Preparation of the culture. In all experiments the mutagenic treatment was carried out on suspensions of conidio harvested from 125-ml Erlenmeyer flasks containing 20 ml of glycerol complete medium (10 ml glycerol per liter instead of 20 ml) + adenine sulfate (25 mg per liter). The flasks were incubated for 1 day at 30°C and then for 6-9 days at 25°C. The conidio were harvested by first shaking the cultures with glass beads (4 mm diameter) to break up the chains of conidio; they were then suspended in saline (0.9%), filtered through a platinum strainer, washed twice by centrifugation and then resuspended in saline. Purple mutants differ from each other with respect to the amount of the Purple pigment in the mycelium. However, when conidio are grown on glycerol complete, supplemented with 250 mg adenine sulfate per liter, purple pigment accumulation is essentially eliminated. The density of the conidial suspensions was measured on a colorimeter (Spectronic 20, Bausch and Lomb, Rochester, New York) at 750 mμ, where the absorption is maximal.

Treatments. All the treatments were carried out with conidiol suspensions (ca. 2 x 10^7/ml) in Erlenmeyer flasks in a water bath at 25°C on a rotary shaker to keep the conidia in suspension during the treatment. Five minutes before quenching, the conidio were centrifuged and the supernatant was decanted. At the time of quenching, after treatment with either NA, EMS or ICR-70, the conidio were resuspended in a solution of Fries' minimal medium adjusted to pH 8 with NaOH. This procedure was repeated twice. The solution of Fries' minimal medium adjusted to pH 8 has been found to stop the reaction of alkylating compounds and NA with conidia immediately.

Standard conditions for treatment with NA, EMS, HA and ICR-70.

NA treatment: The conidio were suspended in 0.05 M sodium acetate buffer at pH 4.5. One volume of freshly prepared 0.02 M sodium nitrate solution was added to 3 volumes of conidial suspension. The final concentration was 0.005 M NaNO₂, and the treatment was quenched as described above 40 minutes after the start of the treatment.

EMS treatment: The conidio were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by adding enough EMS to bring the final concentration to 0.1 M; the treatment was quenched 300 minutes later.

HA treatment: Before the HA treatment the conidio were suspended in 3 M NaCl and then further diluted five times in the reaction mixture of Strock et al. (1964 Mutation Res. 1: 10) which is composed of NH₄OHCl 2.6 g, H₂O 10 ml, 4 M NaCl 17 ml, and 10 M NaOH 2.3 ml, giving a pH of 6.2. The final HA concentration is 1 M. Five minutes before the treatment was quenched, the conidio were centrifuged and decanted and at the quenching time, i.e. 300 minutes after the start of the treatment, the conidio were resuspended in 3 M NaCl. This washing procedure was repeated twice and then the conidio were suspended in the solution of Fries' minimal medium adjusted to pH 8.

ICR-170 treatment: ICR-170 is the code number assigned to 2-methoxy-6-chloro-9-(3-ethyl-2-chloroethyl -aminopropylamino) ocrinidine dihydrochloride by H. J. Creech and co-workers of the Institute for Cancer Research, Philadelphia. Forward mutations induced by ICR-170 in Neurospora have been analyzed by Brockman and Goben (1965 Science 147: 750). The conidio were suspended in a 0.067 M phosphate buffer at pH 7.0. The treatment was started by adding 1 volume of a freshly prepared solution of ICR-170 (250 mg/liter of water) to 49 volumes of the conidial suspension, which gave a final concentration of 10.58 μM/liter. The treatment was quenched as described above 130 minutes after the start of the treatment. The treatment and other manipulations involving ICR-170 on conidio were performed under red light to eliminate the photodynamic effects associated with the ocrinidine ring. Plates were also incubated in the dark for at least 24 hours to allow sufficient time for the conidio to give rise to small colonies.

Plating medium. To estimate the viability of the treated and untreated conidio, they were plated in Westergaard's minimal medium supplemented with sorbose (15 g/liter), glucose (0.5 g/liter), fructose (0.5 g/liter), Casamino acids (200 mg/liter), a vitamin solution as in glycerol-complete medium (1 ml/liter) and adenine sulfate (25 mg/liter). To estimate the number of revertants the conidio were plated in the same substrate used for scoring survivors but supplemented with 0.2 mg adenine sulfate per liter instead of 25 mg/liter.

In the plates used to determine survival the density of the conidio was 5-10/μl of substrate in a total volume of 100 ml. For scoring of mutants revertants after NA, EMS or ICR-170 treatment, the conidio were plated to a density of 10⁶ conidia/ml and 2 x 10⁷ conidia/ml each in a total volume of 100 ml. For scoring of mutants revertants after the HA treatment, the density of the conidio was 2 x 10⁹/ml in a total volume of 500 ml. The plating was done in 15 x 200 mm intergrid phage dishes.

Statistical test. The test for significance is done according to Birnbaum (1954 J. Am. Stat. Assoc. 49: 254). In this test the number of revertants is considered as having a Poisson distribution. The probability is calculated by assuming that the
following two ratios belong to the same population:

\[
\begin{align*}
(1) & \quad \frac{\text{Total population (surviving after treatment)}}{\text{Total population (untreated) + Total population (surviving after treatment)}} \\
(2) & \quad \frac{\text{Total number of revertants (in treated population)}}{\text{Total number of revertants (in untreated population) + Total number of revertants (in treated population)}}.
\end{align*}
\]

A probability lower than 5% indicates a significant difference between the number of reversions obtained in the control and in the treated series.

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Isolation of subcellular fractions of Neurospora mycelium.

The following procedure for the isolation of subcellular fractions from Neurospora mycelia has been developed in our laboratory during the past two years.

Cultures: Two 125 ml Erlenmeyer flasks containing 20 ml of Vogel's minimal medium supplemented with 1.5% sucrose and 1.5% agar were inoculated, cultured at first at 30°C for days and then at a temperature of 25°C and transferred to a 5-gallon carboy containing 15 liters of sterile Vogue's minimal medium supplemented with 1.5% sucrose. After 3 days' incubation with continuous aeration at 25°C, the mycelia were collected on a double layer of cheesecloth in a saline 40 cm in diameter. The mycelia were washed on the sieve with a freshly prepared solution of 0.50 M sucrose (Mann Co., Enzyme Grade) containing one mM dipotassium EDTA (Sucrose EDTA) at 4°C and 50 mM Tris Cl, pH 7.4. The mot is passed through a hand blender and then placed in a large sheet of clothes wringer and blotted with paper towels. The yield of wild-type (74A) is generally 200 grams moist weight.

Disruption of cells: Small pieces of mycelium ore placed in a 1-1/2 quart Waring blender with sucrose-EDTA solution at 4°C. About 7 ml of solution per gram fresh weight is sufficient. The mixture is blended for 6 seconds at the "high" speed of the blender and poured into a Rota centrifuge mill (size No. 1) with 2 liters of glass beads. Prior to uk, the beads (Van Waters and Rogers Co., Cot. No. 340708, 1 mm diameter) are thoroughly washed on a 20-mesh sieve with water and sterilized in an oven at 160°C for at least 3 hours. The jar is rotated 288 rpm at a small mill for at least 1-1/2 hours at 4°C.

The supernatant is poured from the jar and set aside. The beads are washed at least 6 times with a total (1 to 1.5 volumes) of sucrose-EDTA and the washes are combined with the supernatant.

Isolation of subcellular fractions: Large pieces of unbroken mycelium and residual beads are removed from the homogenate by vacuum filtration through a single layer of double-napped pajama flannelette on a 30-cm Buchner funnel. The filtrate, designated as N1, is centrifuged at 2,000 x g for 10 minutes in the VR rotor of a Lourdes Beta centrifuge. The crude nuclear precipitate is set aside (N1) and the supernatant is centrifuged at 16,000 x g for 20 minutes. The mitochondrial precipitate is centrifuged at 16,000 x g for 20 minutes. The slight precipitate is then centrifuged at 120,000 x g for 2 hours in the No. 50 rotor of a Spinco Model L-2 ultracentrifuge. The microsomal precipitate (P) is suspended in 5-10 ml of sucrose-EDTA with the aid of a glass-telefon homogenizer.

Additional purification of nuclear and mitochondrial fractions: Fraction N1 is suspended in 10-12 ml of a solution containing 0.5 M sucrose and 10 mM CaCl2 and filtered through double-napped flannel. The filtrate is centrifuged at 500 x g for 2 minutes and the fluffly layer of nuclei, together with the supernatant, is decanted from the hard pellet of cell-wall debris and unbroken cells. Lawn-speed centrifugation is repeated twice. The crude nuclear fraction (5-10 ml) is layered on 5 ml of 2.3 M sucrose and centrifuged at 5,000 x g for 15 minutes. Nuclei are withdrawn from the density shelf and layered with a syringe on 5 ml of 1.5 M sucrose containing 10 mM CaCl2. After centrifugation at 20,000 x g for 20 minutes, the supernatant is withdrawn from the tube with a syringe and discarded. The nuclear pellet (N2) remains at the bottom.

Additional purification of the crude mitochondrial fraction (M1) is obtained by a "double-shelf" technique. 1.2 ml of 1.2 M sucrose is layered upon 5 ml of 1.5 M sucrose. 5 ml of a suspension of crude mitochondria (in 0.5 M sucrose) are layered above the double layers. After centrifugation at 16,000 x g for 30 minutes, mitochondria are removed from the central layer with a syringe fitted with a wide-gauge, blunt-tipped cannula. The suspension is diluted to 0.5 M sucrose and the double-shelf centrifugation is repeated.

Table 1 shows the distribution of total protein in the various subcellular fractions.

Discussion: Probably no one procedure can be devised that is ideal for the isolation of all of the various subcellular organelles from one cellular homogenate. The procedure described is therefore a compromise. For example, CaCl2 is necessary for the stability of nuclei, (Reich and Tsudo 1964 Biochim. Biophys. Acta 53:574), However, mitochondria tend to aggregate in the presence of divalent cations. Similarly, the preservation of ribosomes with Mg++ leads to the sedimentation of aggregated mitochondrial with the nuclear fraction.