ACTION OF MANGANOUS CHLORIDE ON INDUCED SOMATIC SEGREGATION IN PENICILLIUM CHRYSOGENUM DIPLOIDS

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NITROGEN mustard (HN–2), at doses which bring about almost total inactivation of the treated conidia, has a powerful stimulating effect on somatic segregation in some heterozygous *Penicillium chrysogenum* diploids (MORPURGO and SERMONTI 1959). On plating the surviving conidia, color marker segregants appear as yellow or white entire colonies, or yellow or white sectors emerging from the green phenotype of the heterozygous diploid colonies. The color of the colonies and sectors is due to the color of the conidia which they produce. Some colonies with colored sectors obtained after HN–2 treatment give rise to clones whose conidia produce colonies almost all of which show sectors segregant for color. Each one of these "unstable" clones displays its own individual segregation pattern (MORPURGO and SERMONTI 1959). Similar unstable clones have been obtained from X-ray irradiated conidia.

The inactivation produced by HN–2 is strongly reversed by the action of manganous chloride ($MnCl_2$) added to the plating medium; however, this salt has no reactivating effect on conidia inactivated by X-ray irradiation (SERMONTI and MORPURGO 1958). The object of the present research was to establish the effect of $MnCl_2$ on the somatic segregation processes induced directly or indirectly by HN–2. Some further experiments concern the same problem in connection with X-ray-induced segregation.

MATERIALS AND METHODS

Strains: The strains used have already been described in MORPURGO and SERMONTI (1959), of which the present paper is a continuation. They are all descended from heterozygous diploid XXXIV of *Penicillium chrysogenum*, of which the phenotype is green and the genotype may be symbolized as follows:

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

The strains used were:

Strain XXXIV S, pyridoxineless, diploid, of genotype $Y\ py\ w\ cy/y\ py\ W\ CY$;

Unstable strain B, whose conidia produce green colonies with extensive yellow pyridoxineless and/or white cysteineless sectors;

Unstable strain D, whose conidia produce green prototrophic colonies with extensive yellow prototrophic sectors;

Unstable strain F, with colonies of similar phenotype to strain D;

Unstable strain N, with green colonies producing white cysteineless sectors later.

All these strains were obtained from conidia which had survived HN–2 treatment. Unstable strain 3X was obtained from a colony of strain XXXIV S which had survived X-ray irradiation. It produces yellow pyridoxineless sectors and stable dark-green pyridoxineless sectors.

Media: The complete medium described in the earlier work was used in the present tests.

Use of nitrogen mustard: Methyl-bis(β-chloroethyl)amine (HN–2) was used in the manner described in the previous paper. Treatment was interrupted after one minute or after four minutes, giving 95 percent and 99.9 percent inactivation respectively of the treated conidia.

Use of X-rays: X-ray irradiation was carried out on conidia in aqueous suspension in glass test tubes. A 245KV X-ray tube was used at 15 mA, filtration 1 mm Al, exposure 50,000r.

Use of manganous chloride: $MnCl_2.4\ H_2O$ was added to melted complete agar at a concentration of 0.8 percent (w/v), unless otherwise stated. It corresponds to 40 mM.

RESULTS

Effect of $MnCl_2$ on induced somatic segregation

Immediate segregation: This takes the form of the appearance of some completely yellow or white colonies directly from conidia which have survived HN–2 treatment or X-ray irradiation. The white colonies have been disregarded in this part of the work because their phenotype is easily confused with that of the conidialess colonies which sometimes appear after treatment with mutagenic agents.

After HN–2 treatment the conidia of heterozygous diploid XXXIV S were plated on Petri dishes containing complete medium with and without the addition of $MnCl_2$. Since conidia inactivated by HN–2 are strongly reactivated (SERMONTI and MORPURGO 1958) in the presence of $MnCl_2$, the conidium suspensions were suitably diluted before plating on the agar containing the salt.

Untreated conidia gave rise exclusively to green colonies, with no yellow or otherwise colored colonies either in the presence or in the absence of $MnCl_2$. Conidia treated with HN–2 gave rise to a high proportion of yellow colonies on unsupplemented medium and a proportion 3–5 times lower of yellow colonies on medium supplemented with $MnCl_2$ (Table 1).

Similar experiments were performed with conidia which had survived X-ray irradiation (50,000r). They were plated at the same density in the presence and in the absence of $MnCl_2$, since the salt has no reactivating effect on irradiated conidia (SERMONTI and MORPURGO 1958). The proportion of yellow colonies is

TABLE 1

Effect of $MnCl_2$ on induced segregation of the marker "yellow" from diploid XXXIV S

Mutagenic agent	Exposure	Supplement to the plating medium	Surviving conidia (Percent)	Colonies observed (No.)	Yellow colonies (No.)	Yellow colonies as percentage of total observed (Percent)
....	....	.....	100	107	0	0
....	....	$MnCl_2$ (40 mM)	106	114	0	0
HN–2	1 min	.....	4.7	813	51	6.31±0.85
HN–2	1 min	$MnCl_2$ (40 mM)	36.7	1,098	20	1.82±0.40
HN–2	4 min	.....	0.09	103	12	11.66±3.16
HN–2	4 min	$MnCl_2$ (40 mM)	14.0	601	14	2.33±0.62
X-rays	50,000r	.....	0.42	269	23	8.55±1.70
X-rays	50,000r	$MnCl_2$ (40 mM)	0.22	143	3	2.09 – 1.43

strongly reduced in the presence of $MnCl_2$ (Table 1, last two lines). $MnCl_2$ therefore acts as a corrective of chromosomal aberrations induced by X-rays although this effect is not accompanied by any restoration of vitality such as was observed in HN–2 treated conidia.

Reconstruction experiments have been carried out to check whether $MnCl_2$ had any selective effect against yellow segregants induced by HN–2. Conidia were harvested from a number of yellow segregant colonies and from an equal number of green nonsegregant colonies surviving HN–2 treatment. The mixed suspension was plated on agar supplemented or unsupplemented by $MnCl_2$. As shown in Table 2 a feeble selective effect against the yellow segregants was

TABLE 2

Reduction by $MnCl_2$ of the rate of yellow induced segregants from diploid XXXIV S, as compared with the effect of $MnCl_2$ in reconstruction experiments

Media	Exp.	Colonies from plating of green conidia treated with HN–2			Colonies from plating of yellow and green conidia*			χ^2‡
		Green (No.)	Yellow (No.)	Ratio Yellow/Green (Corrected)†	Green (No.)	Yellow (No.)	Ratio Yellow/Green (Corrected)†	
Complete (C)	1	595	43	(1)	342	260	(1)	....
Complete (C)	2	272	28	(1)	111	104	(1)	....
C+$MnCl_2$ (20 mM)	2	538	29	0.497	138	112	0.867	13.16
C+$MnCl_2$ (30 mM)	2	612	18	0.286	105	67	0.734	18.77
C+$MnCl_2$ (40 mM)	1	1,798	34	0.261	324	169	0.687	42.71

* Harvested at random from about 20 yellow and 20 green colonies grown on complete medium from HN–2 treated conidia.
† Ratios expressed relative to those on C medium, in corresponding experiment, taken as unity.
‡ Comparison of the corrected ratios: yellow/green, on the same medium. (d.f. = 1).

actually observed. This has been confirmed by the observation that induced yellow segregants grown on $MnCl_2$-agar display a significant selective advantage over the green nonsegregant colonies when their conidia are tested in reconstruction experiments as above. The selective effect explains only partially the reduction of the segregation by $MnCl_2$. A significant specific effect on the segregation rate must be invoked to explain the residual diminution.

Action of other bivalent-cation salts on the induced somatic segregation: $MgCl_2$ and $CaCl_2$ have been tested, in the same condition as $MnCl_2$, at the following doses: 50 mM, 100 mM and 200 mM, and $ZnCl_2$, which is more toxic to Penicillium, at doses of 3 mM, 6 mM and 10 mM. The test strain was XXXIV S treated with HN–2, with a survival rate of 0.03 in the absence of the salts. No significant reduction in the frequency of the yellow segregants was observed with any of the tested salts. $MnCl_2$, at the doses of 30 mM and 50 mM, gave a threefold reduction of yellow segregants in the same experiment.

Effect of $MnCl_2$ on spontaneous segregation from unstable clones: Conidia of four different unstable clones set up after HN–2 treatment were plated on complete medium in the presence and in the absence of $MnCl_2$. The proportion of colonies with and without sectors which had grown on the dishes was observed after at least ten days' growth. The general picture is the same for all the strains (Table 3): in the absence of $MnCl_2$ almost all the colonies show the characteristic mosaic pattern (Figure 1) while in the presence of $MnCl_2$ almost all the colonies are green, with no trace of sector formation (Figure 2), even when the colonies have reached the size associated, in colonies grown in the absence of the salt, with the regular appearance of colored segregant sectors.

In some experiments the conidia plated consisted of a mixture of green conidia from an unstable strain and conidia obtained from color segregant sectors of the same strain. The relative sizes of colonies derived from green and segregant conidia were substantially the same in the absence (Figure 3) and in the presence (Figure 4) of $MnCl_2$ for each of the strains observed. Furthermore, the relative frequency of occurrence of the two types of colonies shows no great difference on the two media, although perhaps a greater relative rate of survival of segregant colonies may be observed on the medium with $MnCl_2$ than on the unsupplemented medium (Table 4). These observations make it very improbable that the effect

TABLE 3

Effect of $MnCl_2$ on spontaneous segregation of the markers "yellow" and "white" from unstable strains

Strain	Color of sectors	Supplement to the plating medium	Colonies observed: Mosaic colonies (No.)	Without sectors (No.)	Unclassified (No.)
B	Yellow and white	.....	288	24	22
		$MnCl_2$ (20 mM)	78	251	16
D	Yellow	.....	262	0	0
		$MnCl_2$ (40 mM)	0	97	0
F	Yellow	.....	125	7	1
		$MnCl_2$ (40 mM)	6	250	0
3X	Yellow and dark green	.....	71	0	59
		$MnCl_2$ (40 mM)	0	38	7

of $MnCl_2$ in suppressing the spontaneous segregation of unstable clones may be explained by the occurrence of a selective effect against the segregants.

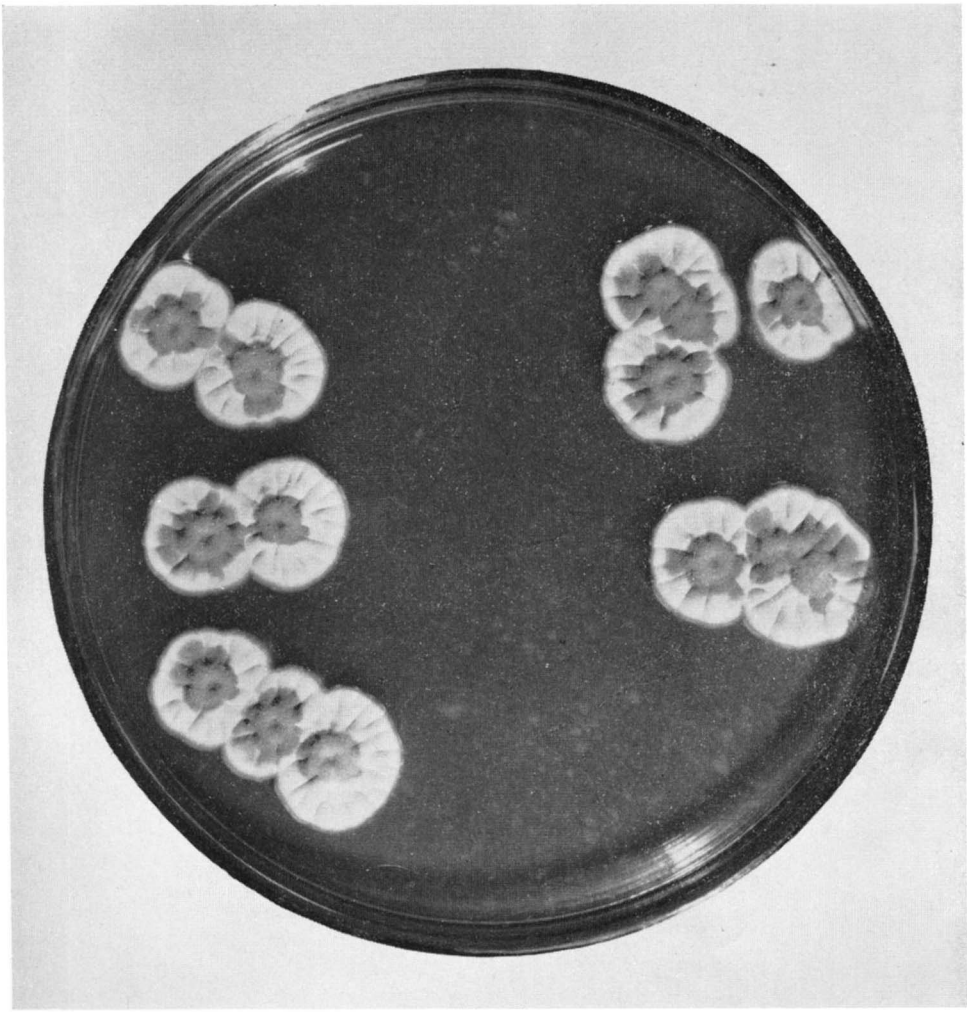

FIGURE 1.—Colonies of unstable strain D grown on complete medium showing wide yellow sectors.

The effect of $MnCl_2$ in inhibiting spontaneous segregation was also observed with one clone whose instability had been set up by X-ray irradiation, clone 3X (Table 3, last two lines).

Action of other bivalent-cation salts on the spontaneous segregation of unstable clones: A series of experiments was carried out in which different bivalent-cation salts were added to the complete medium in the hope of observing some effect similar to that of $MnCl_2$. The test strains used were strain D and in some cases strain B. All the salts were used at doses sufficient to limit growth in the colonies tested, except in the case of calcium and magnesium salts, which were used in concentrations equimolecular with the 0.8 percent $MnCl_2$ solution (40mM). The following salts were tested: $(CH_3COO)_2Cu$, $ZnCl_2$, $FeSO_4$, $NiSO_4$, $CoSO_4$,

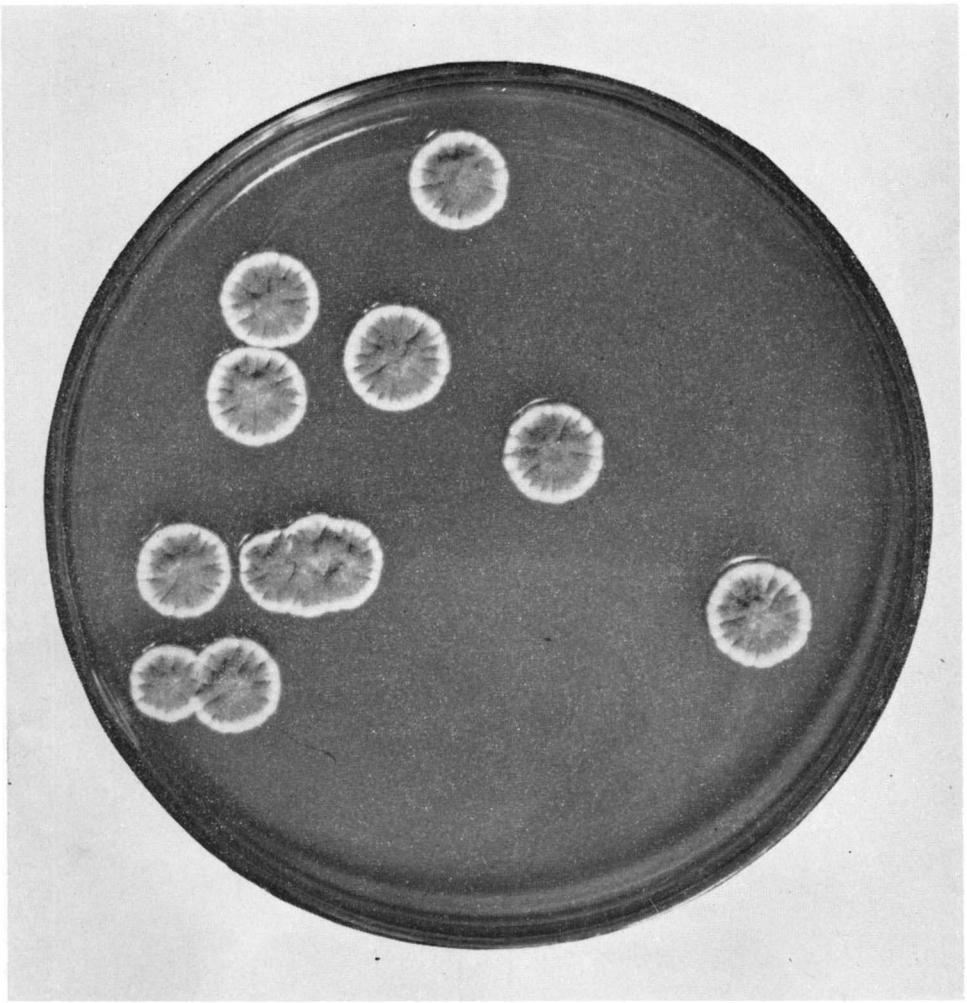

FIGURE 2.—Colonies as in Figure 1 on complete medium supplemented with $MnCl_2$ (40 mM). No sectors are produced.

$CaSO_4$, and $MgSO_4$. None of these produced any reduction in the rate of occurrence of mosaic colonies in the unstable strains, although at the doses used some of them had a marked effect on the size, morphology, and color of the treated colonies.

Progeny of unstable clones grown in the presence of $MnCl_2$: Conidia were collected from six colonies grown in the presence of $MnCl_2$ belonging to unstable strain D (yellow sectors) and seeded separately in the absence of the salt at a density of about 30 conidia per dish. The same operation was carried out on six colonies of the same strain grown in the absence of $MnCl_2$. In the latter case the conidia were taken from the green central zone, avoiding the sectors, while in the

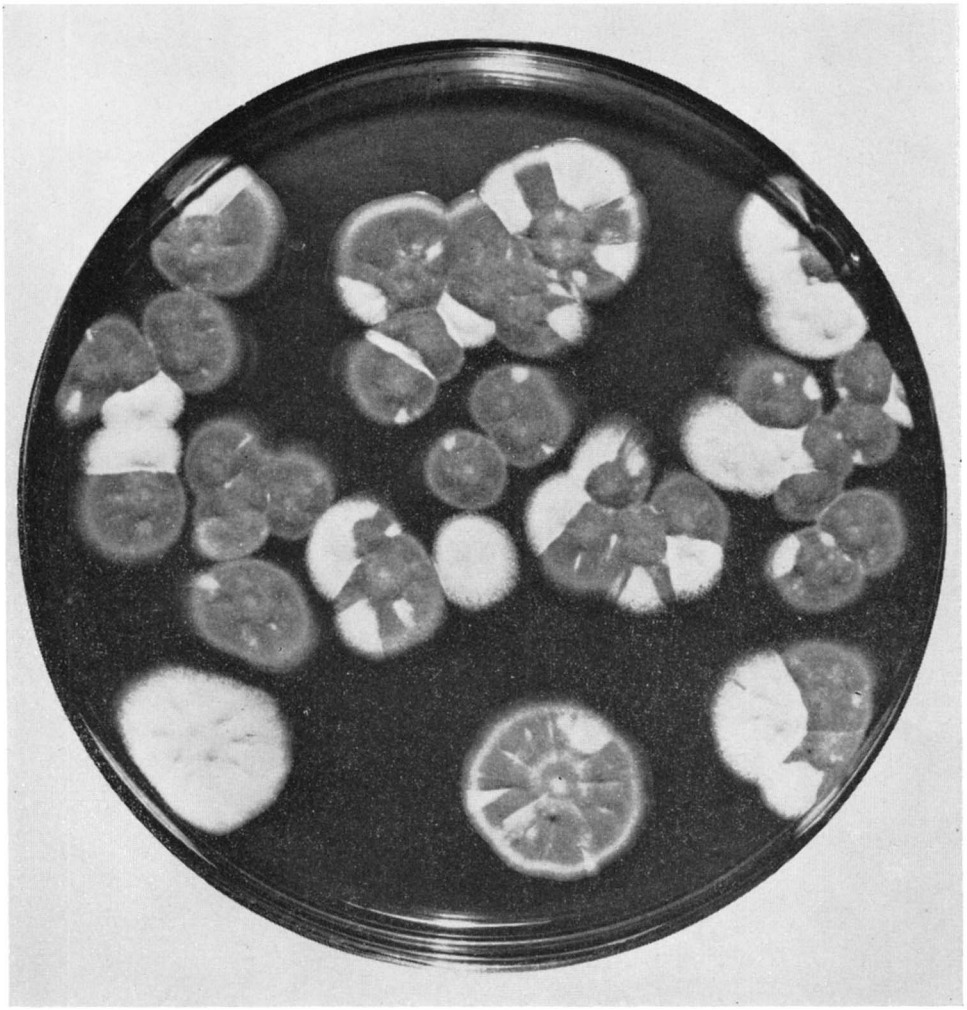

FIGURE 3.—Colonies of unstable strain N grown on complete medium, showing white sectors. The white colonies derive from plating of segregant conidia.

first case they were taken from over the whole spore-bearing surface, there being no sectors at all. All twelve populations of conidia obtained in this way produced 100 percent of characteristic mosaic colonies, independently of the origin of the conidia used for plating. It is worth mentioning that not a single completely yellow colony grew from hundreds of conidia taken from colonies grown in the presence of $MnCl_2$; this suggests that segregation is completely inhibited by $MnCl_2$. However, this suppression is reversible.

An exactly analogous result was obtained on plating conidia from two colonies of unstable strain N (white sectors) grown in the presence of $MnCl_2$. A parallel plating was carried out from the green part of two colonies grown on a medium

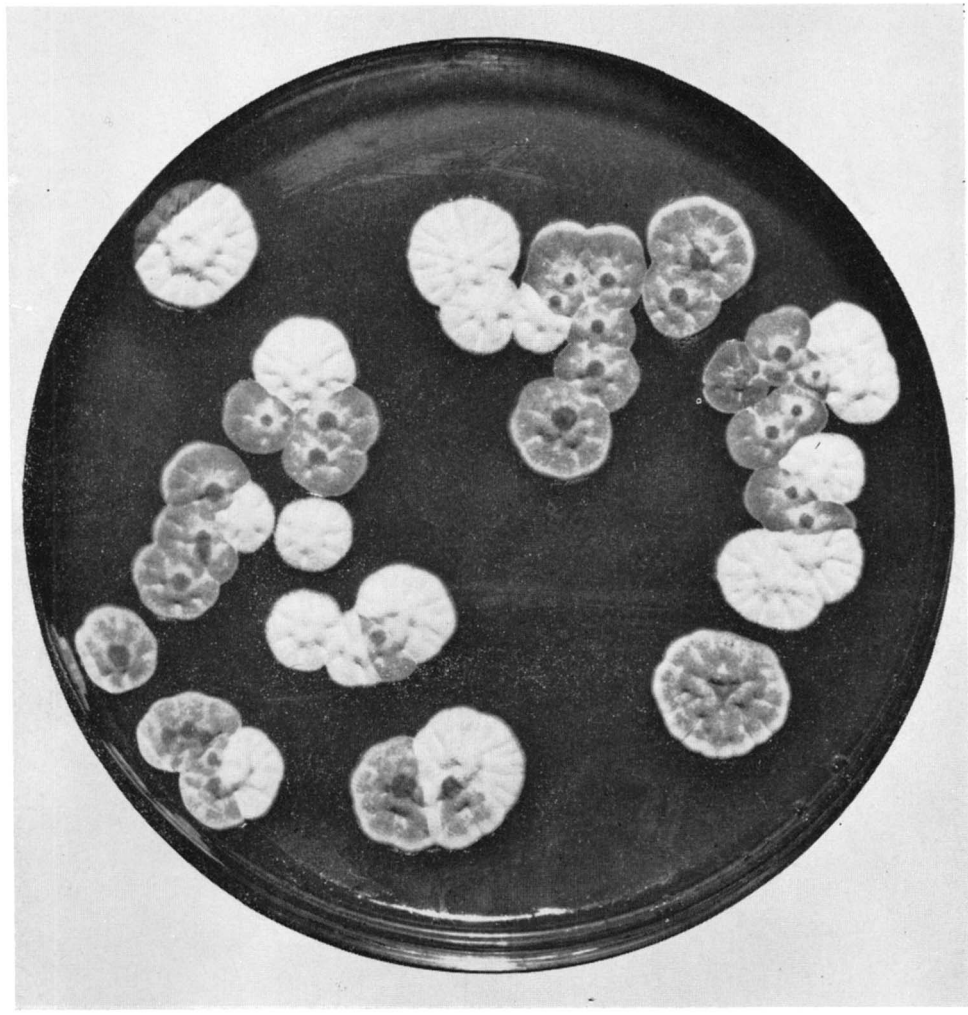

FIGURE 4.—Colonies as in Figure 3 on complete medium supplemented with $MnCl_2$ (40 mM). No sectors are produced. The relative size of the unstable and white colonies are the same as in Figure 3.

unsupplemented with $MnCl_2$. The appearance of the platings obtained from the two types of seeding was exactly analogous. With this strain again the conidia derived from colonies grown in the presence of $MnCl_2$ never gave rise to completely white colonies.

DISCUSSION

Alongside the reviving effect of $MnCl_2$ on conidia treated with nitrogen mustard (HN–2) (SERMONTI and MORPURGO 1958), a second marked effect of this salt has appeared in the course of the present work. $MnCl_2$ added to the plating

TABLE 4

Effect of $MnCl_2$ on survival in unstable clones and their spontaneous segregants, after mixed plating

Strain	Color of segregants	Supplement to the plating medium	Total numbers of colonies observed: Green (unstable) (No.)	Yellow (segregants) (No.)	Ratio Yellow/Green	χ^2 (d.f.=2)
		.....	37	117	3.16	
D	Yellow	$MnCl_2$(20 mM)	31	143	4.61	8.16
		$MnCl_2$(40 mM)	18	123	6.83	
		.....	127	39	0.30	
F	Yellow	$MnCl_2$(20 mM)	54	23	0.43	5.29
		$MnCl_2$(40 mM)	71	40	0.56	
		.....	284	68	0.24	
N	White	$MnCl_2$(20 mM)	104	39	0.37	7.96
		$MnCl_2$(40 mM)	256	99	0.39	

medium strongly reduces the rate of occurrence of the chromosome rearrangements induced both by HN–2 and by X-ray irradiation; this is deduced from the rate of occurrence of phenotypes showing recessive markers of the heterozygous diploid treated. The antisegregant effect of $MnCl_2$ appears in two different types of experiment. On the one hand $MnCl_2$ partially neutralizes the direct effects of HN–2 and X-ray irradiation; this appears when the treated conidia are plated in the presence of adequate concentrations of the salt. On the other hand, it almost completely (although reversibly) neutralizes the indirect effect of these mutagenic agents, *viz.* the chromosome rearrangements which appear as a consequence of a hereditary condition of nuclear instability set up by the mutagenic treatment and preserved indefinitely. This effect is only observable when conidia of unstable strains are plated in the presence of $MnCl_2$. It takes the form of an almost complete absence of mosaic colonies among the colonies grown from these conidia.

Nothing can be deduced at the moment from the present authors' work concerning the mechanism of action of $MnCl_2$, but an interpretation is strongly suggested by the experiments of ERNSTER *et al.* on the action of Mn^{++} on mitochondrial organization. According to these authors (ERNSTER 1956) the change of mitochondrial organization elicited by factors which induce a release of mitochondrial ATP may take the form of a disconnection of the coupling of phosphorylation to electron transfer. This effect can be reversed by supplementing the uncoupled system with suitable levels of ATP and Mn^{++} (LINDBERG and ERNSTER 1954).

Uncoupling of oxidative phosphorylation has been observed in animal tissues after X-ray irradiation (VAN BEKKUM 1956) and, granted the close analogy between the actions of HN–2 and X-rays, it may be postulated that it also takes place after HN–2 treatment. Oxidative phosphorylation is the principal source of ATP in aerobic organisms.

WOLFF and LUIPPOLD (1956) consider, on the basis of a number of observations, that "the chemical bonds formed in rejoining of radiation induced chromosome breaks require ATP as a source of energy for their synthesis". Dinitrophenol inhibition of ATP formation inhibits the rejoining of chromosome breaks (WOLFF and LUIPPOLD 1955) while the addition of exogenic ATP accelerates the rejoining process (WOLFF and LUIPPOLD 1956).

The simplest explanation of the phenomena described in this paper on the basis of the results quoted is as follows. In the case of the immediate effects of the mutagenic agents used and their cancellation by $MnCl_2$, it is reasonable to suppose that HN–2 and X-ray irradiation cause chromosome breaks with consequent loss of the acentric fragments. These phenomena would be observable in heterozygous diploid Penicillium as the appearance of segregants showing the recessive markers lying on the segment of chromosome corresponding to the lost segment.

The action of the mutagenic agents would also bring about a change in the system which controls oxidative phosphorylation, *viz.* ATP synthesis. Two independent effects of X-ray irradiation, one direct effect on chromosome breaks and another on the "rejoining system", have already been postulated (WOLFF and ATWOOD 1954). ATP deficiency would delay the rejoining of the chromosome breaks and probably reduce its efficiency. Restoration of oxidative phosphorylation by means of $MnCl_2$ could bring ATP production back towards normal and thus reduce the proportion of unrepaired chromosome breaks.

The effect of $MnCl_2$ on the spontaneous segregation of unstable strains is more difficult to interpret, on account of the problematic nature of the phenomenon itself (MORPURGO and SERMONTI 1959).

SUMMARY

Nitrogen mustard (HN–2) produces a sharp increase in somatic segregation in a heterozygous diploid of *Penicillium chrysogenum*. This takes the form of the appearance of colonies displaying recessive markers of the diploid treated. HN–2 also sets up unstable clones showing a high rate of occurrence of spontaneous segregation for particular markers (MORPURGO and SERMONTI 1959). The addition of manganous chloride ($MnCl_2$) to the plating medium of conidia treated with HN–2 appreciably lowered the rate of appearance of the segregants induced by HN–2.

Similar phenomena were observed when X-ray irradiation was used as the stimulus of somatic segregation.

$MnCl_2$ is known to be a powerful reactivating agent for conidia inactivated by HN–2, although it is without effect on conidia inactivated by X-ray irradiation (SERMONTI and MORPURGO 1958).

Unstable clones grown on medium supplemented with $MnCl_2$ practically ceased to produce segregant sectors. This effect was reversible: plating of conidia of colonies of unstable clones grown in the presence of $MnCl_2$ on an unsupplemented medium gave rise once again to colonies displaying the instability characteristic of the clone.

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