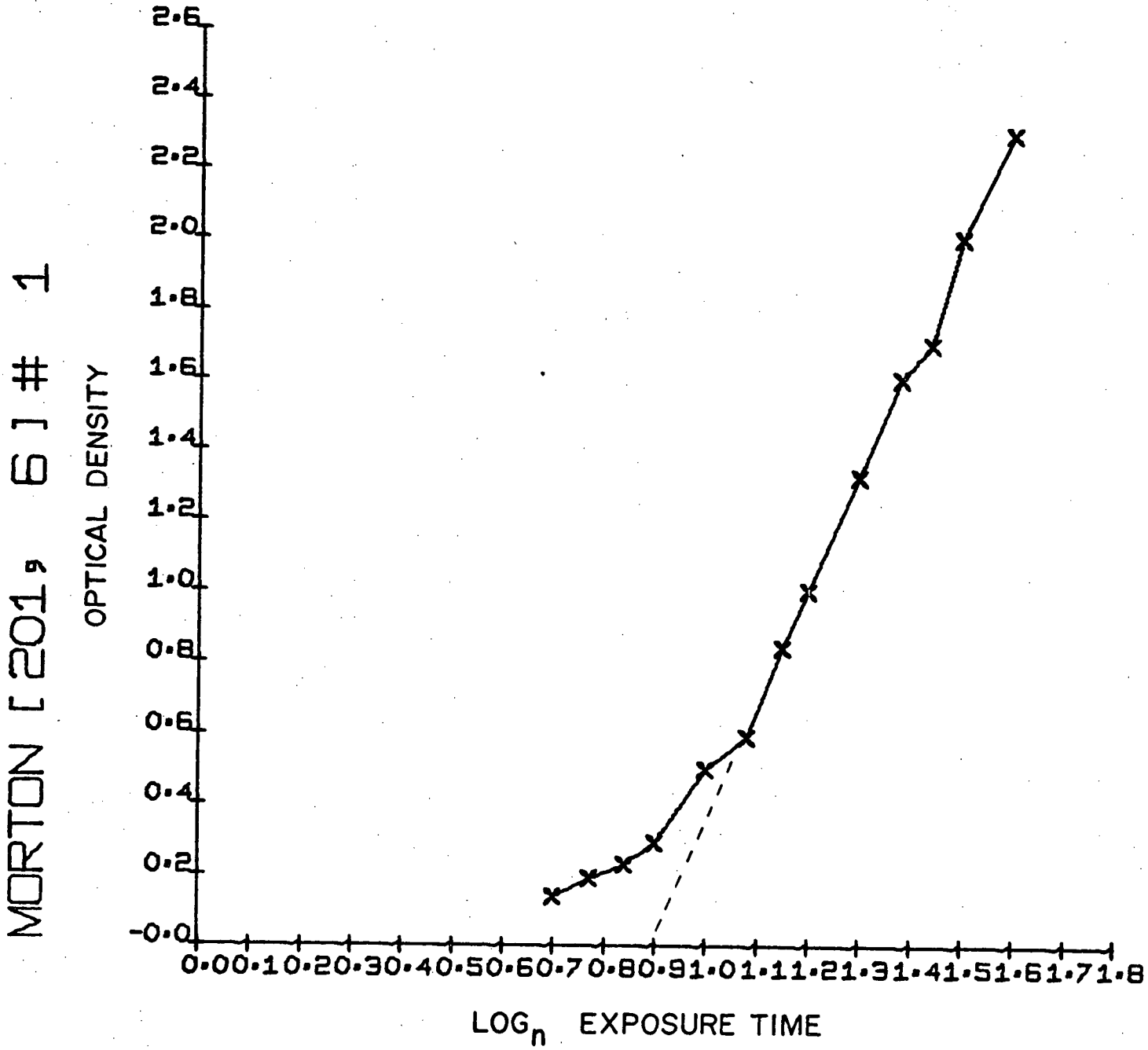

MORTON [201]9 61 # 1
OPTICAL DENSITY
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8
LOG$_n$ EXPOSURE TIME

FIGURE 15

Since output currents from the photocell are directly proportional to the light intensity:

$$A_{object} = \log I_{image} - \log I_{bkg} \tag{6}$$

The total extinction of a series of image elements x_1 to x_n is:

$$E._{tot} = \sum_{x=1}^{n} A_x \Delta X \tag{7}$$

For a two dimensional array, n by m:

$$E._{tot} = \sum_{y=1}^{m} \sum_{x=1}^{n} A_{x,y} \Delta X \Delta Y \tag{8}$$

This is the variable measured by the instrument and is the cumulative extinction at the image plane at the time of scanning. To express the total extinction of the object in terms of the distance between elements at the level of the cell, rather than in terms of the magnified negative: unit = Absorbence $\cdot$ um^2

$$E._{tot} = \sum_{y=1}^{m} \sum_{x=1}^{n} A_{x,y} \Delta X \Delta Y / MP^2 = E._{tot}/MP^2 \tag{9}$$

Thus to correct for differences in magnification of the plate or for differences in the distance between elements when scanning:

$$E_2 = E_1 (MP_2 MP_1)^2 (\Delta X_1 \Delta Y_1 / \Delta X_2 \Delta Y_2) \tag{10}$$

Where: A = absorbence

I = intensity

I_o = incident intensity

bkg = background (fully exposed portion of negative representing optical free space)

btg = brightground = unexposed portion of negative

object = object photographed

image = photographic image of object

$E._{tot}$ = cumulative extinction

ΔX = horizontal distance between elements

A_x = absorbence of the element (x)

A_y = absorbence of the element (y)

ΔY = vertical distance between elements

APPENDIX II

DETERMINATION OF MACHINE ERROR IN MICRODENSITOMETRY

The importance of calculating experimental error is obvious where the experimental apparatus used to collect the raw data may give erroneous measurements. Before starting this study, the consistency of values for cumulative extinction obtained by the summation of individual point measurements of light intensity (as described in Appendix I) were compared by making 20 repeated scans of a photograph of a wild-type singlet cell stained with Napthol yellow S. As the cumulative values for each scan should have been the same, any differences should have resulted from either machine error or processing error (i.e. errors in estimating light absorbence).

Results showed that the coefficient of variation among scans was four percent. Thus errors in measurement attributable to the scanning device or to computer analysis of the data was about four percent.

Mean number of increments/mm = 3.5.