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EFFECT OF N_2 ON THE MUTAGENIC AND LETHAL ACTIVITIES OF ICR-170 IN *NEUROSPORA CRASSA*

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Summary

The nature of the N_2 effect for ICR-170, i.e., the greater mutagenic and lethal activities of this agent in the presence of N_2 than O_2, has been studied at the *ad-3* region of *Neurospora crassa*. The characteristics of the N_2 effect for ICR-170 were that (1) the N_2 effect with ICR-170 was displayed in conidia when N_2 was administered during, but not before or after, ICR-170 treatment, (2) the highly increased mutagenic and lethal activities of ICR-170 in the presence of N_2 were due to an anoxic condition rather than to the presence of N_2 per se, (3) the high killing activity of ICR-170 under N_2 was due largely to increased cytoplasmic inactivation, (4) the N_2 effect was a general phenomenon at the *ad-3* region of *N. crassa*, and (5) the highly ICR-170-induced mutation in conidia under N_2 was attributable to an actual enhancement in the mutagenic activity of ICR-170 rather than to selective killing. With regard to the mechanisms of the N_2 effect with ICR-170, results indicate that this effect (1) was not due to more extracellular oxidative degradation of ICR-170 molecules in the presence of O_2, or to a greater uptake of ICR-170 by conidia under N_2, but (2) was due to the inhibition of conidial respiration under an anoxic environment.

ICR-170 has been synthesized at the Institute for Cancer Research by Creech and co-workers as an antitumor agent [8]. This agent is a monofunctional acridine mustard containing an intercalating nucleus and an alkylating nitrogen half-mustard. The mutagenicity of this agent has been demonstrated in a variety of organisms [1,3,17]. Using the *ad-3* system, Brockman and Goben [3] first

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Abbreviations: *ad-3*, adenine-3; DNP, 2,4-dinitrophenol; TNP, 2,4,6-trinitrophenol; ICR-170, 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]acridine · 2HCl.

described the mutational specificity of ICR-170 in *Neurospora crassa* and proposed that mutants induced by ICR-170 were predominantly of the base-pair addition and deletion type. In addition, it has been reported that ICR-170 also causes a low frequency of mutations that are base-pair substitutions in the same fungus [13]. In *N. crassa*, the mutagenic activity of this agent is a function of experimental conditions. One interesting feature is that the mutagenic and killing activities of ICR-170 in *N. crassa* can be enhanced in the presence of N_2 [5]. The present report describes the nature of the effect of N_2 on the mutagenic and killing activities of ICR-170 in this microbial eukaryote and therefore provides further information on the mutagenicity of this mutagen.

Materials and methods

Strains

A heterokaryotic strain (74-OR60-29A + 74-OR31-16A) of *N. crassa* was used for the study of forward mutations from *ad-3*$^+$ to *ad-3*$^-$. The genetic characteristics of this heterokaryon have been described previously [9]. An *ad-3* presumptive base-pair addition or deletion mutant (12-9-17) [3] and four *ad-3* presumptive base-pair substitution mutants (593, 3-7, 2-17-33, and 2-17-130) [14,16] were used for the reversion assay.

Chemicals

ICR-170 was kindly donated by Dr. H.J. Creech of the Institute for Cancer Research, Fox Chase, PA. ICR-170 stock solution was prepared in distilled water. O_2, N_2 and He were purchased from Union Carbide Corporation, Linde Division, New York, NY. The minimum purities of the gases were at least 99.9%.

Vegetative cultures and conidial suspensions

Procedures for the preparation of vegetative cultures have been described elsewhere [9,16]. Conidial suspensions were prepared in sterile water or phosphate buffer (0.1 M, pH 7) depending on experiments. Concentrations of conidial suspension were adjusted to 2.5×10^7 conidia/ml and 10^7 conidia/ml for reverse- and forward-mutation experiments, respectively.

Reverse mutation

In the study on the exposure to O_2 or N_2 during the mutagen treatment, conidial suspensions of mutant 12-9-17 were prebubbled with N_2 or O_2 for 30 min before addition of the mutagen. At the end of the prebubbling, ICR-170 stock solution was added to experimental tubes to give certain ICR-170 concentrations. At the same time, equal amount of water was added to the control tube. Bubbling with the appropriate gas was continued at 25°C. Unless otherwise indicated, all the experiments with ICR-170 were done under yellow or red light to eliminate possible photodynamic effects of its acridine moiety [15]. At various treatment times, samples from each tube were removed and washed twice with cold water by centrifugation at 4°C. These samples were employed immediately for determining the survivals and mutation frequencies as described by Brusick [7].

In the prebubbling experiment, conidial suspensions were prebubbled separately with O_2 or N_2 at 25°C for 3 h. At the end of prebubbling, the conidial suspensions were centrifuged and the supernatants were decanted. Each pellet containing 10^8 conidia was then resuspended and treated with ICR-170 in a water bath shaker at 25°C for 3 more h. The rest of these procedures were the same as those described above.

In the postbubbling experiment, conidia which had been treated with ICR-170 in the presence of O_2 or N_2 for 3 h were washed and resuspended. Each conidial suspension was then divided into two tubes and further postbubbled with N_2 and O_2 individually. After various postbubbling times, samples were withdrawn for determining population sizes and reversion frequencies.

Forward mutation

The forward-mutation experiment was conducted essentially as described for reversion mutation. However, the gaseous bubblings were only administered during the mutagen treatment. The *ad-3* mutation frequency and survival were determined by the direct method of the *ad-3* system in *N. crassa* [4,9].

Characterization of the killing activity of ICR-170 in the presence of N_2

The heterokaryotic strain, 74-OR60-29A + 74-OR31-16A, produces three kinds of conidia — heterokaryotic and homokaryotic for either of the two components [9]. Conidia (5×10^6/ml) of the heterokaryon were incubated with 5 μg ICR-170/ml in the presence of N_2 or O_2 at 25°C. At 1, 2 and 3 h of treatment, samples were removed and used for the determination of the viability of three conidial fractions [9].

Reconstruction experiment

20 revertants were isolated from an experiment in which conidia from mutant 12-9-17 had been treated with ICR-170 in the presence of N_2. Two conidial suspensions, each containing 10^8 conidia prepared by mixing a one to two ratio of conidia from mutant 12-9-17 and conidia from pooled suspensions of the 20 revertants, were treated with 5 μg ICR-170/ml in the presence of N_2 or O_2 for 3 h at 25°C. Adenine-supplemented medium and minimal medium were used for measuring the survivals of mutant 12-9-17 and its revertants, respectively [7].

Uptake of ICR-170 by conidia

Conidia from strain 12-9-17 (2.5×10^7/ml) were exposed to an ICR-170 solution (5 μg/ml) in the presence of bubbling N_2 or O_2. After various incubation times, a 4-ml sample was withdrawn from each conidial suspension and was centrifuged. The absorption of each supernatant was measured spectrophotometrically at 425 nm with a Spectronic-20 colorimeter. To test whether there was any different loss of ICR-170 between N_2 and O_2 bubblings, ICR-170 solutions (5 μg/ml) were determined as described above.

Respiratory inhibition and uncoupling of phosphorylation

Conidial suspensions prepared from mutant 12-9-17 were divided into two sets. Among one set, 6 different concentrations of NaCN were added sepa-

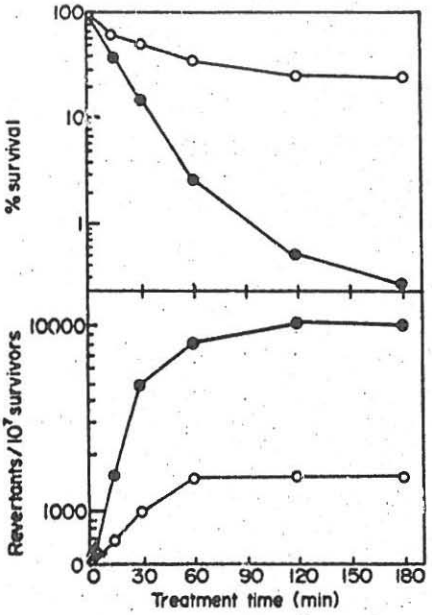
rately. In the same way, 6 different concentrations of NaCl, equimolar to the 6 concentrations of NaCN, were added to the other set. All of these conidial suspensions containing NaCN or NaCl were incubated for 30 min in a water bath shaker at 25°C before the addition of ICR-170. After this preincubation ICR-170 was added to each of conidial suspensions to give an ICR-170 concentration of 5 μg/ml. The treatment was then continued for 3 h. The determination of the reversion frequency and survival was done as described previously [7].

As to testing the effect of the uncoupling of oxidative phosphorylation from respiration on the genetic effect of ICR-170, DNP (an uncoupler of phosphorylation from respiration) and TNP (an analog of DNP that is not an uncoupler) [19] were used during ICR-170 treatment in a reversion assay in mutant 12-9-17. The experimental procedures followed those described for the respiratory inhibition above.

Results

General properties of the N_2 effect with ICR-170

The reversion frequencies and survivals from a kinetic study of the N_2 effect with ICR-170 in *N. crassa* are shown in Fig. 1. The percent survival of ICR-170 treated conidia in the presence of O_2 decreased slowly at early treatment times and became constant at 2 h. However, after exposure to ICR-170 in the presence of N_2, a rapid, linear decline in survival of the conidia was observed up to 1 h, and a continued but slower decrease in survival occurred throughout the rest of the time of exposure to ICR-170. The increase in reversion frequencies



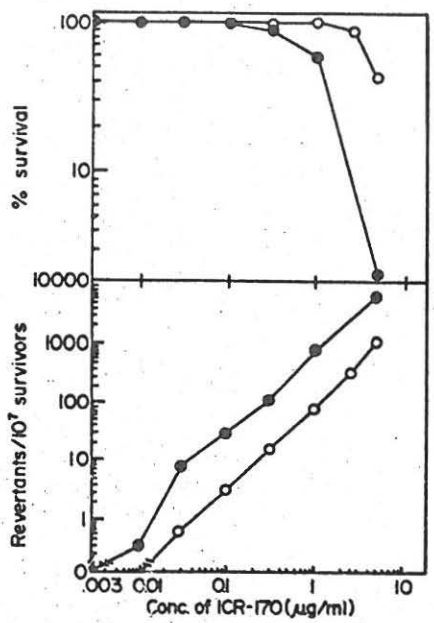


Fig. 1. Effect of N_2 or O_2 on the mutagenic and lethal activities of ICR-170 in strain 12-9-17 as a function of treatment time. Conidia were treated with ICR-170 (5 μg/ml) for 3 h in the presence of N_2 (●) or O_2 (○).

Fig. 2. Effect of N_2 or O_2 on the mutagenic and lethal activities of ICR-170 at various concentrations in strain 12-9-17. Conidia were treated with ICR-170 for 3 h in the presence of N_2 (●) or O_2 (○).

induced by ICR-170 was linear for the first 30 min in the presence of both O_2 and N_2, but the slope was much greater in the presence of N_2 than in the presence of O_2. After 30 min, the reversion frequency increased more slowly and became constant at 1 h under O_2 and at 2 h under N_2.

As to the dose–response curves shown in Fig. 2, the ICR-170-induced reversion in the presence of N_2 first appeared at 0.01 μg ICR-170/ml, whereas the mutagenic activity of ICR-170 was about 6–7 times lower at the concentration range of 0.03–5 μg/ml under O_2 than under N_2. A small N_2 effect for the killing activity of ICR-170 was observed at 0.3 μg ICR-170/ml. Moreover, with further increase in the concentration of ICR-170, the N_2 effect on the killing of conidia increased as ICR-170 concentrations were increased.

In the Fig. 3 from the postbubbling experiment, data clearly indicate that the N_2 effect with ICR-170 did not occur when N_2 was applied after ICR-170 treatment in the presence of O_2. Furthermore, the N_2 effect, which was induced during ICR-170 treatment in the presence of N_2, was not eliminated by O_2 postbubbling, whereas it was somewhat synergized by N_2 postbubbling. Table 1 shows the results obtained from conidia exposed to N_2 only before the ICR-170 treatment. There was essentially no difference either in the reversion frequencies or in the survivals of ICR-170-treated conidia which were prebubbled with N_2 or with O_2. Data obtained from the above two pre- and post-bubbling experiments demonstrate conclusively that an obvious N_2 effect with ICR-170 in *N. crassa* conidia occurred when the conidia were exposed to N_2 during the ICR-170 treatment, but not when N_2 was administered only before or after exposure of the conidia to ICR-170 in the presence of O_2.

Generality of the N_2 effect with ICR-170 in N. crassa

The effect of the presence of N_2 on the activities of ICR-170 was first ob-

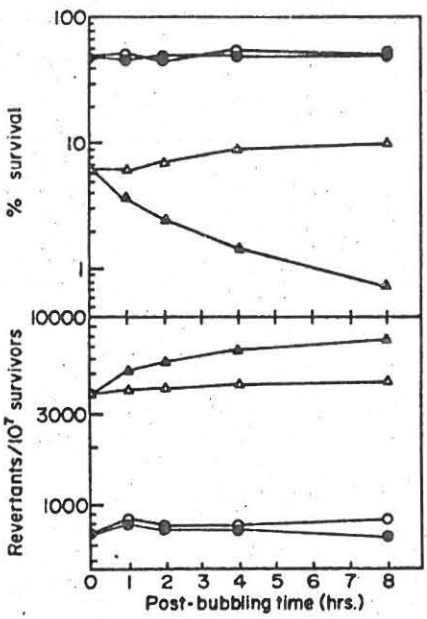


Fig. 3. Effect of postbubbling treatments on the survival and reversion frequency of strain 12-9-17 treated with ICR-170 in the presence of N_2 or O_2. Conidia were treated with ICR-170 (5 μg/ml) in the presence of O_2 (○, ●) or N_2 (△, ▲) and postbubbled with O_2 (○, △) or N_2 (●, ▲).

TABLE 1

REVERSION FREQUENCY AND SURVIVAL OF STRAIN 12-9-17 PREBUBBLED WITH AIR, O_2 OR N_2 AND THEN TREATED WITH ICR-170

Concn. of ICR-170 (μg/ml)	Pre-bubbled with	Percent survival	Number revertants	Revertants per 10^7 survivors
0	Air	100	0	0.0
5	O_2	57.1	3074	646.2
5	N_2	58.8	3177	668.1

served in the presumptive base-pair addition or deletion mutant 12-9-17 [5]. The question arose as to whether the N_2 effect is a common effect for ICR-170 in *N. crassa*, and also, more specifically, whether this phenomenon is also found with ICR-170-induced forward mutations and basepair substitution mutations. As indicated in Table 2, the forward-mutation frequency induced by ICR-170 was approximately 9 times higher in N_2 than in O_2. In testing for whether or not the N_2 effect also occurs in ICR-170-induced base-pair substitution mutations, results show that such effect was exhibited for ICR-170 in all four presumptive base-pair substitution strains tested (Table 3).

Characterization of the lethal activity of ICR-170 in N. crassa in the presence of N_2

The increased killing activity of ICR-170 in the presence of N_2 might be due to cytoplasmic and/or nuclear inactivation. The distinction between these two inactivations can be made in a heterokaryon of *N. crassa* such as 74-OR60-29A + 74-OR31-16A [2,9]. This heterokaryotic strain produces three kinds of conidia which are heterokaryotic and homokaryotic for either of the two components. Therefore, if the high lethality observed in ICR-170-treated conidia in the presence of N_2 results from nuclear inactivation, a more rapid decline in the survival of heterokaryotic conidia than of homokaryotic or of total conidia is expected. On the other hand, if ICR-170 causes cytoplasmic inactivations, an equal decrease in the viabilities of homokaryotic, heterokaryotic, and total conidial fractions would be observed [2,9]. The data from this study (Fig. 4) show that the decrease in the survivals of the three conidial fractions (heterokaryotic, homokaryotic and total) treated with ICR-170 in the presence of N_2

TABLE 2

MUTATION FREQUENCY AND SURVIVAL OF THE HETEROKARYON (74-OR60-29A + 74-OR31-16A) TREATED WITH ICR-170 IN THE PRESENCE OF AIR, O_2 or N_2

Concn. of ICR-170 (μg/ml)	Bubbled with	Percent survival	Number of mutants	Mutants per 10^6 survivors
0	Air	100	0	0.0
2	Air	75.2	432	275.3
2	O_2	69.0	795	386.9
2	N_2	48.7	6402	3529.0

TABLE 3

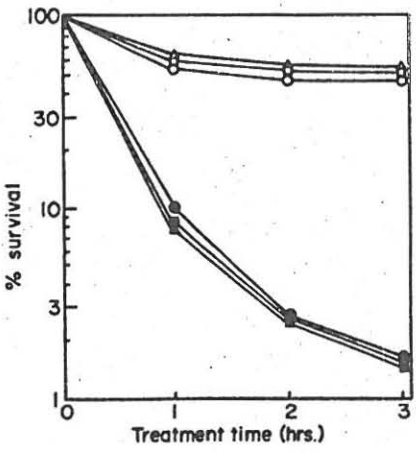
REVERSION FREQUENCY AND SURVIVAL OF PRESUMPTIVE BASE-PAIR SUBSTITUTION MUTANTS TREATED WITH ICR-170 IN THE PRESENCE OF O_2 OR N_2

Strain	Concn. of ICR-170 (μg/ml)	Bubbled with	Percent survival	Number of revertants	Revertants per 10^7 survivors
3-7	0	Air	100	0	0.0
	5	O_2	63.7	45	8.3
	5	N_2	4.9	19	45.6
593	0	Air	100	2	0.3
	5	O_2	70.2	7	1.5
	5	N_2	0.4	1	27.7
2-17-33	0	Air	100	4	0.4
	5	O_2	38.5	173	44.8
	5	N_2	0.5	15	280.7
2-17-130	0	Air	100	3	0.4
	5	O_2	40.1	23	8.9
	5	N_2	0.3	2	47.1

were fairly similar throughout the entire 3-h treatment. Therefore, the results indicate that the greater lethality in conidia treated with ICR-170 in the presence of N_2 resulted from increasing cytoplasmic inactivations.

A selective process was not involved in the increased mutagenicity of ICR-170 under N_2

As shown in Table 4 from the reconstruction experiment, the sensitivities of the mutant and its revertants to the killing activity of ICR-170 in the presence of N_2 were fairly similar. Moreover, in the data given in Table 5, there was a much higher number of ICR-170-induced revertants under N_2 than under



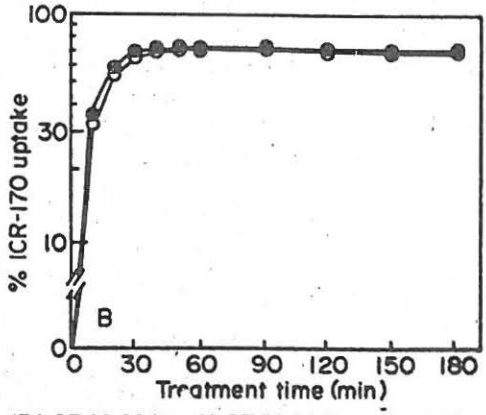


Fig. 4. Sensitivities of conidial fractions of heterokaryon (74-OR60-29A · 74-OR31-16A) to the killing activity of ICR-170 under O_2 or N_2 bubbling. Total fraction (□, ■), Los karyotic fraction (△, ▲) or heterokaryotic fraction (○, ●) was treated with ICR-170 in the presence of N_2 (■, ▲, ●) or O_2 (□, △, ○).

Fig. 5. Uptake of ICR-170 by conidia of strain 12-9-17 after various times of O_2 or N_2 bubbling. Conidia were exposed to ICR-170 in the presence of N_2 (●) or O_2 (○).

TABLE 4

SURVIVAL OF STRAIN 12-9-17 AND ITS REVERTANTS TREATED WITH ICR-170 IN THE PRESENCE OF O_2 OR N_2

Experiment number	Strain	Percent survival			Experimental survival (O_2/N_2)
		Control	Experimental		
		In air	In O_2	In N_2	
1	Mutant	100	45.6	3.84	11.8
	Revertants	100	57.5	3.65	15.7
2	Mutant	100	46.9	3.98	11.8
	Revertants	100	54.1	4.08	13.2
3	Mutant	100	49.2	1.71	28.7
	Revertants	100	47.0	1.69	27.8

O_2 at ICR-170 concentrations that caused no detectable killing (0.03—0.1 μg/ml). The results from the above studies indicate that the greater mutagenicity of ICR-170 observed in the presence of N_2 actually resulted from a potentiation of the mutagenic activity of ICR-170 in a N_2 atmosphere rather than from selective killing.

Mechanisms of the effect of N_2 on the activities of ICR-170 in N. crassa

It is possible that the difference in ICR-170 activities in the presence of N_2 or O_2 may stem from an extracellular event, in which degradation of the mutagen by oxidation is greater in the presence of O_2 than in N_2 before the mutagen molecules penetrate into the conidia. Table 6 clearly shows that about two-thirds of the ICR-170 molecules were lost during the 2-h bubbling with either O_2 or N_2. The equal loss of ICR-170 in both O_2 and N_2 bubblings indicates that the N_2 effect was not due to a difference in the oxidative degradation of ICR-170 between these two bubblings. Furthermore, the data (Fig. 5) indicate that there were no differences in the uptake of ICR-170 by conidia in the presence of the two gases. Therefore, the N_2 effect with ICR-170 was not due to a change in cell permeability under N_2.

TABLE 5

REVERSION FREQUENCY OF STRAIN 12-9-17 TREATED WITH ICR-170 AT DOSES OF NO DETECTABLE KILLING ACTIVITY

Concn. of ICR-170 (μg/ml)	O_2			N_2		
	Percent survival	Number of revertants	Revertants per 10^7 survivors	Percent survival	Number of revertants	Revertants per 10^7 survivors
0.00	100	0	0.0	100	0	0.0
0.01	100	0	0.0	100	4	0.4
0.03	100	5	0.5	100	56	7.8
0.10	100	30	3.0	100	209	27.2

TABLE 6

LOSS OF ICR-170 AFTER BUBBLING WITH O_2 OR N_2

Treatment condition	Percent ICR-170 lost
No bubbling	0.0
O_2 bubbling	65.9
N_2 bubbling	67.1

The N_2 effect observed for the activities of ICR-170 could be due to different modification of the ICR-170 molecules by the oxidative metabolism of conidia in the presence of N_2. For example, the N_2 effect might involve processes that are affected when respiration is inhibited in the absence of O_2. If this is the case, an enhancement of the activities of ICR-170 should be expected when respiration of conidia is inhibited experimentally by means of respiratory inhibitors. The results of the effect of NaCN on the activities of ICR-170 are presented in Fig. 6. The greatest synergistic effect of NaCN on the mutagenic activity of ICR-170 occurred at the NaCN concentration of 2 mM. There was a 7.5-fold increase in the ICR-170-induced reversion frequency at 2 mM of NaCN when compared to conidia co-treated with ICR-170 in the same concentration of NaCl. The data from this study strongly suggest that the great potentiation of the ICR-170 activities in the presence of N_2 may be attributable to the inhibition of respiration in the conidia by N_2.

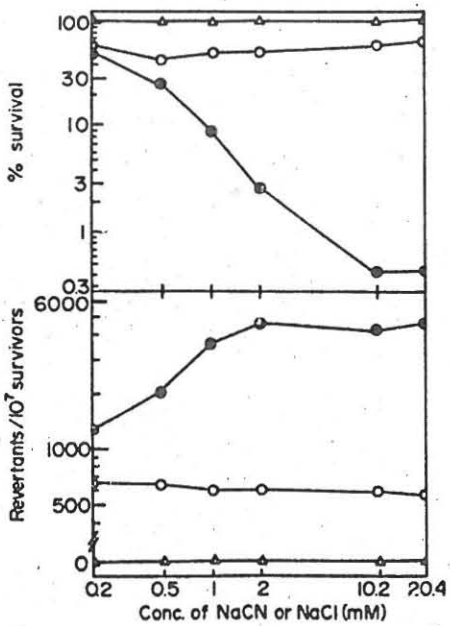


Fig. 6. Effect of NaCN or NaCl on the mutagenic and killing activities of ICR-170 in strain 12-9-17. Conidia were treated with (○, ●) or without (△) ICR-170 in the presence of NaCN (●, —) or NaCl (○, △).

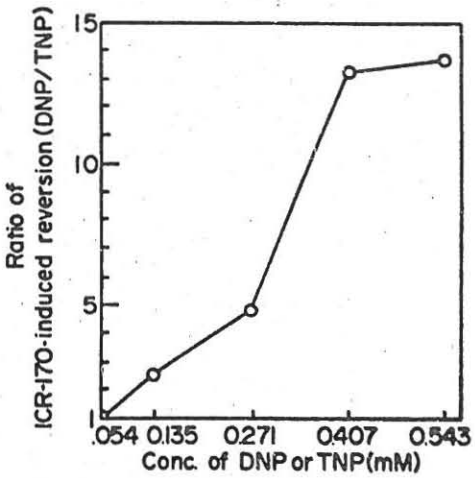


Fig. 7. Ratio of ICR-170-induced reversion in strain 12-9-17 treated with ICR-170 in the presence of DNP or TNP.

In order to gain further insight into oxidative metabolism (respiration), the possibility of oxidative phosphorylation being involved in the N_2 effect with ICR-170 was investigated. Results from this study (Fig. 7) show that in the presence of DNP or TNP from 0.407 to 0.543 mM, ICR-170 mutagenesis was 13 times greater with DNP than with TNP. The data indicate that oxidative phosphorylation may be involved in the N_2 effect with ICR-170.

Discussion

It is apparent from Fig. 2 that the N_2 effect for the mutagenic activity of ICR-170 was detectable at much lower doses than was its effect on lethal activity. This effect was more noticeable for mutagenicity at low doses and for lethality at high doses of the drug. It is conceivable that at a very low doses the N_2 effect occurred only for the mutagenic activity of ICR-170, because ICR-170 induces mutations with no detectable killing at very low doses [21]. As shown in the results of the time course study of ICR-170 (Fig. 1), the high activities found at the early treatment times and the fast reaching of a plateau during the ICR-170 treatment probably correlate to the kinetics of the uptake of ICR-170 by conidia, which follow the same pattern (Fig. 5).

A great enhancement in the mutagenic and killing activities of ICR-170 in the presence of another inert gas, He, was also observed (Table 7). Hence, the N_2 effect with ICR-170 could be attributable to an anoxic environment produced by the bubbling of N_2 rather than to an effect of N_2 per se. The term "N_2 effect" as used in this report is a synonym for the anoxia effect.

Conidia of *N. crassa* respire very effectively when they are incubated in water or in saline solution [12]. The respiration rate of conidia is directly proportional to temperature and inversely proportional to the age of the culture [12]. Oxygen could be depleted from a conidial suspension in a closed container at 22°C by endogeneous respiration of the conidia so that only 1/200 of the O_2 remained after 30 min [12]. Kølmark [11] also demonstrated that an exogenous anoxic condition in the conidial suspension of *N. crassa* is achieved by N_2 bubbling for 2 min and results in protection of conidia from X-ray-induced damages. Therefore, it can be assumed that the potentiation of the mutagenic and killing activities of ICR-170 by bubbling with N_2 was due to an inhibition of respiration by the anoxic condition. This is supported by the finding of an enhancement of the mutagenic activity of this

TABLE 7

EFFECT OF N_2, O_2 OR He ON THE MUTAGENIC AND KILLING ACTIVITIES OF ICR-170 IN STRAIN 12-9-17

Concn. of ICR-170 (μg/ml)	Bubbled with	Percent survival	Number of revertants	Revertants per 10^7 survivors
0	O_2	100	0	0.0
5	O_2	51.9	4784	1100.6
0	N_2	100	0	0.0
5	N_2	3.7	1478	4706.6
0	He	100	0	0.0
5	He	1.5	846	6731.3

drug in conidia in which oxidative respirations were inhibited by NaCN (Fig. 6). Furthermore, the fact that the uncoupling of oxidative phosphorylation in conidia treated with the uncoupler DNP resulted in a high mutagenic activity of ICR-170 indicates that metabolic energy might be involved in the N_2 effect with ICR-170. It is possible that an interference with energy metabolism by anoxia or by metabolic inhibitors might retard the repair of premutational lesions. However, the N_2 effect with ICR-170 might not be simply due to the inhibition of repair mechanisms.

It has been suggested that the anoxia effect with ICR-170 could be due to the higher degradation of ICR-170 molecules in conidia by mixed function oxidases in the presence of O_2 than in the presence of N_2 [18]. However, results from a cell-free system study show that the degradations of ICR-170 molecules by conidial homogenates under either O_2 or N_2 are the same (data unshown). Therefore, this suggested mechanism for the N_2 effect seems to be unlikely.

Because of the inhibition of respiration of conidia by anoxic condition did not influence the uptake of ICR-170 (Fig. 5) or the spontaneous mutation frequency (Table 7), it is likely that the effect of inhibition of respiration on the activities of ICR-170 is also partly due to a direct effect of this inhibition on the ICR-170 molecules. It has been reported that nitrogen mustard is converted rapidly into active reactants in neutral or alkaline aqueous solution [10]. The nature of this conversion is an intramolecular transformation, resulting in release of a chlorine ion and formation of a highly reactive chemical, a cyclic ethylenimonium derivative [10]. ICR-170 contains an acridine ring and a nitrogen half-mustard side-chain. It is possible that the addition of an acridine nucleus to the nitrogen half-mustard stabilizes the alkylating group and thereby reduces the spontaneous transformation of the latter component of the ICR-170 molecule into its active form. However, a certain fraction of the ICR-170 molecules would still be converted spontaneously into the active form. Based on the finding that the N_2 effect with ICR-170 occurred only when N_2 was administered during (but not before or after) ICR-170 treatment, and the suggestion by White and White [20] that inhibition of respiration provides a highly reductive environment in cells, it can be postulated that the reductive process may influence the transformation of a certain component of ICR-170 molecules into active forms and result in a N_2 effect for ICR-170.

An enhancement in the mutagenic and killing activities of ICR-compounds was first observed for ICR-170 in *N. crassa* [5]. The anoxia effect for ICR-170 has been found also in *Saccharomyces cerevisiae* (Brockman, unpublished results). Studies on the effect of N_2 or O_2 on the *ad-3* mutation frequency of *N. crassa* have been extended to 13 other ICR-compounds. Among the ICR-compounds tested, ICR-191 (a well known mutagen in prokaryotes), ICR-340 and ICR-217 (two agents with high antitumor activities) were included [6,22]. The main finding from these studies is that the ICR-compounds that are mutagenic show an anoxia effect and therefore that the anoxia effect is a general property for mutagenic ICR-compounds [6,22].

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