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INDUCTION OF AUTOGAMY BY TREATMENT WITH TRYPSIN IN *PARAMECIUM CAUDATUM*

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SUMMARY

Firmly united conjugant pairs of *P. caudatum* were easily separated by treatment with trypsin, 0·025–1·0 mg/ml in 2 mM phosphate buffer at pH 7·2. Cytological observations showed that pairs separated by this means undergo normal meiosis and subsequent prezygotic divisions. Microspectrophotometric comparisons of G_1 micronuclei in the parent with those in clones derived from prematurely separated conjugants indicate usually the same DNA content in both. The stock d^m -13, heterozygous for mating type gene loci, showed the definite ratio of segregation to 2 mating types in clones derived from prematurely separated conjugants. Those results suggest that the prematurely separated cells usually undergo autogamy.

INTRODUCTION

Autogamy can occur periodically at relative short intervals in species of the *Paramecium aurelia* complex (Sonneborn, 1939, 1942). At autogamy, fertilization is accomplished by the fusion of 2 sister haploid nuclei bringing about homozygosity for all genes. Thus, the occurrence of autogamy enormously simplifies genetic analysis. Erdmann & Woodruff (1916) and Diller (1936) reported the characteristic crescent stage of the first meiotic division in single cells of *Paramecium caudatum*. So far, however, autogamy has not been found to occur spontaneously in *P. caudatum*. Therefore, artificial induction of autogamy in *P. caudatum* would be of special value in genetic work. Miyake (1958) suggested that abnormally united cells from tandem-type unions induced by chemical agents can probably undergo autogamy, and this technique was used for genetical analysis in *P. caudatum* (Hiwatashi, 1967). Miyake (1968) succeeded in inducing autogamy in *P. multimicronucleatum*. Ito (1969) and Tsukii & Hiwatashi (1977) also briefly reported that autogamy could be induced artificially in *P. caudatum* by chemical agents. The present paper demonstrates cytologically that tight pairs of *P. caudatum*, separated prematurely by trypsin treatment (Yashima & Koizumi, to be published elsewhere) undergo normal prezygotic divisions, and indirect evidence suggests that they undergo autogamy.

MATERIALS AND METHODS

Stocks and culture methods

Paramecium caudatum syngen 3, mating type V (stock d^m-11 and d^m-11ami), and mating type VI (stock d^m-13) were used. They were offspring of a cross d^m-1 × d^m-3, both derived from a cross of stocks Kok1 × Koj. The culture medium was fresh lettuce juice diluted with Dryl's solution (DS) (Dryl, 1959) at pH 7·0, and inoculated with *Klebsiella aerogenes* 1 day before use (Hiwatashi, 1968). The cultures were kept at 25 °C.

Enzyme treatment

The method employed was essentially the same as that described by Yashima & Koizumi (to be published elsewhere). Conjugating pairs were exposed to trypsin solution at 25 °C. The trypsin solution was prepared by stirring 0·01–1 mg/ml of trypsin 1:300 (ICN Pharmaceuticals), in 2 mM phosphate buffer at pH 7·2 for 5 min with a Micro Thermo-mixer (Thermonics Co. Ltd, Japan), 30 min before use. After 2 h of treatment, separated cells were transferred to culture medium to free them of trypsin. Cells were kept in the medium for 1–2 h and then transferred into DS.

Cytological observation

Each cell was placed on an albuminized coverglass bearing a small amount of culture fluid and was fixed with Schaudinn's solution (Kirby, 1950). Preparations were stained by the Feulgen reaction and counterstained with Fast green FCF (0·5 %). Temporary stain with aceto-orcein (1 g/100 ml in 45 % acetic acid) was also used to observe nuclear events.

Microspectrophotometry

The relative micronuclear DNA content was determined microspectrophotometrically by using an Olympus MMSP-TU. Cells at stage G_1, produced by dividing cells which had been isolated into DS, were placed on clean slides. The slides were air-dried and fixed in aceto-ethanol (1:3) for 15 min. Subsequently, the slides were Feulgen stained and allowed to age at room temperature for a week prior to measurement. The intensities of absorption at 560 and 450 nm were measured by scanning with a 2-μm monochromatic beam. The values obtained by subtracting the values at 450 nm from that at 560 nm were recorded and considered to be proportional to the DNA content (Fukuda & Fujita, 1971).

RESULTS AND DISCUSSION

Separation of conjugating pairs

When mating reactive cells of stocks d^{m}-11 and d^{m}-13 are mixed and kept at 25 °C, tightly conjugating pairs are formed by 2 h and separate about 15 h later. Firmly united pairs, which could not be separated mechanically by squirting repeatedly from a micropipette, were easily separated by trypsin treatment. Treatment of such pairs with trypsin at 0·05 mg/ml (optimal concentration), beginning 2 h after they became tightly united, resulted in separation of the conjugants in up to 73 % of the pairs. The effect of various concentrations of trypsin (0·01–1·00 mg/ml) on frequency of separation of mating pairs is shown in Fig. 1. Pair separation began in a few minutes, but occurred mostly 40 min to 1 h after start of the treatment.

The next experiment was designed to discover how late in the conjugation process conjugating pairs can be separated by 2-h exposure to trypsin (0·1 mg/ml). Samples were tested beginning 3, 5, 7, 9 and 11 h, respectively, after mixing the 2 reactive mating types. As illustrated in Fig. 2, when treatment began 3 h after mixing, 71 % of the conjugants were separated by the treatment and 63 % of them later underwent fragmentation of the prezygotic macronucleus, indicating that normal nuclear processes associated with fertilization continued after the induced premature separation of mates. A small proportion of conjugants separated even when trypsinization began 7 h after mixing, but not when it began later.

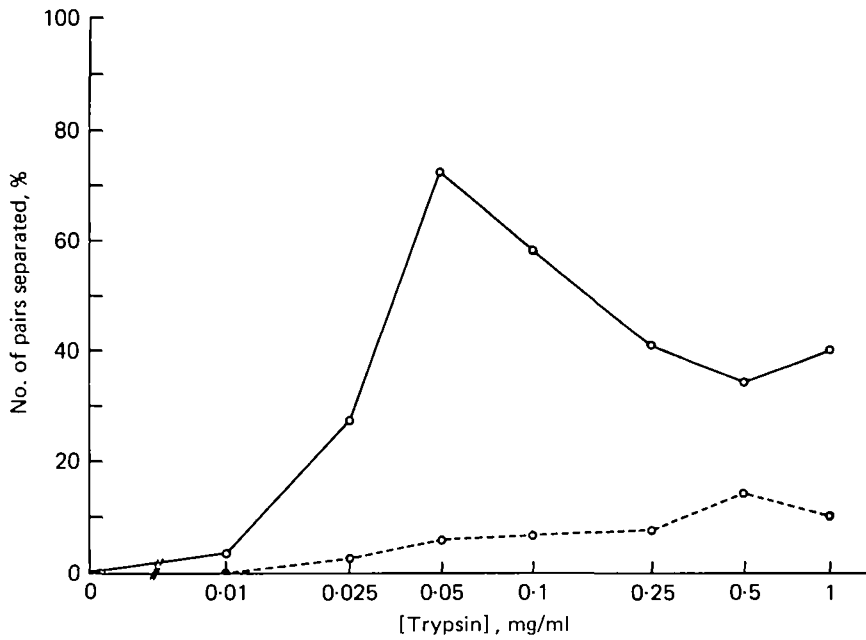

Fig. 1. Relation between concentration of trypsin and frequency of premature separation of conjugating mates. For each concentration, 60 conjugating pairs were treated with trypsin beginning 4 h after mixing the complementary types. Observations for pair separation were made 2 h after the start of the treatment. ○——○, % separated pairs; ○- - -○, % that died during the treatment.

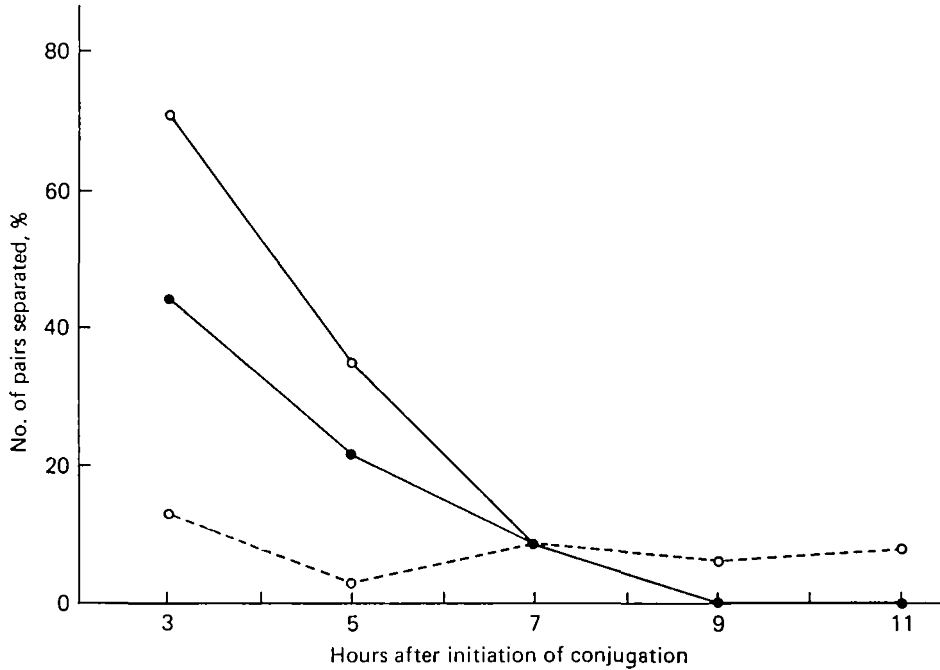

Fig. 2. Relation between time after conjugation when exposure to trypsin began and frequency of separated pairs. At each starting time, 50 pairs of conjugants were treated with 0·1 mg/ml trypsin for 2 h. The horizontal axis shows time of initiation of treatment. ○——○, separated pairs; ●——●, separated pairs in which fragmentation of prezygotic macronucleus was observed 24 h after mixing of the 2 mating types; ○- - -○, cells that died during the treatment.

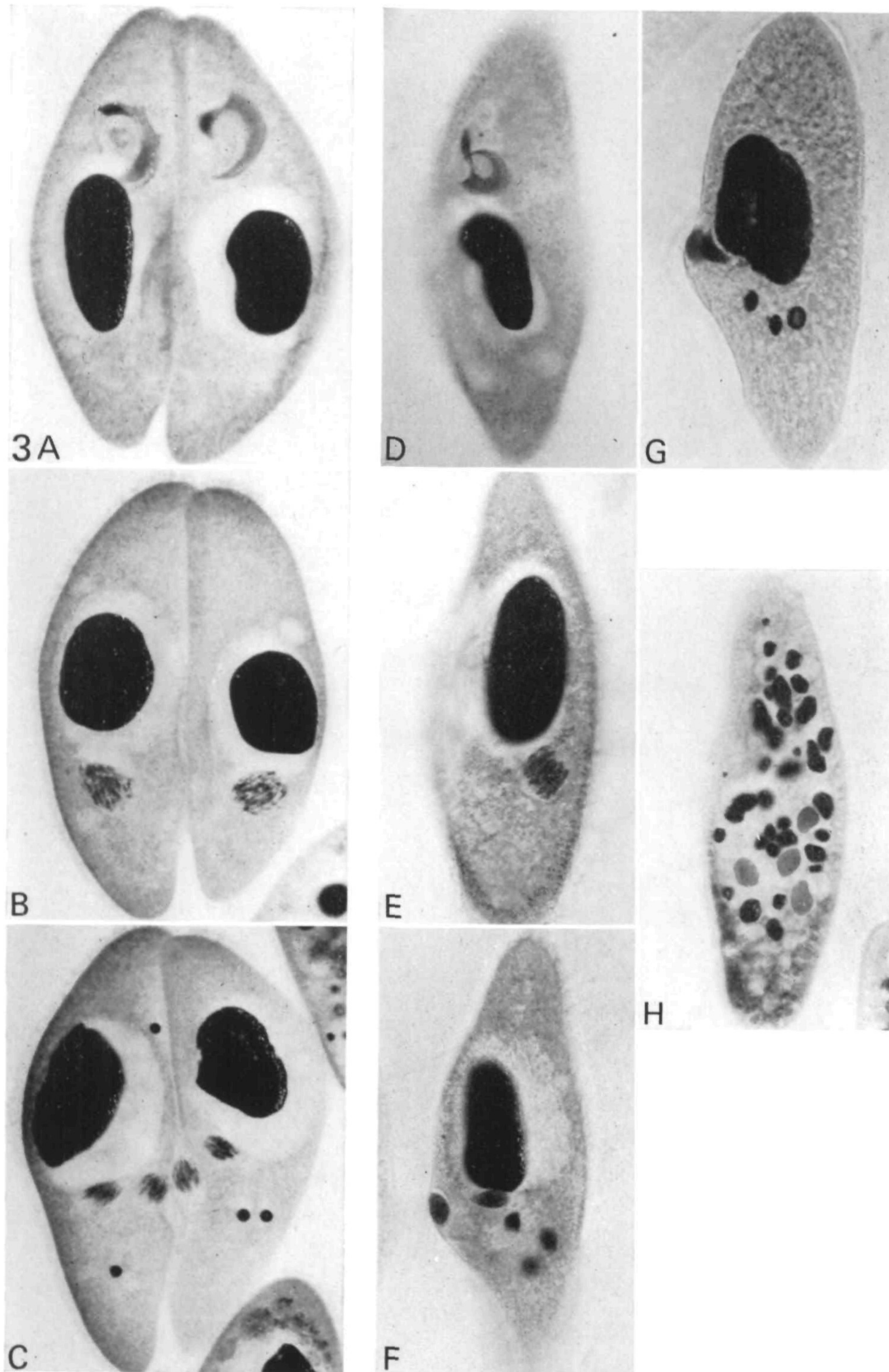

Fig. 3. Nuclear events in conjugating pairs and prematurely separated pairs after trypsinization. A–C show normal conjugating pairs and D–H show separated pairs. A, D, micronucleus in the characteristic crescent stage of the first meiotic division. B, E, micronucleus in meta- or anaphase of the first meiotic division. C, F, G, micronucleus at the stage of the third pregamic division in the paroral cone. Three degenerating micronuclei are seen as pycnotic bodies. H, development of 4 macronuclear Anlagen.

Cytological observations

Mass matings were obtained by mixing 200 ml of cultures (about 1000 paramecia/ml) of each type. More than 80% of the cells formed tight pairs. These mass matings containing some unpaired cells were concentrated to 2 ml by gentle centrifugation and then treated with 400 ml of trypsin (0·1 mg/ml) for 2 h at 25 °C. They were then transferred into DS after suspension in culture medium for 1 h. Starting at 9 h after mixing, samples were randomly taken from them every 1 or 2 h and Feulgen stained. Each sample of about 200 pairs which were not separated prematurely by trypsin treatment and about 200 single cells which were undergoing the prezygotic nuclear process, were recorded for nuclear condition. The typical meiotic enlargement of the nuclei, with the characteristic long crescent stage of the first meiotic division, was observed in cells separated by trypsin treatment (Fig. 3D). Following the crescent stage, the nucleus becomes contracted into a spindle-like form (Fig. 3E). The 2 nuclei produced by the first division proceed directly to the second division. Then 2 or 3 of the 4 nuclei degenerate into small condensed bodies which stain deeply and sooner or later disappear (Fig. 3F–G). All stages of meiotic division in split pairs exactly correspond to the micronuclear figures of the meiotic division in normal conjugation. A bulge (paroral cone) on the ventral surface of the cell bodies also appeared in the cells which were artificially separated, as in autogamous cells of the *Paramecium aurelia* complex (Diller, 1936; Sonneborn, 1954). After meiosis, one nucleus enters the paroral cone and there divides mitotically, producing two nuclei assumed to be the gamete nuclei that fuse to form the synkaryon (Fig. 3F–G), although actual fusion of gamete nuclei was not observed. Meanwhile, the other nuclei degenerate. In so far as observed, the nuclear events in these cells were identical to those of conjugation. Hence, if fertilization also occurs in these separated cells, it would have to be autogamous. Figure 4 shows the time course of nuclear events during the prezygotic stages of conjugation. Although the same sequence of micronuclear divisions was followed both in united and separated pairs, it proceeded somewhat more slowly in the latter (Fig. 4). Moreover, both sets of pairs showed a delay when compared with untreated normal conjugation (Mikami & Hiwatashi, 1975), the delay amounting to 2 or 3 h by the third prezygotic division. It is not clear at present whether the delay was due to a slowing after-effect of trypsin or to a block in the nuclear processes while exposed to trypsin or to some other feature of the treatment. However that may be, most of the surviving cells underwent the normal course of conjugation or autogamy because abnormal nuclear figures were not observed.

Postzygotic nuclear changes of prematurely separated cells were followed by using isolated pairs; most of these cells developed the normal number of 4 macronuclear Anlagen with the fragmentation of prezygotic macronucleus (Fig. 3H).

DNA content of the micronucleus after autogamy induced by trypsinization

If autogamy were induced by trypsinization, it would yield a normal micronucleus containing the same amount of DNA as the parental strains. Conjugants were treated with trypsin (0·1 mg/ml) during 3–5 h after mixing and the members separated in 129

(out of 170) pairs. Each of the separated cells was isolated and fed at 25 °C and one product of its first fission stained with aceto-orcein. Macronuclear fragmentation was observed in 170 (66 %) of these cells. Each unstained sister cell was then induced to undergo macronuclear regeneration by transferring it into culture medium (Mikami & Hiwatashi, 1975). This was done to keep the cell and the clone derived from it under the control of the parental genotype because survival at autogamy after trypsin treatment

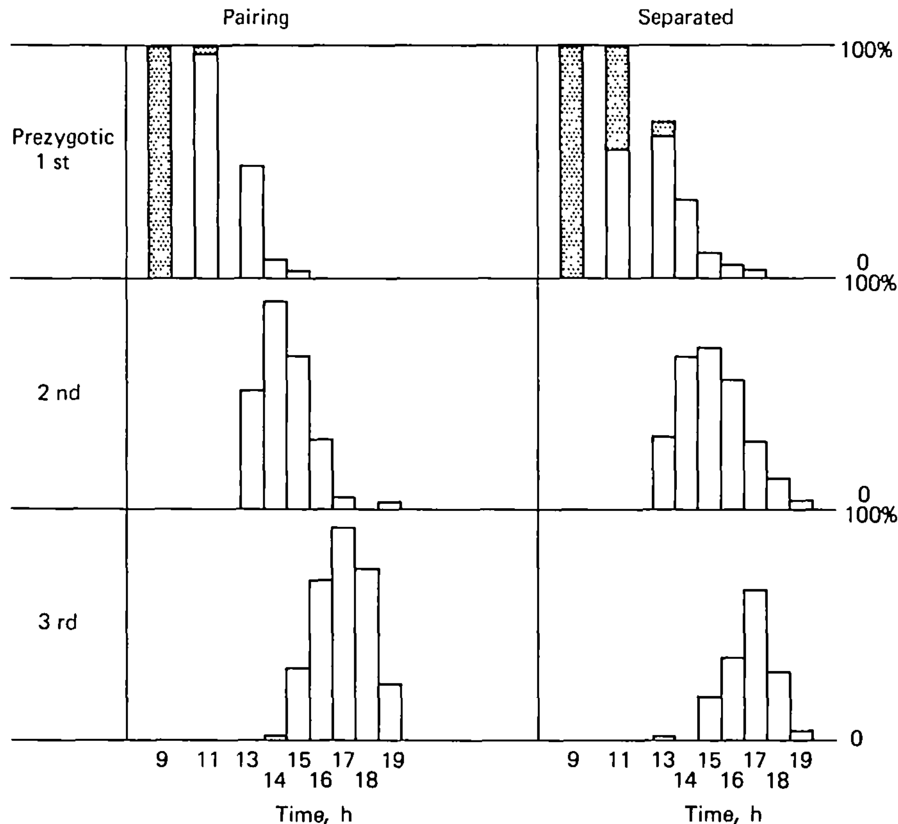

Fig. 4. Histogram representing the time course of nuclear events during prezygotic nuclear divisions in conjugating and prematurely separated pairs. For the first meiotic division, dotted bars show the fraction in the crescent stage and white bars show the fraction in meta- and anaphase stages. The horizontal axis shows hours after mixing of the 2 mating types. Data based on about 200 pairs and about 200 prematurely separated pairs at each point of time.

was only a few per cent (presumably due to homozygosity for recessive lethal genes present in heterozygous condition in the parent). The amount of DNA in the G_1 micronuclei of 19 clones (randomly sampled from the 170 clones) was measured by microspectrophotometry. Mating type showed V in 14 of the 19 clones and VI in others (Table 1). As shown in Table 1, 15 of the 19 clones gave values in the range 4·92–8·09 as compared with 6·07 and 6·66 for parent stocks. This seems to suggest diploid nuclei. Some fluctuations in the values may be partly due to errors in measurement and to differences in nuclear compaction. Moreover, these 15 clones were also

characterized by their viability when conjugation was obtained between them, as expected from restoration of heterozygosity. Therefore, trypsinization of conjugants seems usually to yield in the prematurely separated cells micronuclei with the same amount of DNA as the parental stocks. Hence, if the parents were diploid, so were these clones. However, the data do not distinguish whether this was due to self-fertilization or to diploidization of a reduced gamete-nucleus.

Table 1. *DNA content of G_1 micronuclei in clones from conjugant cells induced to separate prematurely by trypsinization*

Clone	Absorption (± S.D.)	DNA content estimated	Mating type
1	7·28 ± 0·14	2C	V
2	7·09 ± 0·09	2C	VI
3	8·03 ± 1·23	2C	V
4	6·78 ± 0·84	2C	V
5	7·07 ± 0·43	2C	V
6	6·95 ± 0·60	2C	VI
7	2·76 ± 0·17	1C	V
8	6·44 ± 0·38	2C	V
9	12·85 ± 0·75	4C	V
10	6·24 ± 0·71	2C	V
11	6·55 ± 1·44	2C	V
12	5·64 ± 0·16	2C	V
13	1·60 ± 0·11	0·5C	V
14	4·92 ± 0·41	2C	VI
15	5·17 ± 0·07	2C	V
16	5·80 ± 0·28	2C	VI
17	12·17 ± 0·82	4C	V
18	8·09 ± 0·49	2C	V
19	6·28 ± 0·43	2C	VI
d^m-11	6·66 ± 0·26	2C	V
d^m-13	6·07 ± 0·22	2C	VI

d^m-13 and d^m-11 are control strains, the parental strains. The assigned C value is based on the assumption that the G_1 micronuclei of the parental strains are diploid.

Segregation of mating type

The stock d^m-13 employed here is heterozygous for the mating type gene locus. We could, therefore, investigate whether the segregation of mating type expected from autogamy occurred in the prematurely separated pairs of d^m-13. Prematurely separated cells of stock d^m-13 were obtained as follows; isolated pairs of stock d^m-13 and d^m-11ami (an amicronucleate strain derived from d^m-11) were treated with trypsin 4 h after mixing the 2 mating types. Then, 708 separated pairs containing d^m-13 and d^m-11ami were isolated and kept in DS for 48 h so that a high frequency of macronuclear regeneration did not occur. Afterwards, these cells were grown in fresh culture medium at 25 °C. These clones were then tested early, at 10–15 fissions after premature separation, for their mating reactivity. Amicronucleate cells can undergo the fragmentation of the prezygotic macronucleus as in micronucleate cells after induc-

tion of conjugation and autogamy. However, such an 'exconjugant' or 'exautogamous' cell is non-viable (Mikami, unpublished). Cells with a new macronucleus developed from a division product of the synkaryon have no mating reactivity for 50–60 fissions after conjugation even under appropriate conditions. This period is called immaturity. Cells with macronuclei regenerated from prezygotic macronuclear fragments do not show this immaturity period. Therefore, clones which underwent macronuclear regeneration and which did not develop new macronuclei could be excluded by early testing for their mating reactivity. Consequently, most of the d^m-13 clones which seemed to undergo normal macronuclear reorganization died, and only 17 exautogamous clones of d^m-13, which became mature after a period of immaturity, were finally obtained. The segregation of mating types of these clones was examined; 6 of the 17 clones expressed mating type V and the other 11 were VI. These results are consistent with the occurrence of autogamy in cells prematurely separated by trypsinization. However, they still do not distinguish whether assortment was due to self-fertilization or to diploidization of a reduced gamete nucleus.

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