CHEMICALLY-INDUCED INSTABILITIES IN A HETEROZYGOUS DIPLOID OF PENICILLIUM CHRYSOGENUM

GIORGIO MORPURGO AND GIUSEPPE SERMONTI

International Research Centre for Chemical Microbiology, Istituto Superiore di Sanità, Rome, Italy

Received February 24, 1958

SOMATIC sergregation from heterozygous diploids in molds has been analyzed in detail for *Aspergillus nidulans* by PONTECORVO *et al.* (PONTECORVO, TARR GLOOR and FORBES 1954; PONTECORVO and KAFER 1956). Basically the same processes seem to take place in *Penicillium chrysogenum* (SERMONTI 1956, 1957), and probably also in *Aspergillus oryzae* (ISHITANI, IKEDA and SAKAGUCHI 1956). Somatic segregation analysis is comparatively straightforward where it concerns visible or easily selected markers (PONTECORVO and KAFER 1956; ROPER and KAFER 1957), but it becomes extremely laborious for invisible markers like nutritional deficiency, and impracticably difficult when the genes involved are those quantitatively controlling the production of antibiotics or other metabolites. Even segregation analysis of color markers is made laborious, in *Penicillium chrysogenum*, by the impossibility of microscopic identification of the color segregants as single yellow or white heads on the sporulating surface of heterozygous diploids (PONTECORVO 1953; SERMONTI 1957). Color segregants in this species appear, either as entire colonies or as macroscopic sectors, after plating conidia of the heterozygous diploid on complete medium. The rate of spontaneous appearance is sometimes so low that the collection of a modest number of segregants demands the observation of many thousands of colonies.

Attempts to increase the rate of somatic segregation of heterozygous diploids were made by IKEDA, ISHITANI and NAKAMURA (1957) on *Aspergillus oryzae* by ultraviolet irradiation of heterozygous diploid conidia, and by MORPURGO and SERMONTI (1958) on *Penicillium chrysogenum* by ultraviolet irradiation of conidia and by treating them with various chemical mutagenic agents. Somatic segregation was strongly stimulated in almost every case by the action of the mutagenic agents. In MORPURGO and SERMONTI's paper (1958), the delayed appearance of segregating sectors in colonies derived from conidia which had survived mutagenic treatment was very striking, suggesting a delayed effect of the mutagenic agent. An analogous delayed effect of chemical mutagenic agents had been noticed by AUERBACH (1951) in *Drosophila melanogaster*.

The present paper was begun as an analysis of this effect in the material under discussion.

MATERIALS AND METHODS

Strain: The strain used throughout the work was heterozygous diploid XXXIV of *Penicillium chrysogenum* from the Istituto Superiore di Sanità collection. The

diploid was synthesized by ROPER's method (ROPER 1952; PONTECORVO and SERMONTI 1953) between strains *173 y py* (derived from strain Wis. 49.133) and *176 w cy* (derived from strain Wis. 50.1247). The genotype of heterozygous diploid XXXIV may therefore be symbolized as:

$$\frac{Y\ PY\ w\ cy}{y\ py\ W\ CY}$$

No information was available at the beginning of the experiments about the linkage relations between the markers concerned. An attempted mapping will be discussed in the last section on the basis of the results reported. The symbols used are to be understood as follows : Y/y, green *versus* yellow conidium color; PY/py, nutritional independence *versus* requirement of pyridoxin; w/W, white *versus* green condium color; cy/CY, requirement of methionine or cysteine *versus* nutritional independence. The phenotype of the diploid is prototrophic and has green conidia.

Use of nitrogen mustard: The only agent used for stimulation of somatic segregation was a pharmaceutical preparation of the nitrogen mustard methyl-bis(β-chloroethyl)amine (Cloramin) in sterile 5 mg vials (SIMES, MILAN). It will be referred to hereinafter by the code name HN-2. Two ml of a sterile aqueous solution of $NaHCO_3$ (6.3 percent, w/v) were added with a Pasteur pipette to one vial of HN-2, shaken until the mustard was dissolved and then mixed with two ml of an aqueous suspension of 4.10^7 conidia of the strain to be treated. After 3–4 minutes the suspension was diluted ten times in a sterile decontaminating solution (0.6 g glycine, 0.7 g $NaHCO_3$, one liter distilled water.) The decontaminated suspension was kept at +5°C until required for use. The survival rate of the conidia at the end of treatment was 0.008–0.050, with no appreciable variation during the first 3–4 weeks of storing at +5°C.

In the last part of the experiments the method was modified; the HN-2 was dissolved in distilled water, and the conidia were suspended in the aqueous solution of $NaHCO_3$. Only in a very few cases was ultraviolet irradiation used as a mutagenic agent (PONTECORVO and SERMONTI 1954).

Media: The media used were a complete medium (CM), based on corn steep-liquor, nucleic acid, vitamins, etc. (SERMONTI 1957), and Czapek-Dox's minimal medium (MM).

Plating of conidia: The method adopted as standard in this laboratory was used in the first part of the experiments, i.e. 0.1 ml of conidium suspension of known density was spread with a glass rod over the agar surface of each petri dish. A less cumbersome method was later employed for carrying out platings starting from a single colony: conidia are harvested from the surface of the colony with a small drop of water held in a calibrated loop, which is then shaken in 2 ml of sterile water. The suspension is then diluted 1,000 times. Suspensions obtained in this way contain 10–30 conidia per 0.1 ml and can be plated by the standard method. *Penicillium chrysogenum* conidium chains immediately break up when placed

in water and gently shaken, so that the conidium suspensions always consisted of isolated conidia. Cultures were incubated at 24–27°C.

Terminology: Single colonies presumably developed from single conidia will be regarded as individuals in this paper, with a life cycle extending from germination of the original conidium to conidium production in the colony derived from it. Colonies derived from plating of the latter conidia will be regarded as belonging to the "generation" following that of the colony producing such conidia. This convention simplifies the record of the life history of colonies, by simply mentioning the "generation" to which they belong. Colonies obtained from conidia of heterozygous diploid XXXIV will be regarded as belonging to the parental generation (P), those from conidia of the P colonies as belonging to the first filial generation (F_1). Later generations will be recorded as F_2, F_3, etc. This terminology is purely conventional.

RESULTS

Effect of HN-2 on somatic segregation

HN-2-treated and untreated (control) diploid conidia were plated on complete agar at density of about 30 living conidia per dish. The colonies were observed for about ten days and on the last day of observation the number of colonies segregating for color or showing segregant sectors was recorded. The proportion of colonies displaying markers heterozygous in diploid XXXIV was greatly increased by the action of HN-2 on the conidia (Table 1). An analogous effect has

TABLE 1

Somatic segregation from diploid XXXIV: y py / w cy *of* Penicillium chrysogenum: *spontaneous segregation and segregation after HN–2 treatment*

Phenotype	Symbol	Number of segregants observed		
		Whole colonies	Sectors	Total
A. Spontaneous segregants				
Yellow prototrophic	*y*	10	7	17
pyridoxineless	*y py*	..	..	..
White prototrophic	*w*	..	..	..
cysteineless	*w cy*	..	1	1
cyst.-pyrid.-less	*w cy py*	..	..	..
Green (non tested)		706	7	713
B. Segregants after treatment				
Yellow prototrophic*	*y*	11	16	27
pyridoxineless†	*y py*	5	2	7
White prototrophic	*w*	5	..	5
cysteineless‡	*w cy*	22	6	28
cyst.-pyrid.-less‡	*w cy py*	1	2	3
Green prototrophic	+	141	21	162
pyridoxineless	*py*	2	1	3

* Conidia sometimes of haploid size, sometimes of diploid size.
† Conidia always of haploid size.
‡ Among 14 white segregants from another clone, 7 were *w cy* and 7 were *w cy py*.

been obtained with diploid VII (*me w/y thi pr*) (results not published in this paper). What is particularly striking is the increased proportion, among colonies derived from conidia surviving HN-2 treatment, of colonies exibiting segregation of color markers in the form of sectors. In such sectors the segregation process is certainly subsequent to the plating of the conidia. The rate of such sectoring colonies among untreated colonies is about 0.01, while among treated colonies it is about 0.1 (Table 1). The proportion of complete yellow or white colonies is also increased after HN-2 treatment, from 0.014 to 0.18 (Table 1). These could have been produced by a segregation process previous to conidium formation, but the great increase in their number among colonies derived from treated conidia makes it highly probable that in this case the majority of the segregant clones have originated subsequent to conidium collection, as a result of the action of HN-2. If it is assumed that each segregation process gives rise to two segregant nuclei in such cases one of the segregant nuclei must be supposed to have been eliminated, either by a disadvantageous mutation or as a result of genetic drift. Alternatively, some segregation process must be assumed which does not give rise to complementary nuclei, such as deletion, aneuploidization, etc.

Seven different segregant phenotypes out of the ten theoretically possible appeared after HN-2 treatment. Their ploidy is not easy to determine, on account of the irregularity of conidium size within each strain. Some of the yellow colonies (see note to Table 1) produced what were clearly normal conidia of the same size as those of haploid strains, while others produced giant conidia of the same size as those of diploid XXXIV. Giant conidia were also produced by green pyridoxinless (*py*) segregants. An analysis of the second-order somatic segregation from one of these *py* strains, purified by isolation of a single conidium, showed that the strain was heterozygous for the genes *Y/y*, *W/w*, and *CY/cy*.

The number of sectoring colonies among colonies derived from mustard-treated conidia increased as time went on, with the delayed appearance of sectors from some colonies. This phenomenon invited special consideration. It could be attributed to the delayed emergence of a segregant nucleus in the course of growth of a colony; but it could also be due to a delayed effect of the mutagenic agent. This assertion was tested by taking green conidia from a number of color sectoring colonies and plating them on CM in order to observe the appearance of the colonies derived from them. These colonies will be regarded as the first filial generation (F_1) of the sectoring colonies.

Analysis of F_1 of sectoring colonies

A total of 28 colonies with sectors, derived from HN-2-treated conidia, were analyzed by the procedure indicated above. Colonies of the first filial generation (F_1) were observed for about ten days of growth on CM; the proportion of colonies with sectors among them, and the type of each colony, was recorded. Hardly any completely yellow or white colonies were found.

Seventeen of the F_1 populations of colonies examined showed a normal segregation rate, i.e. one not significantly different from that of diploid XXXIV. Three

showed an appreciably higher proportion of sector-producing colonies, but the other eight showed a feature which had never previously been observed: the majority of the colonies, in a few cases almost all of them, produced sectors (Figures 1–4). Clones, or lines, derived from sectoring colonies, in which the property of producing sectors persisted in successive generations, will be referred to by capital letters (A, B, C, etc.). The term "mosaic" colonies will sometimes be used to refer to sectoring colonies, and the term "mosaic" clones, or lines, for the clones, or lines, to which they belong.

The proportion of mosaic colonies in the F_1 of clones with a high rate of segregation varied from 30 percent (D) to 90 percent (G). A particularly striking feature of these populations of colonies with a high rate of segregation was the uniformity of segregation pattern within each clonal population. In the majority, the sectors which appeared were exclusively yellow; in two clones (B and G) the sectors were of both colors, yellow and white, and a fair proportion of the colonies had sectors of both colors. One clone had colonies with white sectors only (L) and one had pale yellow and grey-green sectors in the majority of the colonies (C) (Table 2). The type and size of the sector was also characteristic in

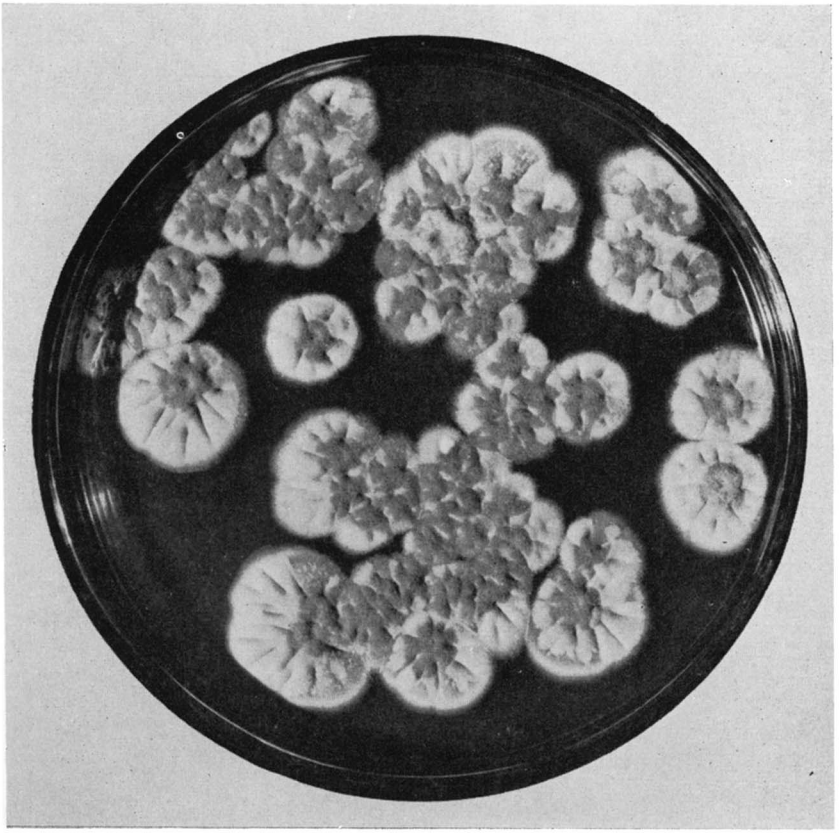

FIGURE 1.—Colonies of the unstable line D, showing yellow zones.

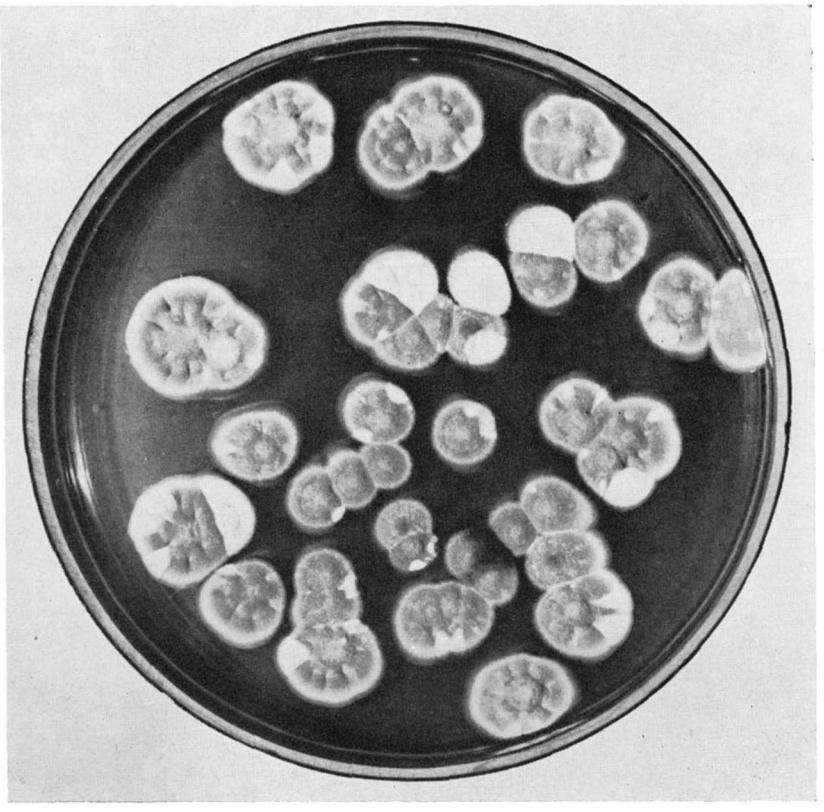

FIGURE 2.—Colonies of the unstable line N, showing white sectors.

TABLE 2

Clonal populations with a high proportion of mosaic colonies, derived from conidia of unstable lines (diploid XXXIV: y py / w cy)

Unstable line (code)	Number of colonies obtained after plating conidia						Sector characterization			
	Colonies showing sectors			Colonies without sectors (no.)	Colonies unclassified (no.)	Totals (no.)	White		Yellow	
	white (no.)	yellow (no.)	white & yellow (no.)				Tested (no.)	Phenotype*	Tested (no.)	Phenotype*
A	0	59	0	5	6	70	..	....	36	*y*
B	15	192	220	42	32	501	28	*w cy*	52	*y (py)*
C	0	120	0	5	3	128	..	....	59	*y*
D	0	69	0	5	4	78	..	....	27	*y*
F	0	41	0	5	0	46	..	....	51	*y*
G	3	50	23	8	1	85	26	*w cy*	68	*y (py)*
H	0	32	0	38	0	70	..	....	22	*y cy*
L	11	0	0	0	14	25	11	*w*	..	..
M	0	13	0	26	2	41	..	....	27	*y*
N	123	0	0	0	161	284	100	*w cy*	..	..

* The allele symbols indicate the phenotype.

each clone. In some populations the segregant zone of the colony was so large that there was only a small central green zone, surrounded by the yellow or yellow-and-white remainder of the colony (Figure 1). The opposite extreme was reached in one population (H), in which the segregant zones appeared as insignificant spots, and in another (E), in which only small sectors appeared, very late and at the extreme edge of the colonies. Clone M, which appeared spontaneously, produced late grey-green sectors, with a few small yellow spots which turned out to be prototrophic (Figure 4).

Three green colonies derived from HN-2-treated conidia were subjected to analysis, and in each case F_1 showed normal color segregation rates.

Ten colonies with sectors derived from untreated conidia of diploid XXXIV were similarly analyzed. Only one of these (M) showed a very high proportion of yellow sector-producing colonies in F_1 (13/41). The sectors were of small size and appeared within a wide grey-green zone protruding from the edge of the colony (Figure 4).

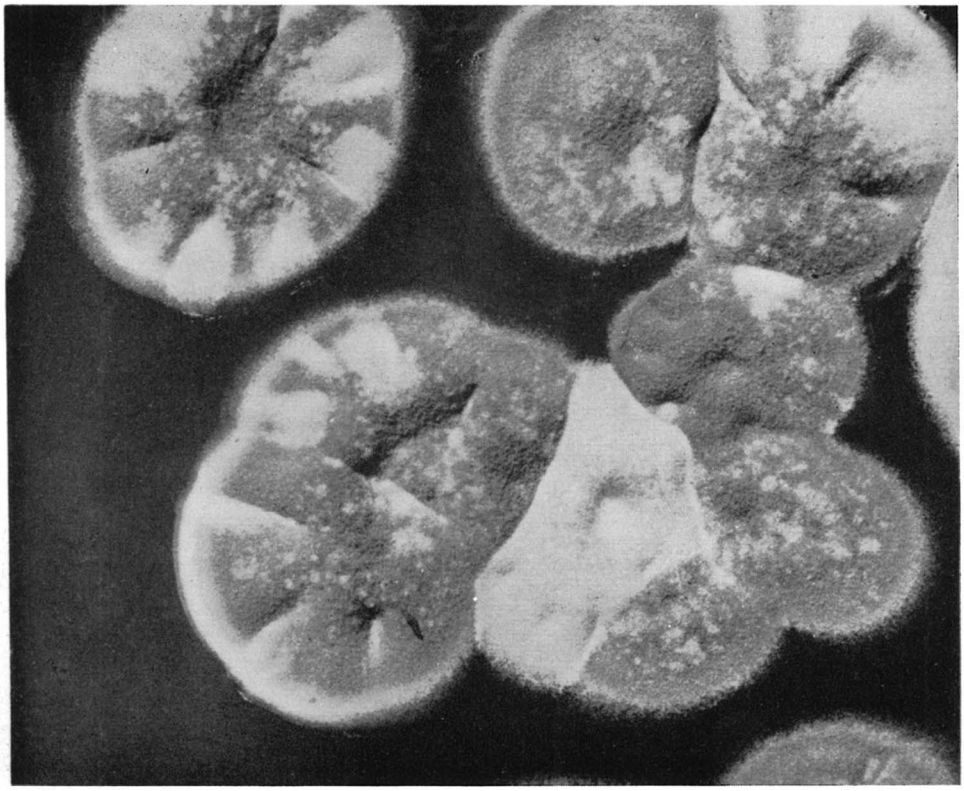

FIGURE 3.—Colonies of the unstable line F, showing yellow sectors and spots.

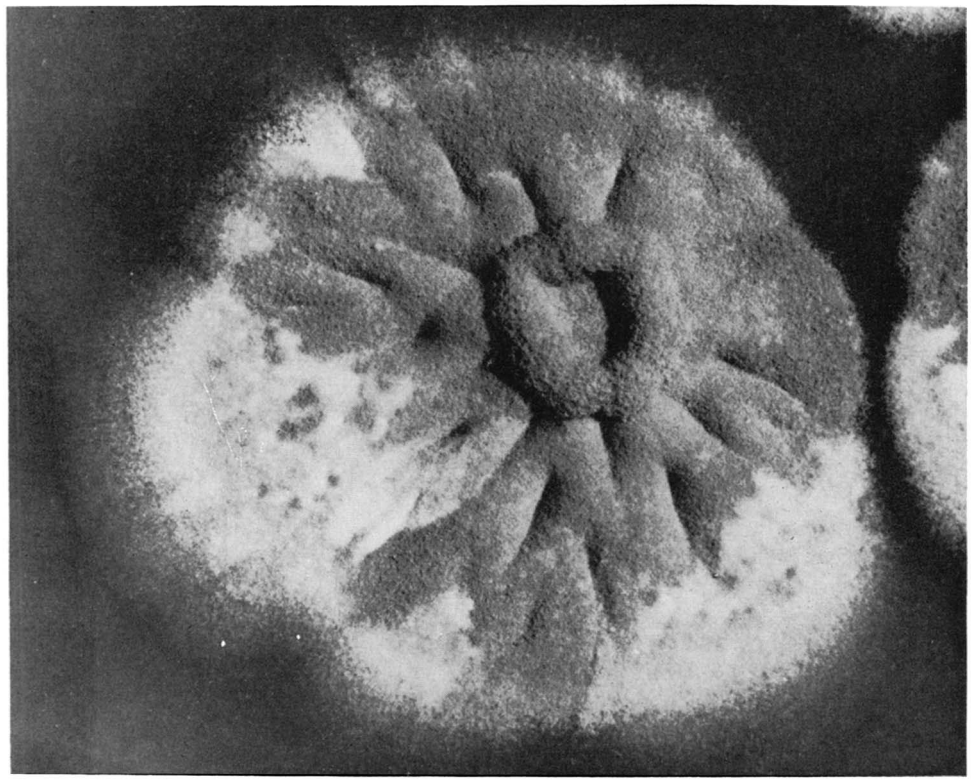

FIGURE 4.—Colonies of the spontaneous unstable line M, showing grey sectors with yellow spots.

Analysis of further generations

A number of F_1 colonies were used for an analysis of the next generation. Plating methods and determinations of the proportion of mosaic colonies in the populations studied were the same as those described in earlier sections.

Eleven sectoring F_1 colonies derived from mosaic clones were studied, and all eleven gave an F_2 with a high proportion of mosaic colonies. All the clones concerned were derived from single HN-2-treated conidia. The mosaic pattern of the F_2 colonies in the various clones was the same in each case as that displayed by the F_1 colonies.

Four different mosaic colonies of the F_1 of clone B (yellow and white sectors) were examined, one with yellow and white sectors only, and two with yellow sectors only. All four produced an F_2 with a high proportion of yellow and white sectors. Moreover, one colony without sectors of the F_1 of clone B (see next section) produced an F_2 with a high proportion of colonies with white and yellow sectors and sectors in both colors. The F_1 colony which had a white sector only gave a higher proportion of white sectoring colonies in the F_2 (0.37) as compared with the two F_1 colonies with yellow sectors (0.16 and 0.18).

Three sectoring colonies of the F_2 of mosaic clones were used to study the F_3; in each case the F_3 populations reproduced the high segregation rate and the mosaic pattern of the clone to which they belonged.

The rates of mosaic colonies in successive generations of several mosaic clones have been recorded. It has been noticed that there is an appreciable increase in segregation rate as the generations proceed. Clone D, for instance, gave 30 percent mosaic colonies in F_1, 45 percent in F_2, 100 percent in F_3 (Figure 1). It should be mentioned that the colonies used for the production of conidia of the next generation were not picked out at random, but chosen from those showing the mosaic configuration.

From the results obtained up to the present it appears that the tendency to produce progeny with a high proportion of mosaic colonies behaves as a hereditary character in the clones analyzed. This character manifests itself in single colonies by the appearance of segregant zones or sectors. The character in question will be referred to by the symbol HSF (high segregation frequency).

Reversion of character HSF in some colonies.

In almost all the HSF populations a certain number of colonies appeared which were completely green, i.e. without the mosaic appearance characteristic of the clone. Three of these colonies, belonging to the F_1 of clone B, were used for an analysis of their respective offspring. One of these (see above section) gave rise to an HSF population, although with a relatively low proportion of mosaic colonies (85/195), and two gave rise to populations with normal segregation rates (0/144 and 1/183). A colony without sectors obtained from line F also showed an almost normal proportion of mosaic colonies in the next generation (5/176).

Although the colonies examined represent only a very small sample, it is clear that the presence of green colonies is due partly to incomplete penetrance of the character HSF and partly to reversion of the character to the normal state. The loss of capacity to produce mosaic offspring does not depend on the occurrence of homozygosis at the locus of the segregant markers, since they continue to appear in the offspring, although very sparsely (Table 3, Clone F).

By repeated transfer under uncontrolled conditions, an unstable strain may lose its characteristic instability, probably because of selection of reverted subclones. This happened with line L; practically no colonies with white sectors were longer detectable after a few transfers. To restore the line an occasional white sectoring colony was selected, which initiated the unstable line N (Figure 2). The white sectors produced by the colonies of the latter line, instead of being prototrophic as the white sectors from the clone L, were regularly cysteineless (Table 2).

Characterization of segregants

From mosaic colonies of some of the HSF clones a few color segregant zones were collected and tested for nutritional requirements. The zones were touched

TABLE 3

Exceptional segregants from conidia of some unstable lines, appearing after mutagenic treatments (diploid XXXIV: y py / w cy)

Unstable line		Exceptional segregants after mutagenic treatment						
			Selected for color			Selected for requirements		
Code	Spontaneous sectors (phenotype)*	Mutagenic agent	Colonies observed (no.)	Segregants (No.)	Segregants (Phenotype)*	Colonies tested (no.)	Segregants (No.)	Segregants (Phenotype)*
A	*y*	u.v.	350	..	..	208	1	*py*
C	*y*	HN-2	3360	..	..	...	..	..
D	*y*	HN-2	859	6	*w*	...	..	
F	*y*	HN-2	740	2	*w*	...	..	..
		u.v.	1104	1	*w*	...	..	..
F†	..	....	176	5	*y*	...	..	..
		u.v.	1360	39	*y*	...	..	..
				1	*w py*	...	..	..
				10	*w*	...	..	..
				1	*w cy*	...	..	..
H	*y cy*	u.v.	602	..	..	120	2	*y*
							1	*y cy py*
L	*w*	HN-2	576	..	..	45	8	*w cy*

* The allele symbols indicate the phenotype.
† From a reverted non-sectoring colony.

with the point of a needle which was then streaked on to complete agar; streaks which gave rise to growth of homogeneous colour were later tested for nutritional requirements, while those which turned out not to be pure were further purified and then tested. The test consisted of transplating conidia of the purified segregant clones on to MM, MM supplemented with pyridoxine (0.07 mg/ml), MM supplemented with methionine (0.05 mg/ml), and in some cases MM supplemented with both compounds.

The right-hand side of Table 2 shows the phenotype of a number of segregants belonging to various clones. It was observed that each mosaic clone usually gives rise to only one colored phenotype (in the case of lines B and G two colored segregant types were observed). Within each clone the colonies of the same color all have the same nutritional requirement. Thus each mosaic clone is uniform within itself in respect not only to the color of the segregant sectors and to their size and shape but also to their nutritional requirement.

Clear distinctions can be drawn between at least six different mosaic patterns. Two clones (B and G) produce large white sectors with cysteine requirement, and large yellow sectors with a pyridoxine requirement; the yellow segregants show slight growth even in the absence of pyridoxine, unlike the component strain *y py* and some other pyridoxine-deficient segregants which show an absolute pyridoxine requirement. One clone (C) produces wide yellow prototrophic and grey-green prototrophic sectors; two clones (D and F) produce wide yellow prototrophic sectors (Figures 1 and 3); one clone (H) produces small yellow

spots with a cysteine/methionine requirement, i.e., a recombinant phenotype; one clone (L) produces wide white prototrophic sectors; one clone (N) produces sharp white cysteineless sectors (Figure 2).

In the majority of the mosaic lines the rate of growth of the isolated sectors was greater than that of the green zone of the colonies. The opposite holds for line H, in which the rate of growth of the isolated sectors was much lower than that of the green zone of the colonies.

No reliable data could be obtained about the conidium size of the mosaic colonies and their segregant sectors, on account of the great variability in shape and size of the conidia of different strains. This was particularly noticeable in the conidia of some segregant sectors. Anyway the conidium size from the nonsegregating part of the unstable colonies is as a rule diploid, while that of the segregant sectors is considerably more irregular but in some cases decisively diploid.

Exceptional segregants from mosaic clones

In order to determine whether the mosaic clones were still heterozygous for the genes for which they did not show spontaneous segregation, green conidia of a number of colonies of various mosaic lines were treated in such a way as to stimulate a somatic segregation.

The details of these experiments are shown in Table 3. The general result was the detection in many HSF clones of markers which showed no appreciable spontaneous segregation. Particularly noticeable were: the occurrence of several white segregants in clone D, which spontaneously gave only yellow segregants; the occurrence of two yellow prototrophic segregants and one yellow cysteine-pyridoxine-deficient segregant in clone H, which spontaneously gave only yellow cysteineless segregants; and the occurrence of white cysteineless segregants in clone L, alongside the spontaneously occurring white prototrophic segregants.

The induced segregation in clone F calls for special mention. The spontaneous segregants in this clone were yellow; some white segregants occurred after HN-2 treatment or ultraviolet irradiation of the conidia. A nonmosaic colony of clone F was also examined, in the filial generation of which occasional yellow sectoring colonies appeared spontaneously (5/176). After conidia of this nonmosaic colony had been subjected to ultraviolet irradiation, a few white segregants appeared alongside the yellow segregants, in a proportion (12/1360) noticeably higher than that (1/1104) in which such segregants appeared after treatment of conidia of a mosaic colony of the same clone F.

DISCUSSION

Methyl-bis(β-chloroethyl)amine (HN-2) turns out to be an effective agent for stimulating somatic segregation in diploids of *Penicillium chrysogenum*, as was already observed by MORPURGO and SERMONTI (1958). The chemical treatment increased the proportion of segregant colonies or colonies with segregant sectors from 0.023 to 0.340 in the experiments recorded here. Practically all the detectable segregants are the result of treatment with the mutagenic agent. This fact

is of particular interest because each segregant obtained after stimulation is the result of an independent chromosomal rearrangement, whereas among the colonies obtained from untreated conidia two segregants of the same phenotype may belong a single segregating clone, originating before conidium formation. This circumstance facilitates genetic analysis of diploids of *Penicillium chrysogenum*, which is, on the other side, simplified by the very high rate of appearance of segregants. Difficulties are of course created by the impossibility at present of stating which particular chromosomal rearrangements are produced by mutagenic treatment of conidia. There is no need to dwell on the practical importance of mutagenic treatment for the purposes of industrial ultilization of the parasexual process. The genetic variability of a population of colonies derived from treated heterozygous diploid conidia is enormously increased, and the breeder can have at his disposal a much greater variety of phenotypes.

A large part of the induced segregants take the form of apparently homogeneous colonies. The remainder appear as yellow or white sectors from green colonies. In about 40 percent of cases, the green part of the colonies gives rise to unstable lines (HSF=high segregation frequency), which are discussed below. In the rest of sector-producing colonies the green part breeds true (not-HSF). Since the number of not-HSF sectoring colonies greatly increased after HN-2 action, in these cases we must assume a delayed effect of the mutagenic agent.

The increased proportion of sectors in colonies of the unstable lines may be explained in two different, but not mutually exclusive, ways. On the one hand, it may be due to a selective advantage enjoyed by the segregant clone as compared with the HSF clone from which it emerges as a sector. In this case there is no need to assume, in the mosaic lines, any increase in the rate of occurrence of the processes which lead to somatic segregation : the increased proportion of sectors could be explained by a normal rate of occurrence of segregation processes with a greater likelihood of sector emergence in the segregant clones. The other possibility is an actual increase in the rate of occurrence of the segregation processes in the mosaic lines.

A model representing the first condition is the occurrence of a dominant semilethal mutation on the chromosome homologous to the one carrying the markers with a high rate of segregation. Segregation of the wild type allele of the semilethal gene would involve segregation of markers linked to it, and possibly of other markers as well, and would give a selective advantage to the segregant clone.

An analogous situation occurs in heterozygous acr_1/ACR_1 diploids of *Aspergillus nidulans* (Roper and Kafer 1957) in the presence of acriflavine. The semidominant allele acr_1 (sensitivity to acriflavine) is harmful to the heterozygous diploid. It is eliminated by haploidization (giving ACR_1) or by somatic crossing-over between this allele and the centromere (giving ACR_1/ACR_1). The derived clones emerge in the form of vigorous sectors from heterozygous colonies of stunted growth. Haploidization leads to segregation of the markers linked to ACR_1, and sometimes to segregation of markers lying on other chromosomes.

Somatic crossing over leads to segregation of the markers distal to ACR_1 and sometimes to segregation of some of the markers lying between ACR_1 and the centromere.

The details of the linkage relations of the markers in diploid XXXIV are still far from completely known, but on the basis of criteria formulated in an earlier paper by one of the present authors (SERMONTI 1957), *py* appears to lie on a different chromosome from that (or those) carrying *y*, *w* and *cy*; *w* and *cy* seem to be on the same chromosome arm with *w* distal to *cy*. Whatever the linkage situation, the elimination of a harmful gene by somatic crossing over or haploidization should give rise to a variety of *noncomplementary* phenotypes, some markers being automatically selected while others are free. No such state of affairs was observed in any of the lines analyzed (see Table 2), and this cannot be attributed to the loss in the HSF clones, of the nonsegregant markers, since in many cases (see Table 3) these turned out to be present in the heterozygous state. Moreover, although better growth of the isolated sectors, as compared with the colonies of origin, was actually observed in many lines, the growth of the isolated sectors in line H was less vigorous than that of the colonies of origin.

A second model may be adduced, again from *Aspergillus nidulans*, to exemplify the first condition. Loss of a complete chromosome behaves as a semidominant disadvantageous mutation; the aneuploid strains grow weakly due to a kind of unbalance (PONTECORVO and KAFER 1957; KAFER 1957). They throw out vigorous sectors which rapidly overwhelm the poorly growing seed colony. Repeated isolation of spores of the strain of the colony usually produces the same configuration. The conidial size from the vigorously growing sectors is haploid, conidia from the center are variable, and range from almost diploid to haploid. It should be observed that, theoretically speaking, aneuploidy could produce, alongside of a selective disadvantage, an actual increase in the rate of occurrence of the process of haploidization, due to some inherent irregularity in the mitotic process.

This hypothesis is open to the same objections as the semilethal-mutation hypotheis: a variety of segregant phenotypes should be expected within each mosaic clone. Besides, the situation with respect to conidium size of the unstable clones is somewhat different from that which appears with aneuploid clones in *Aspergillus nidulans*.

The second explanation of the increased proportion of sectors in the unstable lines is an actual increase in the rate of occurrence of segregation processes. This hypothesis rests on the exclusion of explanations based on selective effects. Although not yet critically proved, there seems at present to be more support for the assumption of an increase in the rate of segregation as an explanation of the phenomena observed.

The occurrence of genetic instability in the material described in this paper is certainly due to a mutational event. The character "Instability" (HSF) is strictly hereditary, its appearance is sudden, and its rate is greatly increased by treatment with mutagenic agents. A small proportion (1:10) of the colonies which produced sectors spontaneously in diploid XXXIV originated unstable lines and

since colonies which produce sectors for color appear with a frequency of approximately 0.01 (7:723), the absolute frequency of colonies producing unstable clones is of the order of 10^{-3}. After HN-2 treatment, mosaic colonies represented about ten percent (23:208) of the surviving colonies, and about 40 percent (11:28) of these were unstable. The absolute proportion was therefore of the order of 4.10^{-2}, an increase of about 40 times over the spontaneous rate.

The results reported here give a strong indication that the induced instability affected different chromosomal regions in the different mosaic clones. Indeed, there is a remarkable specificity: each unstable line is unstable for particular markers only, and at least six different instability patterns have been identified in the nine lines examined. There is high specificity also in the time of appearance and the shape of the sectors in different lines. One could suppose that the various clones are differentiated by the disappearance before sector production of certain markers in some of them, and that the instability itself may be generic, appearing specific only because each clone is only able to segregate for markers which have remained in the heterozygous state. This has turned out not to be the case, since the heterozygosis has been demonstrated in the clones analyzed of some markers which nevertheless did not segregate with high frequency.

The exact nature of the processes of chromosomal rearrangement which give rise to mosaic clones cannot be determined with any certainty in the present state of knowledge of the genetic make-up of the diploid concerned. The existence of clones producing sectors of two complementary types suggests that, in such cases, either the process of somatic crossing over in one or more particular regions or the segregation of whole chromosomes has been stimulated.

It is unfortunate that irregular conidial size of the segregant sectors does not allow of their ploidy classification.

A large number of cases of genetic instability have been observed in maize (McCLINTOCK 1951). In some of these a locus, *Ds*, has been identified which displays a marked fragility. When a rupture occurs at this locus the acentric fragment produced is eliminated during mitotic division. All the dominant alleles in the fragment are removed and any recessive alleles present in the corresponding segment are thus enabled to express themselves in the phenotype. Grains during the development of which such phenomena have occurred are variegated.

This model is more closely applicable than any other as an explanation of the instability in *Penicillium chrysogenum*, in cases in which the segregation concerns a single phenotype.

Cases of radiation-induced genetic instability were observed by NEWCOMBE (1953) in Streptomyces sp. The phenomenon has many points of similarity with the present observations. NEWCOMBE (1953) thinks that the mechanism responsible for the development of unstable lines in maize (McCLINTOCK 1951) might be applicable also to Streptomyces. Unlike the material studied here, the strain from which NEWCOMBE (1953) obtained unstable lines was assumed to be haploid.

Rupture points, with loss of chromosomal fragments, have been suggested in

Escherichia coli K12 diploid (NELSON and LEDERBERG 1954) and haploid (CAVALLI and JINKS 1956).

In 1925 BRIDGES observed, in females of *Drosophila melanogaster* containing the dominant factor Minute-n on one of the X-chromosomes and certain recessive genes on the other X-chromosome, a frequently occurring mosaic condition, i.e. the appearance of small areas in different parts of the body which were not phenotypically Minute-n and expressed the effects of the recessive genes. BRIDGES suggested that the factor Minute-n had the property of occasionally eliminating the X chromosome on which it lay.

STERN (1936) showed that the chromosome elimination theory was not satisfactory as an explanation of the mosaic formation and that the mechanism of the phenomenon in question rests *primarily* on the occurrence of somatic crossing over. The increase in the rate of somatic crossing over must depend on a general "phenotypic Minute reaction" on development.

Hereditary tendencies to delection, loss of complete chromosomes, and somatic crossing over may all play a part in the phenomenon of genetic instability in *Penicillium chrysogenum*, while selective effects probably take part only in so far as they determine the morphological pattern of the mosaic colonies.

SUMMARY

The action of methyl-bis(β-chloroethyl)amine (HN-2) on the somatic segregation of a diploid strain of *Penicillium chrysogenum* heterozygous for color and deficiency markers is studied. The following points are established:

1) HN-2 greatly increase the proportion of segregants observable as whole colonies or as yellow or white sectors from green colonies.

2) About a third of the colonies with sectors ("mosaic colonies") derived from treated conidia gave unstable clones after repeated transfer of the green zone. This instability appears as a capactiy of the conidia to produce a very high proportion of mosaic colonies. The instability persists for an indefinite number of generations.

3) One unstable clone was derived from a mosaic colony which had formed spontaneously.

4) Absolute specificity of segregation pattern was shown by the unstable clones: each clone produces only one or two types of segregant sector. However, tests carried out on clones showed that markers not concerned in the high frequency were present in the heterozygous state.

5) Reverted stable subclones, in which the markers showing a high segregation rate were still present in heterozygous state, were readily obtained from the unstable clones.

6) Possible causes of the instability are discussed. An actual increase in the rate of occurrence of somatic segregations seems to be responsible.

ACKNOWLEDGMENTS

The Authors wish to express their thanks to MR. G. DI GIUSEPPE for his expert technical assistance, to MR. F. G. MILES for the English translation of the Italian text and to MR. A. PICCIRILLI for the photographs. We are indebted to SIMES S.p.A., Milan, Italy, for gifts of Cloramin (HN-2).

LITERATURE CITED

AUERBACH, C., 1951 Problems in chemical mutagenesis. Cold Spring Harbor Symposia Quant. Biol. **16**: 199–213.

BRIDGES, C. B., 1925 Elimination of chromosomes due to a mutant (Minute-*n*) in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. **11**: 701–706.

CAVALLI, L. L., and J. L. JINKS, 1956 Studies on the genetic and mating system of *Escherichia coli* K 12. J. Genet. **54**: 87–112.

IKEDA, Y., C. ISHITANI, and K. NAKAMURA, 1957 A high frequency of heterozygous diploids and somatic recombination induced in imperfecti fungi by ultra-violet light. J. Gen. (appl.) Microbiol. **3**: 1–11.

ISHITANI, C., Y. IKEDA, and K. SAKAGUCHI, 1956 Hereditary variation in koji-molds (*Aspergillus oryzae* and *Aspergillus sojae*). VI. Genetic recombination in heterozygous diploids. J. Gen. (appl.) Microbiol. **2**: 401–430.

KÄFER, E., 1958 An 8 chromosome map of *Aspergillus nidulans*. Advances in Genet. **9**: 105–145.

MCCLINTOCK, B., 1951 Chromosome organisation and genetic expression. Cold Spring Harbor Symposia Quant. Biol. **16**: 13–47.

MORPURGO, G., and G. SERMONTI, 1958 Alta frequenza di segregazione somatica indotta da agenti mutageni in *Penicillium chrysogenum*. Ricerca Sci. (In press).

NELSON, T. C., and J. LEDERBERG, 1954 Postzygotic elimination of genetic factors in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. **40**: 415–419.

NEWCOMBE, H. B., 1953 Radiation-induced instabilities in Streptomyces. J. Gen. Microbiol. **9**: 30–36.

PONTECORVO, G., 1953 The genetics of *Aspergillus nidulans*. Advances in Genet. **5**: 141–238.

PONTECORVO, G., and E. KÄFER, 1956 Mapping the chromosomes by means of mitotic recombination. Proc. Roy. Soc. Edinburgh **25**: 16–20.

1958 Genetic analysis based on mitotic recombination. Advances in Genet. **9**: 71–104.

PONTECORVO, G., and G. SERMONTI, 1954 Parasexual recombination in *Penicillium chrysogenum*. J. Gen. Microbiol. **11**: 94–104.

PONTECORVO, G., E. TARR GLOOR, and E. FORBES, 1954 Analysis of mitotic recombination in *Aspergillus nidulans*. J. Genet. **52**: 226–237.

ROPER, J. A., 1952 Production of heterozygous diploids in filamentous fungi. Experientia **8**: 14.

ROPER, J. A., and E. KÄFER, 1957 Acriflavine-resistant mutants of *Aspergillus nidulans*. J. Gen. Microbiol. **16**: 660–667.

SERMONTI, G., 1956 Complementary genes which affect penicillin yields. J. Gen. Microbiol. **15**: 599–608.

1957 Analysis of vegetative segregation and recombination in *Penicillium chrysogenum*. Genetics **42**: 433–443.

STERN, C., 1936 Somatic crossing over and segregation in *Drosophila melanogaster*. Genetics **4**: 247–265.