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Nitrous acid-induced inactivation and mutagenesis in Neurospora

W. Klingmuller

F. Kaudewitz

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Abstract

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Klingmüller, W. and F. Kaudewitz. Nitrous acid-induced inactivation and mutagenesis in Neurospora.

Nitrous acid is a powerful mutagen and is widely used in mutation studies in Neurospora. A serious problem in these studies, however, is the high variability of results obtained for inactivation rates and for frequencies of induced forward mutations and reversions. We have attempted to identify the reasons for such variability and to establish suitable experimental conditions for routine work with conidial suspensions. From these studies the following standard technique for the induction of mutations in Neurospora was developed.

1. Grow the organism on glycerol complete agar in Erlenmeyer flasks with cotton plugs. Conidial yield is increased by the use of wide-necked Erlenmeyer flasks with plastic foam caps. Incubate at 25°C for 7 days.

2. Suspend the conidia by adding water and stirring with a glass rod. Filter through cotton to remove mycelial fragments. Centrifuge and wash twice. Determine conidial concentration by counting in a haemocytometer (3 fillings are checked, with each count not less than 500 conidia). Adjust the suspension to a concentration of 5×10^7 conidia/ml.

3. Prepare fresh acetate buffer 0.6 M at pH 4.5 (43 ml 2 N NaOH + 100 ml 2 N acetic acid + 190 ml water). Prepare fresh phosphate buffer 0.2 M at pH 7.2 by mixing appropriate volumes of stock M/5 solutions of KH_2PO_4 and Na_2HPO_4. Sterilize both buffers by filtration.

4. Dissolve 82.8 mg of $NaNO_2$ in 10 ml water. This will give a final molarity of 0.04 in the inactivation mixture.

5. Maintain a precision water bath at 25°C ($\pm$ 0.1°). Bring the conidial suspension, buffer and $NaNO_2$ solution to this temperature before starting the inactivation.

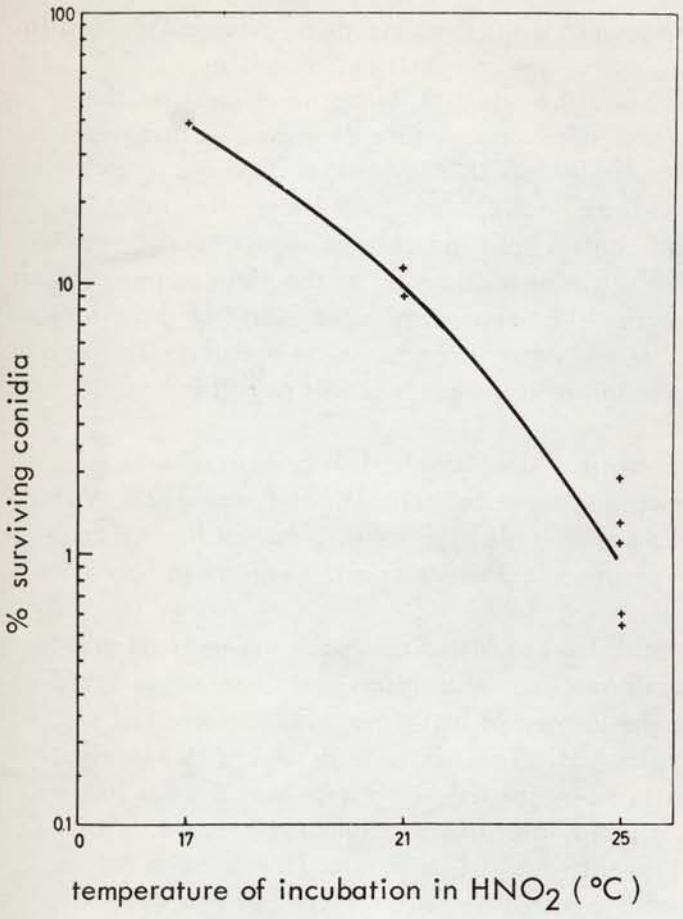
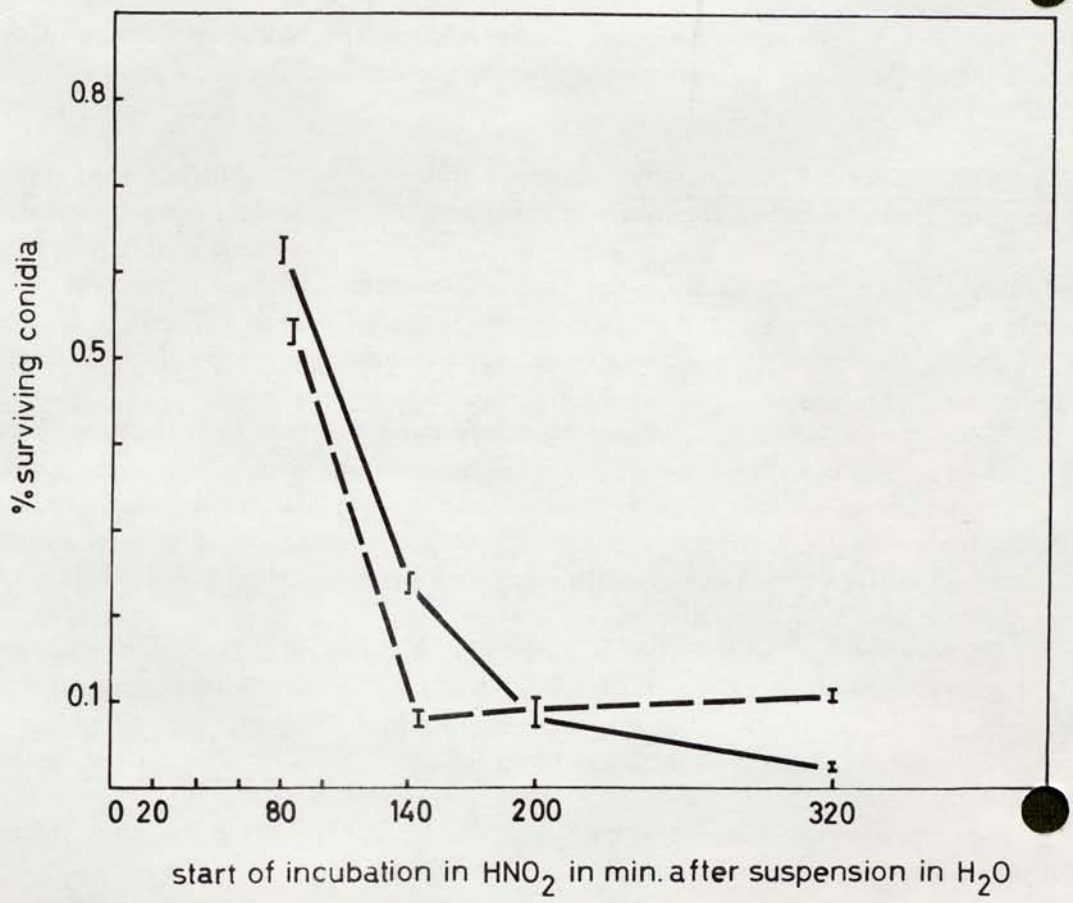


Figure 1 (left). Influence of incubation temperature on the rate of inactivation of conidia by nitrous acid (0.04 M $NaNO_2$, 10 minutes incubation at 25°C, standard incubation technique).

Figure 2 (below). Influence of the length of time interval between suspension of conidia in water and start of incubation with nitrous acid. Solid line: conidia kept in suspension by constant agitation. Dashed line: conidia allowed to sediment to bottom of tube. Standard 10 minute incubation period used.



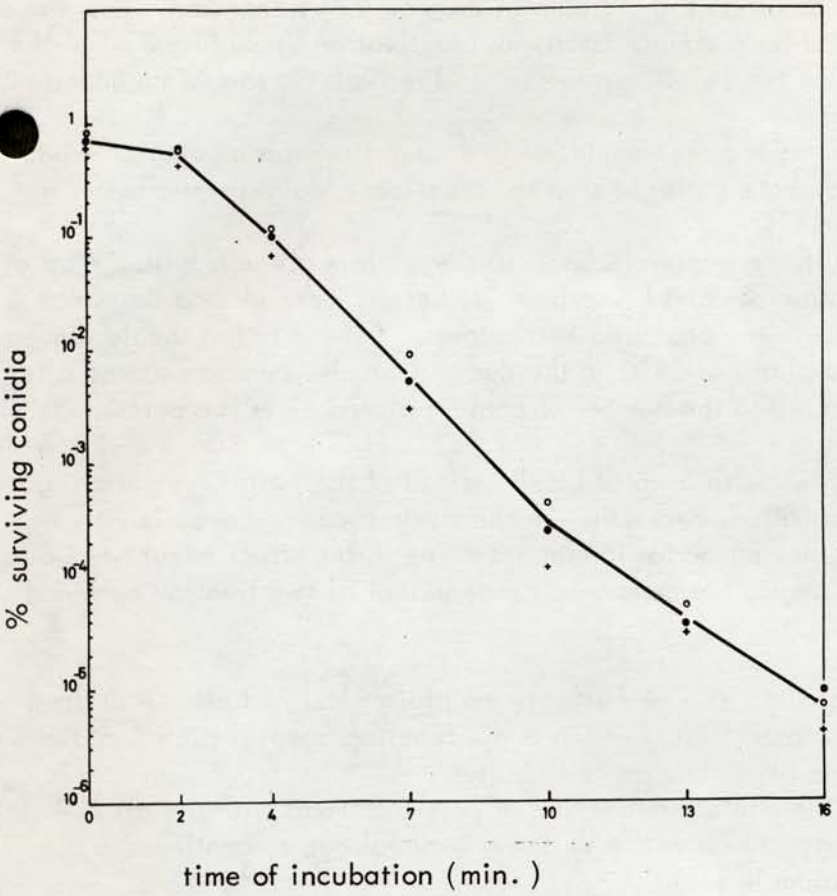
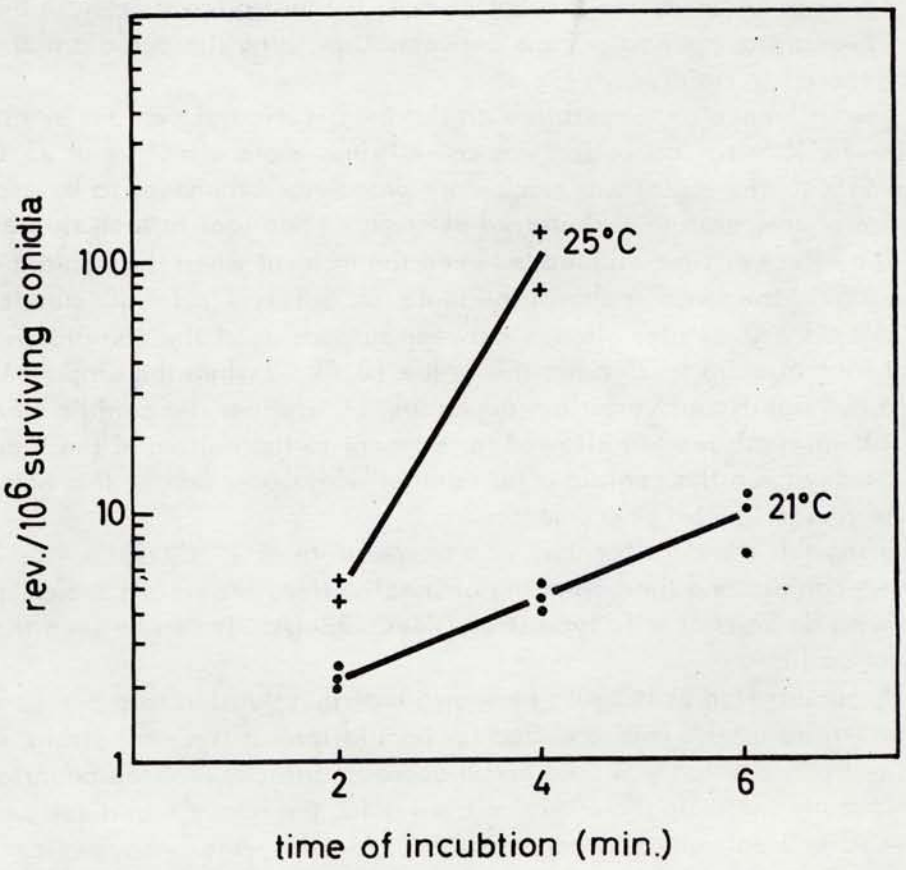


Figure 3 (left). Reproducibility of inactivation data, using standard technique described in text. Three different runs with conidia produced in separate flasks on separate days. Wild type 74-OR23-1A de Serres, 0.04 M nitrous acid, 25°C, 90 min. time interval between suspension of conidia and start of incubation with nitrous acid.

Figure 4 (below). Dependence of reversion rate of ad-4$^-$ → ad-4$^+$ on temperature and time of incubation with nitrous acid. Mutant F 68; standard incubation technique.



6. Carry out the inactivation in 20 ml test tubes containing 3 ml of the conidial suspension. Pass through the suspension an air stream regulated to 200 bubbles per min. Start the inactivation by adding 3 ml of the pH 4.5 acetate buffer and, 10 min. later , 3 ml of the $NaNO_2$ solution. (The $NaNO_2$ should be added 60 min. after suspending the conidia).

7. At intervals of 2-3 minutes up to 16 minutes remove samples of 1 ml each from the incubation mixture. Dilute immediately with 9 ml of the pH 7.2 phosphate buffer to stop the reaction. Maintain the buffer at 25°C and allow it to react for 30 min.

8. Dilute the samples with water to give suitable conidial concentrations. Mix aliquots with 100 ml of Fries minimal agar plus 0.05% glucose, 0.05% fructose and 1% sorbose (de Serres, Kølmark and Brockman 1962 Nature 193:556) cooled to 45°C. Distribute the agar in 5 Petri plates. (The dilution should aim to give ca. 100 survivors per plate.) Incubate the plates at 25°C in the dark. Colonies can be counted after 3-4 days. The number of visible colonies compared to the number of conidia plated gives the percentage of survival.

Control samples run in an identical manner, but with a water blank instead of the $NaNO_2$ solution, gave survivals between 70 and 90% for zero time incubation, depending on the strain used, and occasionally a decrease to between 50 and 60% survival after incubation for 16 minutes. The latter effect might be due to the strength of the buffer used; it is considered negligible compared to the extent of inactivation obtained with nitrous acid over the same period of time.

The following points should be emphasized:

1) The $NaNO_2$ inactivation mixture must contain enough buffer to maintain a defined pH. A defined pH results in a defined concentration of undissociated HNO_2 which is the reacting agent. High conidial concentration, such as 1.6×10^6/ml, will shift the pH of low molarity buffer.

2) A constant period of pre-treatment with the acetate buffer and of post-treatment with the pH 7.2 phosphate buffer is essential. Insufficient post-treatment will give lower survival due to continued action of nitrous acid after plating (pH of plating medium is 5.6).

3) During incubation, the mixture must be stirred constantly and aerated at a constant defined flow rate.

4) Temperature during incubation must be controlled strictly in the range of $\pm$ 0.1°C.

5) A constant period of time between suspending the conidia and starting the incubation has to be chosen and adhered to rigidly.

The influence of temperature on the inactivation of conidia by nitrous acid is shown in Figure 1. Inactivation for 10 min. incubation was ca. 40 times more effective at 25°C than at 17°C,and 10 times as effective as at 21°C. The effect was much more pronounced than was to be expected according to the Q10 rule. Strict control of temperature is therefore extremely important in such studies.

The effect of time elapsed between the moment when the conidia are suspended and the beginning of the nitrous acid treatment is shown in Figure 2. Survival after 10 minutes inactivation treatment was 0.025%, if 320 minutes elapsed between suspension of the conidia and the beginning of the treatment. Survival increased up to 25 times this value (0.62%) when the elapsed time was only 80 minutes. There were slight but significant variations depending on whether the conidia were kept in suspension by agitation during this interval or were allowed to sediment to the bottom of the tube. It is suggested that increased metabolic activity in the conidia after contact with water and/or loss of a protecting substance by diffusion may be the reason for this phenomenon.

Using this standard technique, a temperature of 25°C, and a 90 min. interval between the time of suspending the conidia and the beginning of inactivation, the results shown in figure 3 were obtained from 3 different runs with de Serres's wild type strain 74-OR23-1A. It can be seen that reproducibility is good down to survivals of ca. 10^{-6}.

By similar studies it could be shown that inactivation rate can be influenced by the genetic background of the strains used. It also varied for populations of the same strain, showing an increase during periods when conidial propagation was used and a decrease after sexual reproduction was inserted.

To study the mutagenicity of nitrous acid, the above technique was applied to conidia of ad-4 mutant F68. After treatment as described above, samples were removed from the inactivation mixture at 2,4, and 6 minutes after the addition of $NaNO_2$. From the dilution series, aliquots were plated in adenine-supplemented minimal agar to obtain survival counts. Other aliquots with high conidial concentration (up to 1.4×10^7

conidia / 100 ml medium) were plated in unsupplemented minimal agar and checked for reversions after 5 and 7 days. Occurrence of competitive inhibition (Grigg effect) could be excluded by reconstruction experiments for conidial concentrations up to 10 times the concentration used.

The results are shown in Figure 4. The reversion rate of $\underline{ad\text{-}4^-} \rightarrow \underline{ad\text{-}4^+}$ per surviving conidia increases with incubation time. A positive correlation between reversion rate and inactivation rate can be shown. Reversion rate of controls was between 0 and 0.16/ 10^6 germinating conidia. Thus reversions found after nitrous acid treatment cannot be due to selective inactivation but are induced by nitrous acid.

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