MODIFICATION BY MANGANOUS CHLORIDE OF THE FREQUENCY OF MUTATION INDUCED BY NITROGEN MUSTARD

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THE fact that a posttreatment can abolish the mutations induced by a mutagenic agent can furnish a tool for the study of the metabolic conditions that are necessary for the completion of processes that begin with the mutagenic treatment and lead finally to mutation (WITKIN 1956; DOUDNEY and HAAS 1958, 1960). Different agents can produce a decrease in the mutation frequency after UV treatment: visible light (KELNER 1949), chloramphenicol and, in general, conditions inhibiting protein synthesis (WITKIN 1956), 5-hydroxyuridine (DOUDNEY and HAAS 1960), etc. This type of postirradiation response has also been found with ionizing radiations (KADA, BRUN and MARCOVICH 1960). MORPURGO and SERMONTI (1959) have shown that posttreatment with MnCl₂ of diploid conidia of Penicillium chrysogenum treated with nitrogen mustard (HN-2) produces reactivation of viability and diminishes induced somatic segregation. The period during which the HN-2 effects are reversible lasts several hours. The present report describes the effect of MnCl₂ on HN-2-induced mutation in a haploid strain of Penicillium chrysogenum and contains preliminary indications on the postincubation period that is necessary to make the mutations irreversible. Using the terminology of DOUDNEY and HAAS (1958) we have studied the process of “fixation” by means of the “MnCl₂ challenge” of a mutation induced by HN-2.

MATERIALS AND METHODS

Strains: We have used two strains of the asexual mold Penicillium chrysogenum: a prototrophic haploid strain with green conidia (Wisconsin 47.1564), and a diploid heterozygous strain, also prototrophic and green, named N, 233 w⁻3 thi⁻2/234 y⁻10 ade⁻5. This diploid strain contains two recessive markers for conidial color: w⁻3 for white and y⁻10 for yellow; somatic segregation of these markers from diploid N was studied.

Media: A complete medium (C) described in a previous paper (SERMONTI 1957) and Czapek-Dox Minimal Medium (MM) were used. When specified, 8-azaguanine (8azg) was added to the MM at a concentration of 1.5 mM; in some experiments 7-azaindole was added to the MM in concentrations ranging from 0.2 to 0.8 mM.

Treatment with nitrogen mustard: Methyl-bis (β-chloroethyl) amine (HN-2) was used at a concentration of 6.5 mM in an aqueous solution of NaHCO₃ (3.15
percent w/v). Treatment of the conidia was interrupted by making an appropriate dilution of the suspension in a decontaminating solution containing glycine. In general the treatment lasted about three minutes for the diploid and two minutes for the haploid.

RESULTS

Appearance of mutants on MM+8-azaguanine: 10⁰ conidia from a sensitive haploid strain were treated with nitrogen mustard (HN-2) to give a survival of about ten percent and plated on Petri dishes containing MM+8azg. Some tens of colonies per dish appeared on a clear background (Figure 1), and on further examination they turn out to be resistant to 8-azaguanine (azg-r). Mixing resistant conidia with large numbers of treated sensitive conidia or changing the total quantity of treated sensitive conidia per dish showed that a background of sensitive conidia did not interfere with the appearance of colonies from conidia of a resistant strain. The number of resistant colonies was therefore a good index of the number of resistant conidia present in the plated suspension. The absence of a competition effect (GRIGG 1952) was probably due to the fact that 8azg inhibits completely the metabolism of sensitive conidia.

Incubation of treated conidia in C medium prior to plating on MM + 8azg: The conidia treated with HN-2 (survival 5-10%) were incubated on a liquid C

![Figure 1](image-url)

**Figure 1.**—Number of azg-r mutants obtained by plating a conidial suspension of a haploid strain treated with HN-2. The conidia were inoculated in liquid C medium after the treatment and shaken at 24°C. Every two hours samples were taken and plated on MM+8azg (control) and MM+8azg+MnCl₂ (MnCl₂ added).
medium at 24°C and at intervals samples were plated on MM + 8azg. The number of resistant colonies increased with the time of incubation and the maximum number attainable was two to three times as great as that obtained by plating immediately the treated suspension on MM + 8azg (Figure 1 upper curve). Under the same conditions, no effects were observed on the survival of conidia. This increase could be due to the fact that 8azg prevented expression of the mutation in half or more of the mutant conidia. Alternatively it can be due to a process analogous to that interpreted as “mutation stabilization” (DOUDNEY and HAAS 1958), which would be increased by some factor contained in the complete medium.

Suppression of resistant mutants owing to the presence of MnCl₂: Conidia treated with HN-2 were plated on MM + 8azg to which 5 mM MnCl₂ had also been added. No resistant colonies were obtained from a suspension which gave rise in the absence of MnCl₂ to hundreds of mutants. The effect of the MnCl₂ was not an inhibition of the azaguanine-resistant phenotype because conidia of a resistant strain grew normally on MM + 8-azg + MnCl₂. An inhibition of the phenotypic expression of the induced mutation can also be excluded since MnCl₂ did not diminish the number of mutants induced by X rays or UV light under similar conditions. Moreover nearly half of the mutants were phenotypically expressed immediately after the treatment (see preceding paragraph). From these facts we can conclude that MnCl₂ interferes specifically with the process of genotypic acquisition of the HN-2 induced mutations. A direct action of MnCl₂ on HN-2 is excluded by the fact that the effect of the salt is still observable some hours after the treatment and after decontamination of the conidial suspension (see next paragraph). Furthermore the inactivating effect of HN-2 on the haploids is not suppressed nor is its effect on the induction of mutations for resistance to 7-azaindole, as we should have expected if the salt acted simply by abolishing the action of the nitrogen mustard.

Incubation of treated conidia in C medium prior to plating on MM + 8azg + MnCl₂: Conidia treated with HN-2 were incubated in liquid C at 24°C. Samples were taken at intervals and plated on MM + 8azg + MnCl₂. In the sample taken at time zero no resistant mutants were found. Mutants began to appear later, their number increasing gradually, until after 6–10 hours of incubation they were as numerous as in the absence of the salt (Figures 1 and 2). At this time the mutation has become irreversible. The length of time needed for the mutation to become “fixed” could be due, at least in part, to the large scattering in germination of the conidia, which do not begin their metabolism synchronously.

Fixation of HN-2 effects on diploids: Conidia of a diploid strain are inactivated by treatment with HN-2. Amongst the survivors there is a high percentage of segregants, mainly haploids (FRATELLO, MORPURGO and SERMONTI 1960), their frequency approaching 100 percent in some cases. MnCl₂ added to the medium provokes a strong reversion of the lethal effect and a decrease in the frequency of segregants amongst the survivors (SERMONTI and MORPURGO 1959). Reversion of the lethal effect is much more marked in diploid than in haploid strains.

Diploid conidia treated with HN-2 (survival ca. 0.1%) were inoculated in
Figure 2.—MM agar + 8-azaguanine on which nearly $10^6$ conidia per dish of a sensitive strain treated with HN-2 were plated. Right: with MnCl$_2$. Left: without MnCl$_2$. Above: the treated suspension was plated immediately after the treatment (and elimination of the HN-2). Below: plating after 13 hours of incubation in liquid C complete medium. The mutants are completely suppressed by MnCl$_2$ administered at time zero. After 13 hours the mutation has become irreversible.

liquid C and shaken at 24°C for some hours. At intervals samples were taken and plated on C and on C + MnCl$_2$ (40 mM). In the first 9–12 hours (Morpurgo and Sermonti 1959) the HN-2 effects are reversible by Mn$^{++}$; after the 13th hour the number of lethal events and of segregants begin to increase in the medium containing Mn$^{++}$, and around the 24th hour they are the same as on the medium without Mn$^{++}$ (Figure 3). The effects of the nitrogen mustard have become irreversible. The longer delay in the beginning of the process of fixation of the HN-2 effects, with respect to what is observed for the mutation
**Figure 3.**—The effect of MnCl₂ on conidia of a heterozygous diploid strain treated with HN-2. The conidia were inoculated on liquid C medium after the treatment and shaken at 24°C. At successive intervals samples were plated on C (control) and C+MnCl₂ (MnCl₂ added). Circles indicate survival frequency; triangles indicate frequency of color segregants.

_Induced Mutations_ is, in all probability, due to the more severe treatment the diploid conidia have suffered.

**Incubation of treated conidia in C + MnCl₂ medium, prior to plating on C:** Morpurgo and Sermonti (1959) have shown that the process of reversion of the HN-2 effects in the conidia of a diploid strain plated on C agar + MnCl₂ proceeds gradually for a period of about 20 hours. The conidia were transported from C agar + MnCl₂ to C agar using cellophane discs.

Diploid conidia treated with HN-2 (survival ca. 0.3%) were inoculated into liquid medium containing MnCl₂ (40 mM) and incubated at 24°C. At different intervals samples were taken and plated on C agar with and without the salt. During the first 8–10 hours incubation, the presence of Mn⁺⁺ did not have any effect and the number of conidia that gave rise to colonies was constant and around ten times greater on the plates containing Mn⁺⁺ than on those without. The samples taken after this period began to be reactivated, and after about 24 hours the count of viable colonies on the plates with and without Mn⁺⁺ became the same. During this period of time the effect of the Mn⁺⁺ reached completion.

**Effect of MnCl₂ treatment with other mutagens:** Sermonti and Morpurgo (1958, 1959) have shown that the lethal effects of UV and X rays are not “reversed” by MnCl₂; nevertheless somatic segregation induced by X rays is significantly reduced by the presence of the salt. Frequency of mutation to resistance to 8-azaguanine is not modified by MnCl₂ after UV or X-ray treatment. Some chemical mutagens have been tested, besides HN-2, in the induction of the
azg-r mutants: diethylsulphate, DL-p-N-di(chloroethyl)phenylalanine. All of them were efficient in producing this particular mutation, but never was the MnCl₂ capable of reducing significantly the frequency of the mutation.

**DISCUSSION**

The fact that after mutagenic treatment mutations are not irreversibly stabilized in the genome (DNA) for a certain period of time can be interpreted in various ways. Although the term "reversal" is used for induced mutations it seems that repair of a specific alteration already established in the DNA is a less probable hypothesis. Two types of interpretation can be put forward. One is that at first an intermediate compound (RNA?) is altered, which later transfers the "wrong" information to the DNA; this hypothesis contradicts the "dogma" that daughter DNA receives information only from parental DNA. It has been advanced by DOUDNEY and HAAS (1958).

Alternatively if a mutagen-sensitive structure different from DNA is involved in the early stages of the mutagen process we may suppose that this structure does not act on the transfer of information but on the processes regulating DNA duplication, directly or indirectly disturbed by the action of the mutagen. In the case of induction of phage with UV light it has been supposed that the mutagenic agent acts on a "repressor" (RNA?) whose alteration allows the transition of the prophage (DNA) to the vegetative state (JACOB and MONOD 1961). This effect is photoreversible and is inhibited by chloramphenicol, that is it behaves similarly to UV-induced mutations (WITKIN 1956). The experiments of DOUDNEY and HAAS indicate clearly that alterations in the RNA cause a high frequency of mutation (alterations in DNA) through a process requiring protein synthesis, but are not conclusive enough in establishing whether the RNA implicated acts as a regulator or as a precursor of DNA.

A special regulator system whose functioning determines the fate of X-ray-induced aberrations has been postulated by WOLFF and LUIPPOLD (1955) and called "rejoining system." It requires energy through oxidative phosphorylation processes and is reactivated by MnCl₂ (WOLFF 1960). MORPURGO and SERMONTI (1959) have speculated that a similar system operates in reversing somatic segregation induced by nitrogen mustard in diploids of *Penicillium chrysogenum*. The existence of regulatory mechanisms in this last system is suggested by the fact that in some clones of unstable diploids (MORPURGO and SERMONTI 1959) segregation occurs spontaneously at high frequency as a consequence of a hereditary alteration. MnCl₂ reduces HN-2 effects on induction of somatic segregation and stabilizes temporarily unstable clones.

We cannot at the present time furnish any interpretation of the effect of MnCl₂ on the process of HN-2 induced mutation. However such an effect allows the statement that after HN-2 treatment (as after UV) the process of mutation fixation requires an interval of time and that in all probability it requires also special metabolic conditions that are not fulfilled in the presence of MnCl₂. The occurrence on an unstable condition produced by the nitrogen mustard, has been postulated by AUERBACH (1951); according to her, this condition can end al-
ternatively in a new stable mutated state or in the original stable state, through a process of "stabilization of a metastable molecular equilibrium." MnCl₂ effect provides a more direct evidence of the unstabilizing effect of the nitrogen mustard, and an experimental condition for the reversion of the unstable state to the original stable state.

SUMMARY

MnCl₂ (5 mM) suppresses mutation to resistance to 8-azaguanine (azg-r) induced by nitrogen mustard (HN-2) in a haploid strain of Penicillium chrysogenum. The reversibility of the mutation ceases after a few hours if treated conidia are incubated on a complete medium prior to plating on selective medium. MnCl₂ does not influence the azg-r mutation induced by X rays or UV light or certain other chemical mutagens. HN-2 induced mutation for resistance to 7-azaindole is not reversed by MnCl₂.

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LITERATURE CITED


