REACTIVATION BY MANGANOUS CHLORIDE OF SPORES INACTIVATED BY NITROGEN MUSTARD

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Received July 6, 1959

THE reactivation by visible light of conidia of *Streptomyces griseus* inactivated by ultraviolet irradiation was first observed by KELNER (1949). The phenomenon, photoreactivation, was later observed in several other organisms such as bacteriophages (DULBECCO 1950), bacteria and fungi of the genera *Penicillium* and *Neurospora* (KELNER 1952).

Reactivation of cells inactivated by ultraviolet irradiation was also obtained by means of pyruvic acid and other acids of the Krebs cycle (THOMPSON, MEFFERD and WYSS 1951; HEINMETS *et al.* 1954). Up to the end of 1957, to the present authors' knowledge, no case had come to light of the reactivation of cells inactivated by chemical mutagens. At that time it was observed, again by chance (SERMONTI and MORPURGO 1958), that manganous chloride brought about reactivation in conidia of *Penicillium chrysogenum* inactivated by nitrogen mustard (HN-2). Some of the first results concerning this effect, chemoreactivation, are described at length in this paper.

MATERIALS AND METHODS

Strains: The greater part of the work was carried out on a pyridoxine requiring heterozygous diploid strain of *Penicillium chrysogenum*, XXXIV S (SERMONTI and MORPURGO 1958). Other haploid and heterozygous diploid strains of *Penicillium chrysogenum* were used in particular experiments. Experiments were also carried out on a heterozygous diploid strain of *Saccharomyces cerevisiae* kindly provided by PROFESSOR G. MAGNI of the University of Pavia, Italy, and on a heterozygous diploid strain of *Aspergillus nidulans* kindly provided by PROFESSOR G. PONTECORVO of the University of Glasgow, Scotland.

Media: The complete medium used for all the molds was based on corn steep liquor (SERMONTI 1957), while the medium used for *S. cerevisiae* was based on yeast extract, peptone casein hydrolyzate, and vitamins. Some experiments were carried out with Czapek-Dox's minimal medium.

Nitrogen mustard treatment: Methyl-bis(β -chloroethyl)amine (HN-2) was used (6.5 mM) in an aqueous solution of sodium carbonate (3.15 percent, w./v.). Treatment of the conidia was terminated by diluting the suspensions ten times in a decontaminating solution (0.6 gm glycine, 0.7 gm NaHCO₃, 1 liter distilled water). Details of the procedure are described elsewhere (MORPURGO and SER-

MONTI 1958). Mutagenic treatment in some cases was performed with X-ray or ultraviolet irradiation (SERMONTI and MORPURGO 1958).

RESULTS

If conidia of *Penicillium chrysogenum* are treated with HN-2 and then plated on complete agar supplemented by manganous chloride ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$) their survival rate is regularly higher than that of treated conidia plated on the same medium in the absence of the salt. Whether or not the salt is present, conidia which do not give rise to colonies produce twisted and overbranched promycelia, no more than 100 microns long. A similar picture is shown when conidia are inactivated by X-rays.

Reactivation by different doses of MnCl_2 . (Figure 1): Several series of Petri dishes were plated with conidia of strain XXXIV S, treated with HN-2 so as to obtain a survival rate of about one percent on complete medium. Each series of dishes contained complete agar supplemented with a different dose of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, ranging from zero to 50 mM. Parallel series were plated with untreated conidia of the same strain.

The appearance of colonies derived from both treated and untreated conidia was increasingly delayed with increasing doses of MnCl_2 ; whereas in the absence of the salt colonies became visible after two days' incubation at 27°C, they appeared in the presence of MnCl_2 (40 mM) after four or five days, with a much reduced rate of growth. Sporulation, however, was relatively unaffected. In the case of untreated conidia, the number of colonies per dish noticeably decreased

FIGURE 1.—Survival of treated (HN-2) and untreated conidia of diploid XXXIV S at different concentrations of MnCl_2 ($4\text{H}_2\text{O}$) in the agar.

at the higher doses of MnCl_2 in the agar (40–50 mM). In the case of conidia previously treated with HN-2 the number of colonies per dish increased considerably with increasing doses of MnCl_2 . At doses higher than 50 mM growth was practically suppressed. There was a certain variation from experiment to experiment in the maxima of the survival curves due to conditions which have not yet been brought under control.

Reactivation at different HN-2-inactivation levels (Figure 2): Differences in the intensity of the HN-2 inactivation were obtained by varying the length of time, from the moment of addition of the mutagenic agent to the moment of decontamination, spent by the conidia in the HN-2 solution. The strain employed was XXXIV S. MnCl_2 was added to the medium at a concentration of 40 mM. The *degree of reactivation*, i.e., the number of reactivated conidia $H \times$ percent of treated conidia, decreased as the degree of inactivation increased (Table 1). In some cases where less than 95 percent of the spores were inactivated, the number of colonies obtained from platings of treated and untreated conidia on MnCl_2 -containing medium was approximately the same (Figure 2).

Increasing the degree of HN-2-inactivation increases the *reactivation ratio*, i.e., the ratio between colonies growing from platings of the same conidial suspension in the presence and in the absence of MnCl_2 .

Reactivation of diploid and haploid strains (Table 1, Figures 2 and 3): The first observations on the phenomenon of chemoreactivation (SERMONTI and MORPURGO 1958) suggested that there was a considerable variation in the effectiveness of reactivation with the ploidy of the strain used, the degree of reactivation being much higher with diploid strains. A number of haploid and heterozy-

FIGURE 2.—Reactivation by Mn^{++} (40 mM) of conidia treated with nitrogen mustard (HN-2). Strain: diploid XXXIV S.

TABLE 1

Reactivation by MnCl₂ (40 mM) in diploid and haploid strains of Penicillium chrysogenum

Strain (code)	Ploidy	Percent survival after HN-2 treatment	Average number of colonies per dish		Reactivation ratio [†]	Percent reactivation [‡]
		without MnCl ₂	without MnCl ₂	with MnCl ₂		
XXIX	diploid	0.73	31.5	363.6	11.6	7.74
		0.07	3.1	101.7	32.6	2.21
XL	diploid	0.90	90.0	1,036.0	11.5	9.45
		0.16	16.5	587.0	35.6	5.54
XXXIV S	diploid	6.00	507.0	42.5×100*	8.4	44.40
		2.77	234.3	40.3×100*	17.2	44.87
		1.61	136.0	83.3×20*	12.2	18.03
		0.58	49.0	78.2×20*	31.9	17.92
		0.46	34.0	53.8×10*	15.8	6.81
31 <i>py</i>	haploid	0.08	10.3	36.0§	3.5	0.20
		0.015	1.7	5.2§	3.0	0.03
203 <i>pr</i>	haploid	1.08	7.1	9.4	1.3	0.32
5	haploid	1.92	182.0	534.0	2.9	3.65
206 <i>pr</i>	haploid	3.01	376.6	60.2×10*	1.6	1.81
		2.09	262.0	40.7×10*	1.6	1.25
		1.18	147.3	16.4×10*	1.1	0.12
		0.77	96.2	21.8×10*	2.3	1.00

* In some experiments the MnCl₂ supplemented agar was plated with a more dilute conidium suspension than the unsupplemented agar. In the case of these dishes, the average is shown as the actual figure multiplied by the dilution factor.

† Average number of colonies per MnCl₂ supplemented dish divided by average number per unsupplemented dish.

‡ Reactivated conidia percent of treated conidia.

§ 30 mM of MnCl₂.

gous diploid strains (PONTECORVO and SERMONTI 1957) were treated with HN-2 and reactivated with MnCl₂. Diploid strains showed a much higher ratio and degree of reactivation than haploid.

Persistence of spore reactivability: Experiments were carried out to determine how long the capacity for reactivation persists in the spores after inactivation. A suspension of spores treated with HN-2 which was diluted in the usual way in the decontaminating solution, gave a survival rate on complete medium of 1.3 percent. A sample of the suspension was plated on complete medium and another on complete medium supplemented with MnCl₂ (40 mM), and the remainder of the suspension was kept at +5°C. After three weeks two more samples of the same suspension were plated on the two media. The first pair of samples produced 108 ± 8 and 755 ± 16 colonies per dish on unsupplemented and supplemented medium respectively, and the second produced 235 ± 14 and 710 ± 69 . The proportion of spores capable of reactivation by MnCl₂ had thus not significantly altered during the storage period.

In another experiment, the HN-2 treated spore suspension was incubated in liquid nutrient medium in optimum growth conditions; samples of the suspension were taken at intervals and plated on complete agar in the presence and in

FIGURE 3.—Reactivation by Mn^{++} (40 mM) of conidia treated with nitrogen mustard (HN-2). Strain: haploid 31 *py*.

the absence of $MnCl_2$. The liquid medium contained eight percent corn steep liquor and six percent dextrin, and was used in quantities of 20 ml in 100 ml flasks shaken on a rotary shaker at 24°C. Incubation was discontinued after nine hours, since by this time germination of the conidia had started and they could form clusters and stick to the walls of the flasks. No significant change in the number of spores reactivable by $MnCl_2$ (17.4 ± 1.7 percent in five samples) was observed throughout the whole of the preincubation period. Thus three weeks of storage in distilled water at 5°C on the one hand, and nine hours of preincubation in a fermentation broth on the other, represent the maximum periods of persistence of reactivability so far established.

Duration of contact with $MnCl_2$ required for spore reactivation (Figure 4):

If colonies from HN-2 treated conidia grown on $MnCl_2$ are point-transferred on to medium where the salt is absent, they resume normal growth and once more reach the normal colony size of the strain concerned (SERMONTI and MORPURGO 1958). This implies that once reactivation has taken place the presence of $MnCl_2$ is no longer required by the reactivated clones. It seemed of interest to determine how long a contact with $MnCl_2$ was required by the spores for reactivation to take place. The experiment devised was as follows.

A number of Petri dishes of eight cm diameter were filled with complete agar supplemented by $MnCl_2$ (40 mM). The agar in each dish was then covered with a cellophane film disc covering the whole surface. Some of the dishes were then plated with an HN-2 treated spore suspension, and the remainder, as a control, with an untreated suspension. Beginning immediately after plating, the cellophane disc was removed from each of a series of dishes at regular intervals of

FIGURE 4.—Survival of treated (HN-2) and untreated conidia of diploid XXXIV S after different exposures to agar supplemented by MnCl_2 ($4\text{H}_2\text{O}$) (40 mM). The conidia are transferred from supplemented to unsupplemented agar on a disc of cellophane (see text).

time, transferred first on to Petri dishes containing complete medium and then, after at least half an hour, onto fresh dishes of the same medium. The object of this double transfer was to remove the MnCl_2 from the cellophane discs. Thus the conidia and microcolonies which grew on top of the cellophane where they had been in contact with MnCl_2 , continued to grow in the absence of the salt. Results of a typical experiment are given in Figure 4. Reactivation of the treated spores increased with continued contact with MnCl_2 up to about the 20th hour. Further contact produced no further reactivation. Under the same conditions, no consistent effect was observed on the survival rate of untreated spores due to continued contact with MnCl_2 up to about 33 hours. An interesting point is that contact of the conidia with MnCl_2 for this period does not affect either the time of appearance or the subsequent growth of the colonies, which from this point of view are no different from colonies which have never been in contact with MnCl_2 . The experiment described in Figure 4 was repeated three times with consistent results. The halting of the reactivation process between the 8th and the 12th hour was observed every time (Figure 4). It may be concluded that, under the experimental conditions described, the reactivation process takes about one day from seeding of the treated spores in the presence of MnCl_2 , and that no further reactivation due to the presence of MnCl_2 in the medium takes place after this time.

Action of MnCl_2 on irradiated spores (Figure 5): The results of a typical experiment with X-ray irradiation is illustrated in Figure 5. Conidia of strain XXXIV S were given varying exposures to X-ray irradiation and plated in the presence and in the absence of MnCl_2 . No reactivation due to the salt was ob-

served. Conidia inactivated by ultraviolet irradiation (SERMONTI and MORPURGO 1958) were also not reactivated by MnCl_2 .

Action of other bivalent cations on HN-2 inactivated conidia (Figure 6): Some other bivalent cations were tested as Cl salts under the same conditions in which Mn^{++} produces reactivation, at different concentrations dictated by considerations of toxicity.

FIGURE 5.—Survival of conidia of diploid XXXIV S after different exposures to X-rays, in the presence of MnCl_2 ($4\text{H}_2\text{O}$) (40 mM) and in its absence. No reactivation by Mn^{++} is observed.

FIGURE 6.—Survival of treated (HN-2) and untreated conidia of diploid XXXIV S on agar media supplemented by different concentrations of several salts, added as chlorides.

Mg^{++} : Less toxic than Mn^{++} ; produces no inactivation on untreated conidia at any of the doses used. At higher concentrations it causes a progressive decrease in the size of the colonies. It is the best reactivating agent, after Mn^{++} , of all the ions tested.

Zn^{++} : Highly toxic. The behavior of this ion is apparently paradoxical. At concentrations up to six mM it has no effect on the size of the colonies, and only slightly affects sporulation. At these dosages there is a slight increase in the number of colonies produced by treated conidia, and a slight decrease in the number of colonies produced by untreated conidia as compared in each case with the numbers produced on unsupplemented medium. At ten mM, it produces a slight reduction in the size of the colonies and further depresses sporulation, side by side with a dramatic reduction in the survival rate of the untreated conidia and an appreciable increase in that of the treated conidia.

Ca^{++} : Morphology and size of the colonies unaffected, except (slightly) at concentrations of 200 mM. The highest dose produced a slight reactivation of treated spores. A slight lowering of the survival rate of treated conidia was observed in some experiments at concentrations of Ca^{++} in the agar around 50 mM.

Reactivation by $MnCl_2$ on minimal medium: The inhibitory action of $MnCl_2$ on the growth of *Penicillium* is stronger on minimal than on complete medium, and for this reason it was used at lower concentrations (15 mM) in the reactivation experiments on minimal medium. Reactivation of HN-2 treated conidia of strain XXXIV S was observed on pyridoxine-supplemented minimal medium with a reactivation ratio similar to that obtained by plating the same conidial suspension on a complete medium (40 mM $MnCl_2$) also supplemented with pyridoxine ($160.4 \pm 13.1/19.8 \pm 3.1$ on minimal medium as compared with $606.6 \pm 63.6/66.2 \pm 6.0$ on complete medium, 200,000 conidia per dish). Storage overnight of HN-2 treated conidia of *P. chrysogenum* in distilled water in the presence of $MnCl_2 \cdot 4H_2O$ concentrations up to ten percent (w./v.) produced no significant increase or decrease in vitality.

$MnCl_2$ reactivation in other microorganisms (Table 2): Preliminary experiments were carried out on other genera of Eumycetes. The phenomenon of reactivation in the presence of $MnCl_2$ after inactivation by HN-2 occurred in both

TABLE 2

*Reactivation by $MnCl_2$ in heterozygous diploid strains of *Saccharomyces cerevisiae* and *Aspergillus nidulans**

Species	$MnCl_2$ conc.	Colonies per dish (untreated)		Colonies per dish (HN-2 treated)		Reactivation ratio	Percent reactivation
		(Dilution factor)	(No.)	(No.)	(Percent survived)*		
<i>S. cerevisiae</i>		20	61.4 ± 4.9	102.2 ± 2.8	8.3	..	...
	20 mM	20	43.7 ± 12.6	262.0 ± 7.8	30.0	3.6	21.7
<i>A. nidulans</i>		50	13.7 ± 3.0	8.4 ± 0.7	1.2	..	...
	40 mM	50	16.3 ± 4.2	45.5 ± 6.0	5.6	4.7	4.4

* Percent of the untreated conidia growing on the same supplemented medium.

diploid conidia of *Aspergillus nidulans* and diploid cells of *Saccharomyces cerevisiae*.

MnCl₂ reactivation has been observed in *Escherichia coli* K12 and its non-occurrence in *B. megatherium*, by DR. M. PRINCIVALLE of this Institute (Personal communication).

DISCUSSION

A clear reactivating effect of MnCl₂ on nitrogen mustard (HN-2) inactivated conidia is established by the results presented in this paper. It would be premature to offer any explanation of the processes involved, but certain aspects call for discussion since it is possible they may provide a clue to its elucidation.

MnCl₂ reactivates conidia which in its absence would not produce more than an abortive promycelium, so that it must act on the conidium in the resting state or during germination. The continuous increase in the number of conidia reactivated in the first 20 hours suggests that the reactivation operates on each conidium as it sets out on a germination process which, in the absence of Mn⁺⁺, would abort. It is noteworthy that HN-2 treated conidia kept in a solution of MnCl₂ in distilled water undergo no reactivation.

It remains to be determined what type of action Mn⁺⁺ exerts on the germination metabolism. Since germination of the conidia of *P. chrysogenum* is delayed by Mn⁺⁺ at reactivating doses, its effect might be thought to be aspecifically inhibitory, i.e., to be the same as that of any other germination inhibitor on mutagen treated conidia. DOUDNEY and HAAS (1958) observed reactivation in ultraviolet irradiated cells of *E. coli* when the cells were incubated after irradiation in the absence of nitrogen. ALFER and GILLIES (1958) and GILLIES and ALFER (1959) also observed a reduction in the lethal effect of irradiation in *E. coli* in below optimum growth conditions or with subsequent chloramphenicol treatment. No systematic analysis has yet been carried out of the inhibiting effect on germination and the reactivating effect of different agents, but the work already reported rules out any possibility of a simple relation.

All the same, it must be considered unlikely that Mn⁺⁺ is aspecific in its action, in view of the wide differences in the effectiveness of reactivation by Mn⁺⁺ with different inactivating agents on the one hand and with different strains on the other. Conidia inactivated by HN-2 are the only ones which have so far been shown to be reactivable by Mn⁺⁺; conidia inactivated by X-ray or ultraviolet irradiation are not reactivated at all. This contrast is particularly significant in view of the close analogy between the modes of action of X-rays and HN-2, which is the most radiomimetic of all the chemical mutagens.

Mn⁺⁺ is specific in its action from another point of view, in relation to the ploidy of the strain. Diploid strains are reactivated about ten times more effectively than haploid. No correlation was observed between ploidy and the effectiveness of photoreactivation in ultraviolet irradiated cells with haploid and diploid yeast cells (WARSHAW 1952). One may explain the effect of ploidy in Mn⁺⁺ reactivation on the assumption that inactivation is a two-stage affair, with

a reversible process overlapped by an irreversible process, which follows immediately in haploid strains but only after further mutagen treatment in diploid strains. The haploid nucleus, certainly, is more exposed than the diploid to the lethal action of the mutagen and it may be that the irreversible inactivation takes place in the nucleus, while the reversible inactivation is sited in the extranuclear material.

ORD and DANIELLI (1956) have shown that amoeba cytoplasm is damaged lethally by an appropriate exposure to HN-2 (MBA), and that this damage is not a result of nuclear damage. They have observed that the presence of a nucleus, either during exposure or after exposure, makes no difference to the life of amoeba treated with 0.01 percent HN-2. They suggest that the nucleus is damaged in two stages; one of which is probably the direct action of HN-2, the other is presumably a result of the cytoplasmic damage.

A reduction in the frequency of chromosome rearrangements due to Mn^{++} was observed by SERMONTI and MORPURGO (1959) in *Penicillium chrysogenum* diploids after HN-2 treatment, but the reduction also occurs after X-ray irradiation, and in this case it does not accompany any increase in vitality. A restabilizing effect of Mn^{++} on unstable clones obtained after treatment of heterozygous diploids with HN-2 was also observed (SERMONTI and MORPURGO 1959). No explanation of the relation between the reactivating and the restabilizing effects of Mn^{++} can yet be offered.

No clue to the system involved in the HN-2 inactivation and Mn^{++} reactivation processes is given by the results obtained so far, except for the suggestion that a cytoplasmic site is involved. The role of Mn^{++} (or Mg^{++}) in reactivating enzymic systems inactivated after incubation of subcellular particles *in vitro* has been shown by several authors. ERNSTER (1956) demonstrated that the loss of phosphorylative capacity in isolated rat liver mitochondria can be restored by adding Mn^{++} to the inactivated system. Mg^{++} has the same effect to a smaller degree. GOSSELIN and POPJAK (1959) have shown that the enzymic activity of microsomes in sterol biosynthesis can be restored by the addition of Mn^{++} ions or by an increased concentration of Mg^{++} after inactivation by aging. In both cases the presence of ATP is required. Before attempting any interpretation of the process involved in the reactivation observed by the present authors, work at the biochemical level is badly needed.

SUMMARY

Conidia of *Penicillium chrysogenum* inactivated by nitrogen mustard (HN-2) are reactivated by the addition of $MnCl_2 \cdot 4H_2O$ to the plating agar. Reactivation is much more effective in the case of diploid strains than in that of haploid strains. The reactivability of treated conidia is unchanged after nine hours of culture in the absence of $MnCl_2$. The reactivation process is complete after about one day and further contact of the treated conidia with $MnCl_2$ does not lead to further reactivation. Conidia inactivated by X-ray or ultraviolet irradiation are not reactivated by $MnCl_2$. $MgCl_2$ also has a reactivating effect on conidia treated with

HN-2, but is less effective than MnCl_2 . It is suggested that the site of the inactivation-reactivation process is in the cytoplasm.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Mr. G. GIUSEPPE for his expert technical assistance, to Mr. F. G. MILES for the English translation of the Italian text. We are indebted to SIMES S.p.A., Milan, Italy, for gifts of Clorammin (HN-2).

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