Drug Resistant Mutants and Appearance of Heterozygotes in the Cellular Slime Mould *Dictyostelium discoideum*

By Y. FUKUI

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan

AND I. TAKEUCHI

Department of Botany, Faculty of Science, Kyoto University, Kyoto 606, Japan

(Accepted for publication 10 June 1971)

SUMMARY

Mutants of *Dictyostelium discoideum* resistant to the antifungal antibiotics, Naramycin or Trichomycin, were isolated after treatment with nitrosoguanidine. When two strains differing in pigmentation and drug resistance were cultured together, amoebae resistant to both drugs appeared in underwater cultures and in liquid shake cultures. Such heterozygotes did not appear during the growth period, but only after cultures reached stationary phase. The appearance of the heterozygotes seems to have some relationship to the state of cells. Studies on the ploidy and the resistance of the progeny of these heterozygous isolates revealed that:

1. Diplophase of the original heterozygote is probably transient,
2. All the progeny observed are mononucleate,
3. The ploidy of each progeny settles in a stable state of aneuploidy (between the haploid and the diploid),
4. Genetic segregants for drug resistance and pigmentation appear among the progeny in serial subcultures,
5. Preferential chromosome elimination occurs during the process of chromosomal reduction.

A mechanism of recombination in *D. discoideum* is discussed in relation to the parasexual cycle.

INTRODUCTION

In the life-cycle of the cellular slime moulds, a spore germinates and an amoeboid cell is liberated. This amoeba feeds on bacteria and divides by binary fission with a generation time of 3 to 4 h. (vegetative stage). At the end of this stage, the amoebae cease to feed, and after some delay a large number of cells stream together to form a multicellular organization (aggregation stage). The resulting mass of cells called a pseudoplasmodium crawls on a substratum (migration stage). Finally, the cell mass rises up and forms a fruiting body which consists of a mass of spores and a supporting cellular stalk (culmination stage) (Bonner, 1967).

Haploid and diploid cells of *Dictyostelium discoideum* bear seven and 14 chromosomes respectively (Wilson, 1953; Ross, 1960). From cytological evidence, Wilson (1952, 1953) and Wilson & Ross (1957) proposed that the aggregation stage was the expression of the diplophase and the aggregating amoebae underwent the sexual phenomenon of syngamy and meiosis. But Bonner & Frascella (1952), Sussman (1956), Huffman & Olive (1964) and Huffman (1967) conclude that it is not clear whether a true sexual process exists. No recombination has been observed (Sussman, 1956). However, a diploid strain of yellow (wild-type) pigmentation was obtained from a mixed culture of two haploid mutant strains of white and brown pigmentation (Sussman & Sussman, 1962, 1963).

Recently, Loomis & Ashworth (1968) and Loomis (1969) revealed that when two strains of plaque-size or temperature-sensitive mutants of *Dictyostelium discoideum* were incubated...
together, heterozygous cells bearing both of the parental markers appeared. Sinha & Ashworth (1969) also found heterozygotes using different genetic markers, and from the characteristics of segregation and the ploidy of offspring they proposed the existence of a parasexual, rather than a true sexual cycle.

To elucidate the sexual process in this organism, we have attempted to demonstrate the occurrence of recombination in a genome of two drug-resistant mutant strains. Drug-resistant markers were used so that recombinants could be effectively isolated by selective plating. Heterozygous cells which were resistant to both drugs were isolated in mixed cultures of the two resistant strains under certain conditions. Some characteristics of the progeny of these isolates are described.

**METHODS**

*Chemicals.* Two antifungal antibiotics were used. Naramycin (mixture of Naramycin A and B) was a gift of Tanabe Seiyaku Co. Ltd (Osaka, Japan). Naramycin A is known as actidione or cycloheximide. Trichomycin was a gift of Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was a product of Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.).

*Organisms.* *Dictyostelium discoideum* strain NC4, which was originally isolated by Raper (1935).

*Culture methods.* The amoebae of NC4 were cultivated in association with *Escherichia coli* at 22°C (Bonner, 1947). Drug-resistant mutants were grown on a nutrient poor medium containing lactose and peptone (Raper, 1951). Amoebae were collected and washed in standard salt solution (Bonner, 1947) by repeated centrifugation (100 g, 1-5 min.). The amoebae were counted with a haemocytometer and suspended in standard solution. When the bacteria were pregrown, they were cultivated on a nutrient medium at 22°C for 2 days. They were collected and washed in distilled water by repeated centrifugation (300 g, 20 min.), and the final pellet was resuspended in water.

Liquid shake culture was conducted as follows. A suspension (10 ml.) of amoebae was placed in a 100 ml. Erlenmyer flask and shaken on a reciprocating shaker (120 cyc./min.) at 22°C.

*Isolation of drug-resistant mutants.* The conditions finally adopted for mutagenesis by nitrosoguanidine (NTG) differed from those described by Yanagisawa, Loomis & Sussman (1967). Amoebae were collected at an early stage of aggregation. About 10⁶ washed amoebae were suspended in 10 ml. of M/60 Sörensen’s phosphate buffer (pH 6.4) containing 200 μg./ml. freshly prepared NTG and placed in a 100 ml. flask. After incubation on a reciprocating shaker for 30 min. at 22°C, the amoebae were washed three times and suspended in 10 ml. of buffer and shaken for 24 h. The amoebae were then washed and suspended in buffer at a final concentration of 2 x 10⁶ cells/ml. Supplied with bacteria, surviving amoebae began to grow and increased in number until they reached a final density of 1 to 2 x 10⁷ cells/ml. in 48 h. The yield of mutants was increased several hundred times by this ‘intermediate cultivation’, as observed with *Escherichia coli* by Adelberg, Mandel & Chein (1965). The amoebae were then washed and suspended in salt solution. A suspension (0.1 ml.) containing 5 x 10⁶ cells was inoculated with bacteria on a plate containing either Naramycin (250 μg./ml.) or Trichomycin (1300 units/ml.) for selection of resistant mutants.

*Selective medium.* The selective medium used for isolating double resistant clones was 2% agar dissolved in M/30 Sörensen’s phosphate buffer (pH 7.0) containing 75 μg. Naramycin/ml. and 50 units Trichomycin/ml.

*Genetic notation.* nar, Naramycin-resistant; nar+, Naramycin-sensitive; tri, Trichomycin-
resistant; \textit{tri}^+, Trichomycin-sensitive; \textit{pig}^+, yellow pigments in fruiting bodies; \textit{pig}, albino fruiting bodies.

\textbf{RESULTS}

\textit{Isolation and characteristics of drug-resistant mutant strains}

Two clones of Naramycin-resistant mutants and 10 clones of Trichomycin-resistant mutants were isolated from $2.5 \times 10^7$ and $2 \times 10^8$ NC4 amoebae treated by NTG and were designated as \textit{nar} 21, 22 and \textit{tri} 6, 10, 11, 13, 14 respectively. The two \textit{nar} mutants were stable haploid and contained yellow pigments in their spores (\textit{pig}^+) and grew on plates containing as much as 1000 \textmu g. Naramycin/ml. \textit{Tri} 10, 11, 13 and 14 were double mutants with albino fruiting bodies (\textit{pig}). All of the \textit{tri} mutants were stable haploid and grew on plates containing as much as 5000 units Trichomycin/ml. All of \textit{nar} and \textit{tri} mutant strains retained their resistance throughout subculture in the absence of drug. Detailed description of these mutants has been reported by Fukui (1969). Since \textit{nar} 22 and \textit{tri} 11 showed active growth and normal morphogenesis, they were mainly employed in this study.

\textit{Appearance of double resistant clones from mixcultures of two drug-resistant mutants}

The amoebae of \textit{nar} 22 and \textit{tri} 11 were inoculated together on a lactose-peptone medium in equal numbers. After growth the amoebae were either collected at the preaggregation stage or allowed to form the fruiting bodies. The preaggregation amoebae or amoebae germinated from spores were inoculated on to selective medium at a density of $5 \times 10^6$ cells/plate with bacteria. None of the amoebae inoculated grew, indicating that cells which are resistant to both drugs did not appear at a frequency greater than $10^{-7}$ in standard culture conditions.

Two sets of conditions were found to yield double resistant clones at a frequency of $10^{-4}$ to $10^{-6}$. The first was an under-water culture in which the pregrown amoebae of both mutants were allowed to aggregate but not to form the migrating pseudoplasmodia or the fruiting bodies. Washed amoebae of each mutant were mixed in equal numbers, and incubated at $22^\circ$ in 20 ml. of standard salt solution in a 90 mm. Petri dish. After 24 h., the salt solution was removed, and the amoebae formed fruiting bodies after 3 days. The spores were collected and germinated by shaking for 8 h. in buffer containing peptone (Cotter & Raper, 1966). The germinated amoebae were inoculated on to selective medium with bacteria. Double resistant clones appeared at a frequency of $3 \times 10^{-6}$.

The second was a liquid shake culture. Equal numbers of amoebae of \textit{nar} 22 and \textit{tri} 11 were permitted to grow in the presence of bacteria. After reaching a final density of $2 \times 10^7$ cells/ml. in 28 h., they were further incubated for an additional 20 h. At various times during the incubation, cells were collected and plated out on to selective medium. No cells which were collected during the growth phase or during the early stationary phase could grow, while those in the late stationary phase (48 h.) gave a number of double resistant clones. The frequency of appearance was $2 \times 10^{-5}$. During incubation, the morphology of amoebae was microscopically examined. The amoebae in and after the late growth phase showed variation in size (Fig. 1a). Giant multinucleate cells were observed, constituting 5 to 50 \% of the total cell population. Cell fusions and subsequent separations were occasionally observed (Fig. 1b, c). Similar phenomena of cell fusion (anastomosis) and its separation were previously observed by Huffman, Kahn & Olive (1962) and Huffman & Olive (1964). During the period when cell fusions were frequently observed, however, no double resistant clones appeared from mixed cultures. In the late stationary phase the amoebae agglutinated
Fig. 1. Microphotographs of amoebae in liquid shake, mixed culture of nar22 and tri11. The scale markers indicate 20 μm. (a) Amoebae in late growth phase, showing variety of cell size. (b) Amoebae in the same stage immediately after cell fusion (arrow). They separated again after a while. (c) Separation of giant multinucleate cells after transfer to fresh medium. (d) Cells in late stationary phase. They are round and inactive, forming large agglutinates.

Fig. 2. Microphotographs of the spores of the two parental strains and those of the two double resistant clones. Large size of the spores of the double resistant clones indicates their aneuploidy. The scale markers indicate 20 μm. (a) Parental strain nar22. (b) Parental strain tri11. (c) Double resistant clone 18C2. (d) Double resistant clone 18C1.
Drug resistance in *D. discoideum* and became spherical and motionless (Fig. 1d). This was accompanied by a decrease in the number of viable amoebae. On the other hand, this also coincided with the appearance of double resistant clones.

**Relationship of culture conditions to the appearance of double resistant clones**

To discover whether the coexistence of the two resistant mutants in the growth phase was essential for the appearance of double resistants, *narz2* and *triri* were grown independently and equal numbers (10⁶) of amoebae of the two strains were mixed and incubated without bacteria for 24 h. An aliquot of cells was taken every 6 h. and plated on to selective medium. Double resistant clones appeared at a frequency of 5 × 10⁻⁶, after 24 h., indicating that growth was not essential for the appearance of double resistants. In this culture, giant multinucleate cells appeared after 24 h. and thereafter the amoebae became spherical, and the number of viable cells decreased to 75% of the initial value after 48 h.

The relationship between the state of amoebae and the appearance of double resistants was further examined. Amoebae of *narz2* and *triri* were incubated together on a shaker. After reaching stationary phase, they were washed and resuspended in (a) 10 ml. of buffer containing fresh bacteria, (b) 5 ml. of the medium previously used for culturing the amoebae (centrifuged free of bacteria)+ 5 ml. of the buffer, and (c) 10 ml. of the buffer. After 24 h. incubation, cells were collected and plated on to selective medium. In medium (a) the amoebae multiplied until they reached a density of 4.7 × 10⁷ cells/ml. After a few hours, when almost all the cells became spherical (Fig. 1d), eight double resistants appeared amongst 4 × 10⁵ cells, i.e. at a frequency of 2 × 10⁻⁶. In medium (b) two double resistants appeared amongst 4 × 10⁵ cells, i.e. at a frequency of 5 × 10⁻⁸. The cells became spherical 1 to 2 h. later than those in medium (a). In medium (c), however, no double resistants appeared amongst 2.5 × 10⁵ cells, i.e. at a frequency greater than 4 × 10⁻⁶, and no morphological changes of the amoebae were observed. Thus the appearance of these double resistants was correlated with certain morphological changes of amoebae.

**Characteristics of the progeny of double resistant clones**

Phase contrast microscopy revealed that the double resistants isolated were mononucleate. Ploidies of these clones were investigated by measuring major diameters of spores (Sussman & Sussman, 1962, 1963). The mean diameters of the spores of seven clones which were formed on the original selective medium varied within the range between the haploid and the diploid; 8.4, 8.8, 9.5, 9.6, 10.5, 10.6 and 10.8 μm. They were significantly larger than spores of the two parental strains, *narz2* (7.25 μm.) or *triri* (7.35 μm.), and were probably aneuploid (Sackin & Ashworth, 1969) (Fig. 2a to d).

To study whether ploidies of the progeny derived from the original double resistant cell change through cell divisions, isolated double resistant clones were serially subcultured on selective medium, as described in Table 1. The size of spores formed in each subculture was measured under a microscope with an ocular micrometer. Among seven clones examined at least two types were found in respect to changes in the distribution curves of spore size during subculture (Fig. 3a, b). Two clones were classified as type I, in which the distribution curve shifted almost to that of the haploid after only one subculture and the mean spore diameters decreased. Five clones were classified as type II, in which neither the distribution curve nor the mean spore diameter shifted significantly after 15 subcultures. In either type, however, the value of standard deviation became smaller, indicating that the variation in spore size diminished during subcultures.

Drug resistance of the progenies derived from 17 double resistant clones were examined,
Y. FUKUI AND I. TAKEUCHI

according to the method described in Table 1. Their modes of resistance were classified
into two types, type N and type T. Sixteen clones belonged to type N, all of which stably
retained the resistance to Naramycin (nar) and formed yellow fruiting bodies (pig+) after
15 subcultures, e.g. clone 17 C1 (Table 1). On the contrary, the amoebae resistant to

Table 1. Dictyostelium discoideum: resistance of the progeny of double resistant
clone 17 C1 (type N)

The amoebae of clone 17 C1 were collected with a platinum loop from the original selective medium.
Depending on the level of resistance of the progeny, 50 or 10⁴ amoebae were inoculated with bacteria
on two plates for each of four kinds of test media. Amoebae of the original isolate were, at the same
time, subcultured twice on four kinds of test media and 15 times on the test medium containing both
drugs. In each subculture, amoebae were allowed to divide about 10 times. The numbers of clones
produced on the test media were counted under a dissecting microscope after 2 days incubation at
22°. The clone numbers on the medium containing no drugs indicate the number of surviving
amoebae. The clone numbers on the test media containing Naramycin, Trichomycin and both drugs
indicate the number of amoebae which are nar, tri, and nar tri.

<table>
<thead>
<tr>
<th>No. of</th>
<th>Media used for subculture</th>
<th>Naramycin (75 µg/ml.)</th>
<th>Trichomycin (50 units/ml.)</th>
<th>Trichomycin (50 units/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>subcultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original</td>
<td>—</td>
<td>100</td>
<td>110</td>
<td>&lt;1*</td>
</tr>
<tr>
<td>1</td>
<td>No drugs</td>
<td>100</td>
<td>108</td>
<td>1 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>Naramycin (75 µg/ml.)</td>
<td>100</td>
<td>90</td>
<td>&lt;2 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td>100</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Naramycin (75 µg/ml.) and</td>
<td>100</td>
<td>105</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>No drugs</td>
<td>100</td>
<td>112</td>
<td>2 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>Naramycin (75 µg/ml.)</td>
<td>100</td>
<td>95</td>
<td>1 × 10⁻⁳</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td>100</td>
<td>116</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Naramycin (75 µg/ml.) and</td>
<td>100</td>
<td>102</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Naramycin (75 µg/ml.) and</td>
<td>100</td>
<td>97</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Only 100 cells were inoculated, but no cell grew.

Trichomycin (tri) were effectively selected on the media containing Trichomycin or both
drugs, but were eliminated on the media which did not contain Trichomycin. On the other
hand, only clone 1502 among 17 double resistant clones belonged to type T, in which all of
the progeny formed albino fruiting bodies (pig) (Table 2). When the amoebae obtained
from the original selective medium were inoculated on the medium containing both drugs,
76% of them could grow, in contrast to those of type N. When they were subcultured on the
medium containing both drugs, almost all the amoebae grew on the media containing
Trichomycin or both drugs, indicating that they bore both of the genotypes of nar and tri.
On the contrary, when they were subcultured on the test medium containing no drugs, only
4% of the amoebae were resistant to Trichomycin (tri), and the remaining 96% of the
amoebae were sensitive to both drugs (nar+ tri+). This indicates that tri was relatively stable
when compared with nar, and that nar+ tri+ amoebae were preferentially selected on the
medium containing no drugs.

It should be noted that the growth rates of the progenies of some double resistant clones
decreased during subculture. The growth rate of the progeny of clone 17 C1 decreased to
Drug resistance in *D. discoideum*

Fig. 3. Changes during subculture in spore size distribution of the two double resistant clones derived from *nar22* and *tri11*. (a) Changes in spore size distribution of double resistant clone 1502 belonging to type I. Spore size distribution of the clone shifted nearly to that of the parental haploid strains, after the second subculture. (b) Changes in spore size distribution of double resistant clone 17C1 belonging to type II. Spore size distribution of the clone after the tenth subculture is about the same as that of the original isolate and falls within the range of aneuploidy between haploid and diploid. Orig., spores formed on an original selective medium; 2nd and 10th, spores formed on a selective medium after the second and tenth subcultures. ○, Spore size of parental *nar22* ($\bar{d} = 2.90 \pm 0.41$); ●, spore size of parental *tri11* ($\bar{d} = 2.94 \pm 0.39$). One micrometer division equals 2.5 μm. For reference, mean diameters of haploid strain NC4 and diploid strain RA were taken from Sussman (1962) and are indicated by the arrows.
about one half of that of the original isolate after three subcultures (i.e. about 30 generations) on the medium containing both drugs and was not recovered by culturing amoebae on the medium containing no drugs. A similar decrease in growth rate was also observed with clone 18C1 (type II, N), and clone 18C5 (type I, N). In these strains the germination rates also decreased to 23 and 29%, after a single subculture on the medium containing no drugs.

Table 2. Dictyostelium discoideum: resistance of the progeny of double resistant clone 1502 (type T)

See Table 1 for details of method.

<table>
<thead>
<tr>
<th>No. of subcultures</th>
<th>Media used for subcultures</th>
<th>No drugs</th>
<th>Naramycin (75 µg/ml.)</th>
<th>Trichomycin (50 units/ml.)</th>
<th>Naramycin (75 µg/ml.) and Trichomycin (50 units/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>—</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>No drugs</td>
<td>100</td>
<td>&lt;1*</td>
<td>4</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td></td>
<td>Naramycin (75 µg/ml.) and</td>
<td>100</td>
<td>60</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Trichomycin (50 units/ml.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Only 100 cells were inoculated, but no cell grew.

DISCUSSION

Judging from the spore size (Fig. 3), the cells of each original double resistant clone show a wide variation in their chromosome numbers, within a range between the haploid and the diploid. The distribution curve for spore size changed and became settled only after a certain number of subcultures. This indicates that the amoebae of each double resistant clone became stable with respect to the number of chromosomes they contained. A considerable number (96%) of sensitive cells (nar+ tri+) appeared when clone 1502 (type I, T) was subcultured on the medium without drugs (Table 2). These facts indicate that the original double resistant clone, which is considered to be a diploid, does not revert to a stable haploid through a transient stage of aneuploidy. In fact, all these clones obtained in the present work produced populations which mostly consist of aneuploid cells, and no stable haploid nar tri was obtained after 150 generations.

During subcultures of these double resistant clones, progenies with the genotypes nar tri, nar+ tri+, nar pig, and tri pig+ were segregated. Occurrence of the mononucleate double resistant amoebae of the genotype nar tri pig+ or nar+ tri+ pig from the mixed cultures of nar tri+ pig+ and nar+ tri pig, and appearance of the segregants from these double resistant clones indicate that a double resistant amoeba which is originally formed from the two parental cells is at least a partial heterozygous diploid and that both nar and tri are dominant. Studies on the resistances of the progeny of the original heterozygous diploid also suggest that some chromosomes were eliminated during multiplication of the cell. In spite of such chromosome elimination, the progenies of type N always formed yellow fruiting bodies and stably retained the genotype nar, while those of type T always formed albino fruiting bodies and retained the genotype tri, i.e. the former tended to retain the characters of nar22 while the latter those of triII (see p. 312). These facts suggest that the chromosome constitution of the progenies of type N was the mixture of the whole set of chromosomes of nar22 and a partial complement of those of triII, while those of type T was the mixture of the whole
Drug resistance in *D. discoideum*

set of chromosomes of *tri II* and a partial complement of those of *nar 22* (Fig. 4). How and why such a preferential chromosome elimination occurs must await further studies.

The sexual process of *Dictyostelium discoideum* was interpreted by Sinha & Ashworth (1969) to be a type of a parasexual cycle which was described for Aspergillus by Pontecorvo & Roper (1952) and for Verticillium by Hastie (1967). Although the present study seems to support their interpretation, there are two points which are incompatible with this inter-

---

Fig. 4. Suggested chromosome constitution of the two types of heterozygous clones, derived from *nar 22* and *tri II*. Type N is schematically shown to contain the chromosomes enclosed by a solid line, and Type T contains those enclosed by a dotted line. The location of genetic markers is arbitrary.

---

Fig. 5. Suggested mechanisms of recombination in the cellular slime mould *Dictyostelium discoideum*. For detailed explanation see text.
pretation. In the first place, the heterozygotes appeared only under the two specified conditions, but not under standard culture conditions. This idea is supported by the fact that diploid strains appeared at a relatively high frequency when unaggregated, hence aged amoebae were used as inocula for subcultures (Sussman, 1964). On the contrary, in Aspergillus or Verticillium heterozygotes are formed in a normal developmental process during conidiophore formation. In the second place, the resulting heterozygote of the slime mould did not undergo haploidization as readily as that of the fungi. No complete haploidization of the isolated heterozygotes was observed.

A mechanism for diploidization and subsequent aneuploidization in this organism is shown in Fig. 5. Huffman & Olive (1964) showed that cell fusions in this organism were of short duration and that almost all of the fused cells moved apart without visible cytoplasmic exchange. Our observation confirmed the instability of the fusions which underwent complete separations in 10 to 60 min. This was perhaps the reason why the heterozygotes did not appear during this process, supporting Huffman (1967) and Olive (1963). The occurrence of karyogamy following complete plasmogamy was in fact reported by Huffman et al. (1962), but this was an exceptional case. On our hypothesis, when cells become inactive, separation of the fused cell is presumably inhibited, and this results in formation of a heterokaryon. When the two nuclei in a heterokaryon undergo a nuclear division, they will divide synchronously with a single spindle apparatus, as in animal tissue culture cells (Fell & Hughes, 1949). This brings about the mixing of chromosomes and formation of the heterozygote.

Formation of a heterozygote is followed by reduction of some chromosomes. At least three mechanisms for chromosomal reduction are possible. The first is chromosome elimination which occurs in animal tissue culture cells (Weiss & Green, 1967). This will occur when a heterozygote undergoes mitosis, and during this process certain chromosomes are preferentially eliminated depending on the type of cell. The second is nondisjunction of chromosomes during meiosis, which occurs when the pairing of homologous chromosomes is poor, as suggested by Pontecorvo (1956) in the parasexual cycle. The last possibility is an unequal distribution of chromosomes either by amitosis or due to formation of incomplete spindle apparatus. The decrease in growth rate or in germination rate, we observed, may be related to some chromosomal aberration caused by any one of these mechanisms. Detailed cytological evidence is necessary to establish mechanism involved in chromosomal reduction in this organism.

How regularly the formation of a heterozygote and the exchange of genetic information occur during the development of this organism is not yet known. It is supposed that the sexual process in the cellular slime moulds is more irregular than the parasexual cycle described in fungi (Pontecorvo, 1956, 1969; Käffer, 1960; Hastie, 1967) and is very different from the ordinary sexual processes.

REFERENCES


Drug resistance in *D. discoideum* 317


