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Are macronuclear subunits in *Paramecium* functionally diploid?*

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SUMMARY

The organization of the genetic material in the macronucleus of ciliates has been the subject of considerable controversy. Two of the four models of macronuclear structure predict assortment of the alternative phenotypes of heterozygotes during vegetative growth. Early studies of the phenotypic behaviour of heterozygotes after macronuclear regeneration (Sonneborn, 1947) had supported a diploid subunit model. The availability of quantitative predictions of the rate of assortment for the haploid and chromosomal models (Preer, this volume) and the existence of two alleles controlling a quantitative trait, copper tolerance, in *Paramecium tetraurelia*, has provided an opportunity to test these models. The median tolerance limits to copper of unselected sublines were measured as a function of age. There was no increase in the variance among sublines, as the haploid and chromosomal models predict. Quantitative evaluation shows that subdiploid models with a kinetic complexity of 860 or less are not compatible with the results. This experiment was not sensitive enough, however, to exclude subdiploid models if the kinetic complexity is 2000 or greater. Selection on heterozygotes also failed to provide evidence in favour of assortment. All the results are consistent with and support the diploid subunit model of the *Paramecium* macronucleus.

1. INTRODUCTION

Among the protista the ciliates are a relatively distinct and homogeneous group. Nuclear dimorphism is one of their distinctive features; most ciliates possess both a macronucleus and a micronucleus. Cytological and genetic studies have shown that the macronucleus controls and determines the vegetative phenotype and the micronucleus functions as the germinal nucleus. Only those species or strains which have a micronucleus undergo sexual reorganization. At each sexual reorganization the macronucleus is normally fragmented and destroyed. It is replaced by division products which differentiate and grow from the micronucleus. Genetic studies of a variety of ciliates, especially *Paramecium*, *Tetrahymena* and *Euplotes*, have shown that the micronucleus is diploid. The nature of the macronucleus, the concern of this study, is more enigmatic.

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During cell division the macronucleus divides amitotically. Cytologically the macronucleus of *Paramecium* contains small bodies, each of which has the DNA equivalent of about one chromosome, connected together by fibrils (Wolfe, 1967). These bodies cannot be definitely identified as chromosomes, nor is the behaviour of these networks during division known. Consequently most of the evidence and alternative models of macronuclear structure have been based on genetic and biochemical studies.

Several diverse models, each supported by some experimental evidence, have been put forward as interpretations of the organization of the macronucleus of ciliates. They are as follows: (1) the macronucleus is a collection of diploid subunits which duplicate and assort randomly, (2) the macronucleus is a collection of complete genome haploid subunits, (3) the macronucleus is a collection of chromosomes or subchromosomal units which replicate and assort randomly, (4) the macronucleus is a collection of genes (or subchromosomal particles) among which only the two master copies duplicate, the other copies of genes being non-replicating slaves which assort randomly at division.

Sonneborn (1947) provided evidence that the macronuclear subunits were diploid in *P. aurelia* by studying macronuclear regeneration. In macronuclear regeneration the developing anlage is aborted and instead a new macronucleus develops from one of the fragments of the old macronucleus. Repeating this process of macronuclear regeneration in heterozygous clones did not change the phenotype of the clone and hence Sonneborn concluded that the macronucleus was most likely composed of diploid subunits. Nanney (1964) reviewed the genetic studies of *Tetrahymena pyriformis* and concluded that a model with 45 diploid subunits, each subunit showing stable intralocus differentiations, provided the best fit of the available data. The primary reasons for favouring the diploid over the haploid or chromosomal models were: (1) some loci, but not others, express both alleles *equivalently* for 50 fissions or more, and (2) the proportion of sublines of a heterozygote becoming pure for one allele versus the other allele may be highly eccentric, as much as 9:1.

The haploid subunit model for *Tetrahymena* was revived when Woodard, Kaneshiro & Gorovsky (1972) showed that the amount of DNA in the macronucleus is about 22 times the amount in the micronucleus. Thus, if there were 45 subunits as indicated by the kinetic data, the subunits would be haploid. The haploid model has gained favour by the failure to ever recover expression of a silent allele from a heterozygote and by the fact that induced mutations (except *ts1*, McCoy, 1973) and isozyme loci (Borden *et al.* 1973) show sorting out. For each locus which shows sorting out the diploid model requires a hypothesis of specific mechanisms of interallelic repression. Orias & Flacks (1975) have summarized the support for the haploid model for *Tetrahymena* but have offered no specific mechanisms explaining either the equivalent expression of both alleles at some loci for many fissions or the eccentric output ratios.

Fauré-Fremiet (1953) proposed the idea that the macronucleus was composed of chromosomal or subchromosomal particles in order to explain the phenomenon

of senescence in ciliates. Kimura (1957) made calculations of expected death rates which he interpreted as compatible with the observed ageing of *Paramecium*. Preer (this volume) has reviewed and re-evaluated the chromosomal model and concludes that aneuploidy is not the basis of senescence in *Paramecium*. Much of the recent support for this model has come from biochemical studies of the DNA in hypotrich ciliates. Prescott *et al.* (1971) have found that the macronuclear DNA of *Stylonychia* is found in small pieces approximately the size of a single gene, while micronuclear DNA is found in large pieces. Furthermore Bostock & Prescott (1972) have shown that the micronuclear DNA is more heterogeneous in terms of density profiles than macronuclear DNA. Thus there is selective replication during the formation of the macronucleus. Ammermann *et al.* (1974) have also found differences between micronuclear and macronuclear DNA in *Stylonychia* but were able to isolate large pieces of DNA from the macronucleus.

Allen & Gibson (1972) have proposed a master-slave model of the *Tetrahymena* macronucleus. In this model each allele is represented by a master copy which duplicates itself and also produces many non-replicating slaves. The differentiation of heterozygotes is interpreted as a failure of a master gene to replicate. Until this failure occurs the macronucleus is functionally diploid. Because the macronucleus may remain functionally diploid indefinitely or it may begin sorting out the present experiments can not provide evidence for or against this model. Doerder (1973) and Orias & Flacks (1975) have presented evidence and arguments attempting to exclude this model.

Since much of the recent genetic and biochemical evidence has weakened support for the idea of diploid subunits in the ciliate macronucleus, it seems appropriate to investigate the possibility of sorting out in *Paramecium*. The primary evidence for diploid subunits in *Paramecium* has depended on behaviour of macronuclear regenerates. Unlikely as it may seem, the process of macronuclear regeneration could include a mechanism for balancing out unequal ratios among subdiploid units. An apparently ideal situation to look for assortment in heterozygotes would be to have a quantitative trait in which the heterozygote was exactly intermediate between the homozygotes and to have calculations on the expected distribution of subunits with time. Preer (this issue) has made such calculations and Nyberg (1975) has found a gene for cupric ion resistance. The cu^r allele is almost fully recessive to its sensitive counterpart, cu^s.

If the phenotype of the heterozygote were exactly intermediate between the homozygotes, one could make reasonably sure predictions about the expected phenotype of cells with varying dosages of the two alleles. With an incompletely dominant allele such as cu^s it is not obvious what phenotypic level of resistance would be shown by a cell with, say, three doses of cu^r to one of cu^s. Fortunately this problem could be experimentally approached by creating double cells with two different types of nuclei.

2. MATERIALS AND METHODS

The stocks used and the marker genes they carry are shown in Table 1. Stock 40–136 was constructed and given to me by Dr Mary Austin, Indiana University; it has the stock 51 genetic background. The phenotypes of *hr*^{b}, *paA*, *ndB* and *ts111* have been described by Sonneborn (1974). The phenotypes of *cu*^{r}, *cu*^{s}, *nd203* and *ts203* may be found in Nyberg (1975). Nyberg (1975) also provides the experimental details in determining the tolerance to cupric ion. One prepares a series of solutions of increasing copper concentration and then isolates single cells into aliquots of these solutions. The cells are scored for viability at 24 h and the proportion alive or dead at the different dosages determines a dosage–response relationship from which the median tolerance is estimated. The median tolerance limits are expressed as the $\log_{10}$ of the concentration of Cu^{2+} in micromoles per litre. The log scale is used because the variability is distributed normally on this scale.

Table 1. *The stocks used and the clones which began the experiment*

Stock	Mating type(s)	Marker genes
51	VII, VIII	*cu*^{s}
40–136	VIII	*hr*^{b}, *paA*, *ndB*, *ts111*, *cu*^{s}
203	VII, VIII	*cu*^{r}, *nd203*, *ts203*

Initial clones

Autogamous clones: two clones from each of the mating types of each stock	Subtotal 10 clones
Crosses: two pairs (4 clones) from each of the following crosses: 203 vii × 51 viii, 203 viii × 51 vii, 203 vii × 40–136 viii, 203 vii × 203 viii, 51 vii × 51 viii, 51 vii × 40–136 viii	Subtotal 24 clones
	Total 34 clones

The basic experimental design was to create heterozygotes and measure the median tolerance limits to cupric ion as the clones aged. In addition to the heterozygotes, aged and young exautogamous and exconjugant homozygotes were included as controls. The 34 clones which began the experiment are shown in Table 1. Each of these clones was maintained by daily transfers of a single cell to 0·25% Cerophyl inoculated the previous day with *Klebsiella aerogenes*. Daily transfers are necessary to prevent autogamy (Sonneborn, 1954) and hence to study vegetative assortment. The number of cells remaining in the depression slides after each transfer was counted so that the exact number of fissions undergone since the beginning of the life-cycle could be determined. Despite the daily transfers some lines underwent autogamy or died. These were replaced by other subclones when available. Additional subclones of ageing heterozygotes were established as the cells aged. The time required for the bioassay meant that not all clones and subclones were tested each day the bioassay of copper tolerance was done.

In addition to the main experiment following subclones of heterozygous clones, an attempt was made to increase the sensitivity of this experiment by selecting and expanding those sublines of heterozygotes which showed the greatest tolerance

to cupric ion. Initially cells were rescued from the bioassay test and used to begin new sublines. This procedure would confound assortment with acclimatization and later sublines were expanded without exposure to copper. In Fig. 2 the dotted lines indicate exposure to cupric ion and the straight lines represent expansion without exposure to cupric ion.

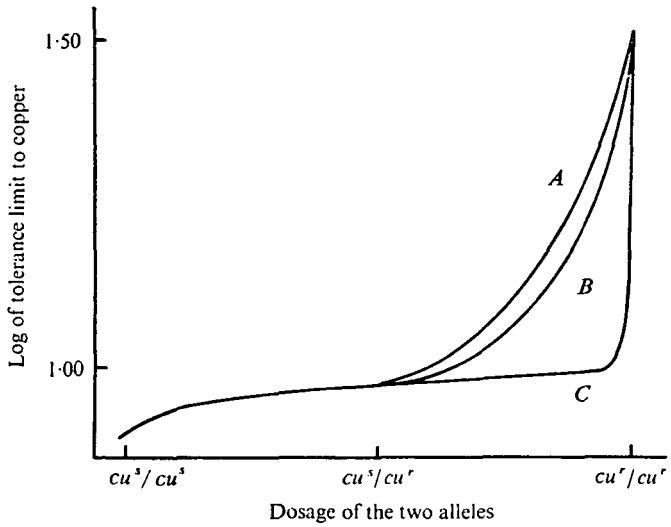

Fig. 1. Hypothetical relationships between the dosage of the alleles, cu^r and cu^s, and the log of the median tolerance limit to cupric ion.

The experiment with double *Paramecium* (Sonneborn, 1963) was done to get some idea of the tolerance level that a cell with, say, 215 cu^s genes and 645 cu^r genes would have. When a double is mated to a single, one side of the double undergoes normal conjugation while the other side undergoes autogamy. Hence it is possible to have two kinds of macronuclei, heterozygous and homozygous, together in the same cell. Double *Paramecium* (provided by Steve Ng, Indiana University) were mated and made homozygous for the cu^r allele. These doubles, genotype cu^r/cu^r; cu^r/cu^r, were mated with a single homozygous for cu^s. After the exconjugant double had divided twice three sublines were initiated. Two of these sublines were further expanded. The copper tolerance levels of various subclones of this double were then determined by the bioassay.

3. RESULTS

The significance of the main experiment depends on the relationship between the phenotypic expression of cupric ion tolerance and the relative dosages of the cu^r and cu^s alleles. Fig. 1 shows three possible relationships connecting the known points. Curve *A* is quadratic, curve *B* is cubic, and curve *C* is a hypothetical relationship in which very few cu^s alleles dominate the phenotypic expression. If something like curve *C* were the true relationship, then the present experiment could not detect sorting out.

The copper tolerance of various subclones of a heterocaryon double are shown

in Table 2. Sublines L and M are presumably $cu^s/cu^r : cu^r/cu^r$ heterokaryons while the R subline apparently lost the heterozygous nucleus. The six expansion lines of both subclones L and M indicate the ratios between the nuclei may vary greatly as L 1 and M 1 quite quickly behaved like cu^r homozygotes while other sublines maintained the intermediate type. Because the balance between the different nuclei is not stable it would be presumptuous to assert that the average tolerance level of these doubles reflects the tolerance of a cell with exactly three doses of cu^r to one of cu^s. But the data do show that extra doses of the cu^r gene clearly enhance the tolerance to cupric ion and indicate that the quadratic curve (*A*) on Fig. 1 is the best estimate of the dosage relationship.

Table 2. *Copper tolerance of heterokaryon doubles*

	$cu^r/cu^r : cu^r/cu^r$			×	cu^s/cu^s	
	Subclones of double				Controls	
Date	L	M	R		cu^r/cu	cu^s/cu^s
6/28						
7/1	1·42	1·38	—		1·59 ± 0·06 (8)	0·94 ± 0·06 (5)
7/3	1·28	1·15	1·55		1·59 ± 0·06 (8)	0·86 ± 0·08 (8)
7/9	1·25	1·18	—		1·50 ± 0·07 (5)	0·76 ± 0·05 (10)

Expansion lines*

	L 1	L 2	L 3	L 4	L 5	L 6
7/7	> 1·45	1·28	1·25	1·22	1·18	1·18
7/9	1·50	1·25	< 1·05	1·25	1·22	1·25

	M 1	M 2	M 3	M 4	M 5	M 6
7/7	> 1·45	1·25	1·22	1·18	1·18	< 1·15
7/9	1·55	1·18	1·35	1·22	1·22	1·28

The $\log_{10}$ of the median tolerance limit to cupric ion in micromoles per litre is shown.

* The expansion of subclones L and M was done on 7/3 from depressions isolated 6/29. After expansion the sublines were maintained by daily transfer.

The cupric ion tolerance of the heterozygotes as a function of age are shown in Table 3. Also shown is the mean and standard deviation of the $\log_{10}$ of the median tolerance limit to cupric ion in micromoles per litre of the two homozygotes. Inspection of Table 3 reveals considerable variability in the medians from day to day as well as a general downward trend. It is believed that this variability, which affects cu^s/cu^s and cu^s/cu^r lines much more than cu^r/cu^r lines, is a consequence of using different batches of Cerophyl. The concentration of the Cerophyl to which the copper is added is important; in higher Cerophyl concentrations the cells of all genotypes show increased tolerance limits (unpublished). The decline in the mean of the medians suggests this might be a function of ageing but it is not. Young cu^s/cu^s and cu^r/cu^r homozygotes were included in the later tests to specifically look for an effect of ageing on tolerance. A regression analysis showed no effect of age on copper tolerance (manuscript in preparation) and the trend must be due to a systematic bias of unknown source.

The haploid and chromosomal models predict an increase in the variance among heterozygous lines as the clones age and, because the heterozygote is not half-way between the homozygotes, an increase in the mean. No such trends are evident in this data. We can compare these results to Preer's (this volume) calculations, if one knows or assumes a dosage–phenotype relationship. I have used curve *A* (Fig. 1) and Preer's Table 3 (this volume) to calculate the increase in the mean and the variance expected at 200 fissions for a kinetic complexity of 860 units (430 of each allele); the change in mean expected is +0·04 and the increase in the standard deviation is +0·09. The data for 180 and 219 fissions show no such increase in the variance. If the kinetic complexity were 2000, however, calculations using Preer's (unpublished) computations show only an expected increase in the mean of +0·01 and an increase in the standard deviation of +0·016. The present data can not exclude the possibility that such a small change has occurred.

Table 3. *The mean and standard deviation of the copper tolerance of unselected lines as a function of genotype and age*

Date	Age of oldest clone (in fissions)	Genotype		
		cu^s/cu^s	cu^s/cu^r	cu^r/cu^r
2/3	8	1·11 ± 0·08 (7)	> 1·25 (6)	1·62 ± 0·03 (4)
2/5	18	1·15 ± 0·13 (7)	1·23 ± 0·09 (6)	1·60 ± 0·06 (4)
2/12	52	0·92 ± 0·08 (11)	1·01 ± 0·04 (12)	1·50 ± 0·04 (5)
2/19	84	0·79 ± 0·02 (3)	0·95 ± 0·07 (7)	
2/23	102	0·96 ± 0·02 (4)	0·98 ± 0·07 (6)	
2/27	119	0·91 ± 0·07 (16)	0·96 ± 0·07 (11)	1·54 ± 0·03 (5)
3/6	147	0·81 ± 0·07 (19)	0·93 ± 0·06 (12)	1·38 ± 0·09 (7)
3/15	180	0·83 ± 0·12 (10)	0·88 ± 0·07 (14)	1·46 ± 0·09 (9)
3/27	219	0·69 ± 0·12 (14)	0·75 ± 0·16 (8)	1·59 ± 0·07 (5)
4/2	248	< 0·75 (4)	< 0·75 (3)	1·45 ± 0·07 (5)

The vegetative pedigree of one heterozygous clone subject to selection for copper resistance is shown in Fig. 2. The median tolerance limit to cupric ion is shown in micromoles per litre, rather than as the $\log_{10}$. This pedigree is of the clone most widely expanded and which showed the greatest response to selection. Some sublines initially show a response to selection, and this was my initial interpretation. But given the assorting units model the mean of the sublines expanded from any subline should equal the parental value. In this pedigree all of the sublines of a line which tested high eventually returned to a level typical of heterozygotes. It is believed that the transitory increase in tolerance that some lines showed is the result of quasi-stable differences which arise within lines. A similar phenomenon with respect to tolerance of $CaCl_2$ has been reported by Genermont (1966). In any case the selection process was clearly unsuccessful in establishing sublines which behaved like resistant homozygotes.

Finally phenotypic scoring of the other traits carried in aged heterozygotes showed no evidence of sorting out. At the conclusion of this experiment 44 sublines heterozygous for *ndB*, *nd203*, or both *ndB* and *nd203* (this includes selection lines)

were tested with picric acid. All 44 sublines discharged their trichocysts as did the initial heterozygotes. The temperature-sensitivity test gave less readily interpretable results. Four of 27 lines heterozygous for *ts111* (21 of these also carried *ts203*) died at 35 °C, but one of the four remaining aged wild-type homozygotes also died. Apparently the capacity to grow at 35 °C declines in aged lines. None of the *paA* or hr^b heterozygotes were observed to behave like their respective homozygotes.

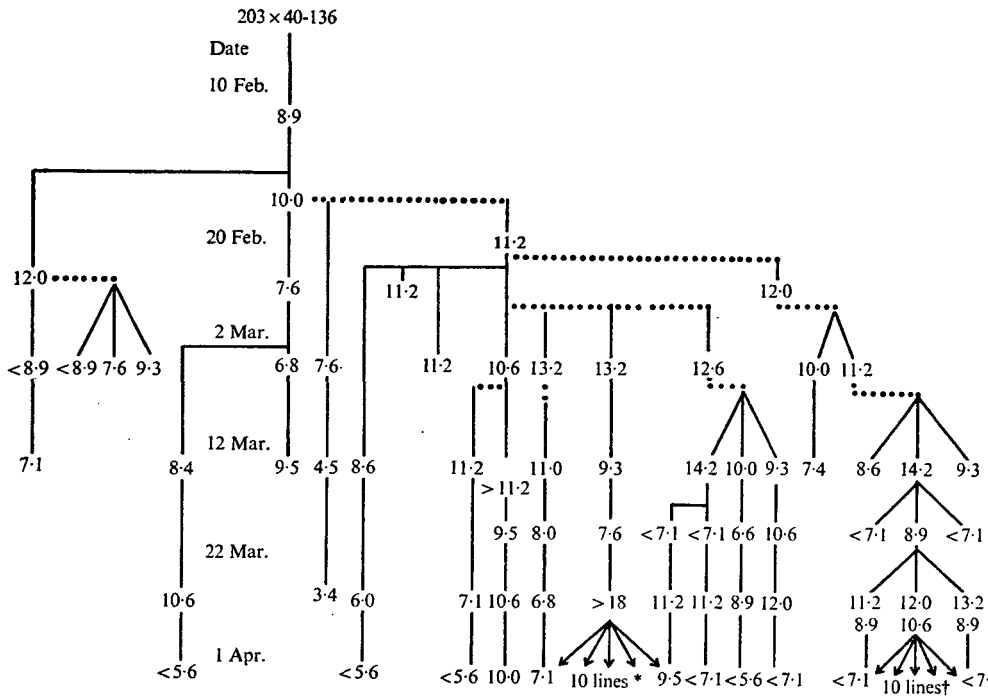

Fig. 2. The pedigree and median tolerance limits to copper of one heterozygous, cu^s/cu^r, clone selected for high resistance to copper. The dotted lines indicate exposure to high sublethal concentrations of cupric ion. The straight lines show transfers in the normal medium. Copper tolerance is given in micromoles per litre. * 11·2 (2), 9·5 (3), < 8·9 (5). † 8·9, 8·4, 8·0 (2), 7·6 (3), < 7·1 (3).

4. DISCUSSION

All of the results in this experiment on ageing heterozygotes have supported Sonneborn's (1947) proposal that the macronucleus of *Paramecium aurelia* is composed of functionally diploid subunits. Since calculations based on Preer's (this volume) simulation of the expected kinetics of assortment lead one to expect an approximate doubling of the average observed environmental standard deviation at 200 fissions and no increase was found, haploid and chromosomal models based on a kinetic complexity of 860 or less may be ruled out. These data do not allow one to exclude these models if the kinetic complexity is 2000 or greater. The expected rate of assortment based on Preer's calculations is conservative, however, for he assumes a constant amount of macronuclear DNA. Schwartz & Meister

(1975) report a regular drop in DNA as a function of age so that at 80 fissions after autogamy the macronucleus has only about 60% as much DNA as it has at five fissions. They also report that after 80 fissions the DNA increases irregularly. If macronuclear DNA is reduced this way one would expect an even greater increase in the variance than that determined from Preer's calculations.

Because the kinetic complexity of *Paramecium tetraurelia* macronuclear DNA may be greater than 860 (e.g. Soldo & Godoy, 1972) and because curve *A*, Fig. 1, may not accurately represent the phenotype–dosage relationship in heterozygotes the present experiments do not allow one to rule out all subdiploid models of the *Paramecium* macronucleus. The failure of this experiment to find any evidence of assortment indicates that the diploid subunit model is still the best available model, and the only model supported by experimental evidence.

Some other ciliates do not have a functionally diploid macronucleus. Macronuclear development is very different in *Euplotes* and *Stylonychia* (Kloetzel, 1970; Ammermann, 1971) from that of *Paramecium* and *Tetrahymena*. Apparently the macronucleus is organized differently in different ciliates. While this idea may be initially discouraging, the variety of genetic and biochemical organizations of the macronucleus is a fascinating evolutionary problem. Comparative biochemical studies of as morphologically similar species as the syngens of *Tetrahymena pyriformis* have already shown considerable biochemical diversity (Allen & Li, 1974; Borden *et al.* 1976) underlying their apparent morphological similarity.

Finally, these experiments draw attention to the potential value of the bioassay approach to quantitative traits like temperature tolerance, drug tolerance and metal tolerance in studying the genetic organization of the macronucleus. Such an approach may be useful in exploring the nature of dominance in a species such as *Tetrahymena pyriformis* syngen 1 which is known to assort.

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