MUTATION INDUCTION BY DIFUNCTIONAL ALKYLATING AGENTS IN *NEUROSPORA CRASSA*

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Manuscript received December 4, 1974
Revised copy received March 17, 1975

ABSTRACT

The genetic characterization of *ad-3* mutants of *Neurospora crassa* induced by two carcinogenic difunctional alkylating agents, 1,2,4,5-diepoxypentane (DEP) and 1,2,7,8-diepoxyoctane (DEO), has shown that point mutations at the *ad-3B* locus have similar complementation patterns. In addition to the induction of point mutations, DEP induces a low frequency (7.5%) of multilocus deletions, whereas DEO induces an extremely high frequency (42.0%). The distribution of the different classes of *ad-3* mutants and the frequency of multilocus deletion mutants among DEP-induced mutants are not significantly different from those induced by the monofunctional alkylating agents EI, EMS and ICR-177 at comparable forward-mutation frequencies. Moreover, the frequencies of DEP-induced *ad-3B* mutants showing allelic completion or having nonpolarized complementation patterns are similar to those of *ad-3B* mutants induced by monofunctional agents. It is suggested, therefore, that the mechanism of mutation-induction by DEP in *N. crassa* is similar to that of monofunctional alkylating agents. Mutation-induction by DEO probably results both from the mechanism of action of monofunctional alkylating agents and from inter-strand cross-linkage of the DNA molecule by the two functional epoxy groups.

SINCE the discovery of the mutagenic activity of nitrogen mustards (AUERBACH and ROBSON 1947), the mutagenicity of many alkylating agents has been investigated. The mechanisms of mutation induction and other biological activities of alkylating agents have been reviewed extensively (FREESE 1963; ORGEL 1965; LAWLEY 1966; LOVELESS 1966). Based upon the number of active groups, alkylating agents are classified as mono-, di- or polyfunctional compounds. It seems likely that the principal difference among these groups with respect to interaction with DNA is that di- or polyfunctional agents *per se* could cause intra- or inter-strand cross-linkage of DNA (BROOKES and LAWLEY 1961, 1963). Inter-strand cross-linkage of DNA could lead to an inhibition of DNA synthesis and a deletion of chromosomal segments (BROOKES and LAWLEY 1964). Hence, monofunctional and di- or polyfunctional compounds might cause different spectra of genetic alterations. Several studies have shown that polyfunctional agents produce more chromosome breaks than monofunctional agents (FAHMY and FAHMY 1960; OBE 1968; NAWAR, KONZAK and NILAN 1971). Other studies, how-

ever, have indicated that mono-and polyfunctional compounds cause similar genetic and other biological changes (NAKAO and AUERBACH 1961; SNYDER and OSTER 1964).

In *Neurospora crassa*, the monofunctional alkylating agent, ethylene oxide (EO), and the difunctional compound, diepoxybutane (DEB), seem to induce mutations and cause lethality by similar mechanisms (KØLMARK and KILBEY 1968). It is of interest to know whether other diepoxides have a mechanism of mutation induction similar to that of monofunctional agents. It is also of interest to determine how variation in the length of the carbon chain between the active epoxy groups affects the spectrum of genetic alterations induced.

The mutagenicity and mutagenic specificity of two carcinogenic diepoxides, 1,2,4,5-diepoxypentane (DEP) (VAN DUUREN, ORRIS and NELSON 1965) and 1,2,7,8-diepoxyoctane (DEO) (VAN DUUREN *et al.* 1967) (Table 1) were studied with the *ad-3* test system of *N. crassa*. With this system, mutants that result from intragenic mutations and recessive multilocus deletions can be recovered and identified (DE SERRES 1964, 1968; DE SERRES and BROCKMAN 1968). It is possible with the *ad-3* system, therefore, to compare the spectra of genetic alterations caused by mutagenic agents by a number of criteria.

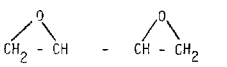
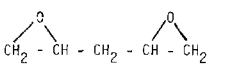
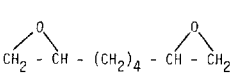
Abbreviations: *ad*, adenine; *al*, albino; *cot*, colonial temperature sensitive; *hist*, histidine; *nic*, nicotinic acid; *pan*, pantothenic acid; EO, epoxide; DEB, diepoxybutane; DEP, 1,2,4,5-diepoxypentane; DEO, 1,2,7,8-diepoxyoctane; EI, ethylanimine; EMS, ethyl methanesulfonate; ICR-177, N-(2-chloroethyl)-N-ethyl-1,3-propylenediamine·2HCL.

MATERIAL AND METHODS

Ad-3 mutants: A genetically marked two-component heterokaryon of *N. crassa* consisting of strain 74-OR60-29A component I, *A*, *hist-2*, *ad-3A*, *ad-3B*, *nic-2*, +; *ad-2*; +; *inos*; +; and strain 74-OR31-16A component II, *A*, +, +, +, +, *al-2*;+; *cot*; +; *pan-2* was used for the induction of *ad-3* mutations. Samples of 201 DEP- and 162 DEO-induced *ad-3* mutants (selected from samples recovered from similar forward-mutation frequencies) from forward-mutation experiments reported earlier (ONG and DE SERRES 1972) were characterized genetically by com-

TABLE 1

Chemical names and structures of diepoxides

Abbreviation	Chemical name	Chemical structure
DEB	Diepoxybutane	
DEP	1,2,4,5-diepoxy-pentane	
DEO	1,2,7,8-diepoxy-octane	

plementation, dikaryon and trikaryon tests. Spontaneous *ad-3* mutants among DEP- and DEO-induced mutants are negligible (<0.5%).

Complementation test: The genotype and the complementation pattern of each mutant was determined by growing each mutant with each complementation tester; the methods are similar to those described by BROCKMAN and DE SERRES (1963). The following nine testers were used: (1) *ad-3B*, complon 1(2-17-258); (2) *ad-3B*, complon 2(2-17-123); (3) *ad-3B*, complons 10-11 (2-31-8); (4) *ad-3B*, complon 15 (2-32-3); (5) *ad-3B*, complon 16-17 (2-32-5); (6) *ad-3A*, (1-68-13); (7) noncomplementing *ad-3B*, (1-112-2); (8) *hist-2, nic-2, al-2* (74-0933-3A); (9) *ad-2, inos* (74-OR60-44A). Testers 1 through 5 determine the complementation patterns of *ad-3B* mutants showing allelic complementation. Based on the complementation patterns, *ad-3B* mutants are classified as noncomplementing, nonpolarized complementing and polarized complementing. Testers 6 through 8 determine the genotype. Tester 9 is used as a control to test the ability of each mutant to form a heterokaryon. Mutants are classified as leaky if they grow by themselves in the complementation test.

Dikaryon test: Dikaryon tests were carried out by plating conidia from each mutant in Fries' minimal medium (HOROWITZ and BEADLE 1943) supplemented with 100 mg adenine sulfate/l and 10 mg calcium pantothenate/l. After incubation at 35° for 2 days, the plates were examined for the presence of *cot* colonies. The *ad-3* mutations were induced in the heterokaryon component that carries the genetic markers *cot, al-2* and *pan-2*. The presence of *cot* colonies indicates that the induced *ad-3* mutants are viable in the medium supplemented with adenine. This type of mutant is classified as having a reparable point mutation (*ad-3*^{R}) (BROCKMAN, DE SERRES and BARNETT 1969). Mutants that failed to give *cot* colonies in dikaryon tests were tested in the trikaryon test.

Trikaryon test: Mutants that do not produce *cot* colonies in the dikaryon test have either a point mutation within the *ad-3A* or *ad-3B* locus and an independent recessive lethal mutation at another locus elsewhere in the genome (*ad-3*^{R} + RL) or have a multilocus deletion covering one or both *ad-3* loci and one or more adjacent essential (irreparable) genes (*ad-3*^{IR}). These two types of mutants can be distinguished in the trikaryon test by the method of DE SERRES (1964, 1968). Trikaryons were formed by growing each mutant with each of three tester strains. The trikaryon tester strains are heterokaryons which carry the same genetic markers as that of the heterokaryon used for the forward-mutation induction experiments, except in component II: tester 12-7-215 (*ad-3A*^{IR}) having a deletion covering the *ad-3A* locus, tester 12-5-182 (*ad-3B*^{IR}) having a deletion covering the *ad-3B* locus and tester 12-1-8 (*ad-3A ad-3B nic-2*^{IR}) having a deletion covering the *ad-3A, ad-3B* and *nic-2* loci. Mutants were classified as *ad-3*^{IR} if no dikaryotic *cot* colonies were found from the trikaryons formed with all of the testers or if *cot* colonies were only found from the trikaryon formed with tester *ad-3A*^{IR} or *ad-3B*^{IR}. Mutants were classified as *ad-3*^{R} + RL if *cot* colonies were found from the trikaryons formed with all three testers. If *cot* colonies were produced by the trikaryon formed with *ad-3A*^{IR} and *ad-3B*^{IR}, but not with tester *ad-3A ad-3B nic-2*^{IR}, the mutant was a reparable *ad-3* mutant with a recessive lethal mutation at another locus closely linked to the *ad-3* region (*ad-3*^{R} + RL^{cl}).

RESULTS

The results of genetic characterization of DEP- and DEO-induced *ad-3* mutants can be summarized as follows:

Complementation test: Among the DEP-induced *ad-3* mutants tested, 35.3% are *ad-3A* and 60.2% are *ad-3B* mutants. The remaining 4.5% are *ad-3A ad-3B* double mutants (Table 2). Among the *ad-3B* point mutations, 52.1% show allelic complementation with 66.1% of the complementing mutants having nonpolarized complementation patterns (Table 3).

Analysis of DEO-induced *ad-3* mutants indicates 27.2% *ad-3A*, 50.0% *ad-3B* and 22.8% *ad-3A, ad-3B* double mutants (Table 2). The *ad-3B* point mutations

TABLE 2

Frequencies of genotypes of ad-3 *mutants induced by DEP and DEO as determined by complementation tests*

Compound	Total *ad-3* mutants	*ad-3A* mutants (%)	*ad-3B* mutants (%)	*ad-3A ad-3B* double mutants (%)
DEP	201	35.3 (71)*	60.2 (121)	4.5 (9)
DEO	162	27.2 (44)	50.0 (81)	22.8 (37)

* The number of mutants is given in parentheses.

TABLE 3

Frequencies of complementation patterns of DEP- and DEO-induced ad-3B *point mutations*

Compound	Total *ad-3B* point mutations	Complementing		Noncomplementing (%)	Nonpolarized complementing *ad-3B* among complementing *ad-3B*'s (%)
		Nonpolarized (%)	Polarized (%)		
DEP	119	34.5 (41)*	17.6 (21)	47.9 (57)	66.1 (41/62)
DEO	59	37.3 (22)	18.6 (11)	44.1 (26)	66.7 (22/33)

* The number of mutants is given in parentheses.

include 55.9% which show allelic complementation, among which 66.7% have nonpolarized complementation patterns (Table 3).

Dikaryon test: In the dikaryon test, 84% of the DEP-induced mutants and 51% of those induced by DEO produce *cot* colonies. These mutants carry point mutations at the *ad-3A* or *ad-3B* locus (*ad-3*^{R}) (Tables 4 and 5). Mutants which did not give *cot* colonies in dikaryon tests were tested further in the trikaryon test.

Trikaryon test: Thirteen (6.5%) DEP-induced mutants and seven (4.3%) DEO-induced mutants are classified as *ad-3*^{R} + RL, because they give *cot* colonies from the trikaryons formed with all three testers. Four (2%) DEP- and four (2.5%) DEO-induced mutants, which gave *cot* colonies only from trikaryons formed with *ad-3A*^{IR} and *ad-3B*^{IR} testers, are *ad-3*^{R} + *RL*^{cl}.

TABLE 4

Results of dikaryon and trikaryon tests

Compound	Total mutants tested	Point mutations				Total multilocus deletions (*ad-3*^{IR})
		Total	*ad-3*^{R}	*ad-3*^{R} + RL	*ad-3*^{R} + RL^{cl}	
DEP	201	92.5*(186)†	84.0 (169)	6.5 (13)	2.0 (4)	7.5 (15)
DEO	162	58.0 (94)	51.2 (83)	4.3 (7)	2.5 (4)	42.0 (68)

* The frequency (%) is based on the total mutants tested.
† The number of mutants is given in parentheses.

TABLE 5

Distribution of point mutations and multilocus deletions among DEP and DEO induced ad-3 *mutants*

Compound	Total *ad-3* mutants	Point mutations				Multilocus deletions			
		Total	*ad-3A*	*ad-3B*	*ad-3A/ad-3B*	Total	*ad-3A*	*ad-3B*	*ad-3A ad-3B*
DEP	201	186	67	119	0.56	15	4	3*	8
DEO	162	94	35	59	0.59	68	9	28*	31

* One of the DEP- and 6 of the DEO-induced *ad-3B* multilocus-deletion mutants also carry point mutations within the *ad-3A* locus and therefore are classified as *ad-3A ad-3B* double mutants in Table 2.

The remaining 7.5% of the DEP- and 42% of the DEO-induced mutants do not give *cot* colonies in the trikaryon test or give *cot* colonies from the trikaryon formed with the *ad-3A*^{IR} or *ad-3B*^{IR} testers. These mutants are multilocus deletion mutants (*ad-3*^{IR}) (Tables 4 and 5).

DISCUSSION

Our previous studies have shown that both carcinogenic diepoxides, DEP and DEO, are mutagenic in *N. crassa* (ONG and DE SERRES 1972). The results of the complementation tests presented in this paper show that the ratios of *ad-3A* to *ad-3B* mutants among DEP- and DEO-induced *ad-3* point mutations are 0.56 and 0.59, respectively (Table 5), which are not significantly different. Only 4.5% of the *ad-3* mutants induced by DEP, however, are *ad-3A ad-3B* double mutants, whereas 22.8% of those induced by DEO are *ad-3A ad-3B* double mutants. These results from the complementation tests are in agreement with the results of dikaryon and trikaryon tests which show that only 7.5% of DEP-induced *ad-3* mutants are multilocus deletion mutants, whereas 42.0% of DEO-induced *ad-3* mutants are multilocus deletion mutants. Genetic characterization of *ad-3* mutants induced by the two compounds also shows that the frequencies of complementing *ad-3B* and nonpolarized complementing *ad-3B* mutants among DEP- and DEO-induced *ad-3B* point mutations are similar (Table 3) and that the frequencies of the different classes of point mutations (*ad-3*^{R}, *ad-3*^{R} + RL and *ad-3*^{R} + RL^{c1}) induced by both agents are not significantly different from one another (Table 4). These data suggest that, except for inducing an unusually high frequency of multilocus deletions, DEO induced a spectrum of *ad-3* mutants similar to that induced by DEP.

In terms of the frequency of leakiness, allelic complementation, nonpolarized complementation patterns and multilocus deletions, observed at comparable forward mutation frequencies, the spectrum of DEP-induced *ad-3* mutants is similar to those of mutants induced by monofunctional alkylating agents (EI, EMS and ICR-177) (Table 6). These results seem to suggest that the mechanisms of mutation induction by DEP in *N. crassa* are similar to those of monofunctional alkylating agents. Studies by KØLMARK and KILBEY (1968) on the induction of adenine reversions in *N. crassa* have shown that EO and DEB are equally efficient

TABLE 6

Comparison between the spectra of genetic alterations in ad-3 *mutants induced by monofunctional and difunctional alkylating agents*

Compound	Leaky mutant (%)	Complementing *ad-3*B's among *ad-3B*'s (%)	Nonpolarized complementing *ad-3*B's among complementing *ad-3B*'s (%)	Multilocus deletion mutants (%)
EI*	13	41	67	3
EMS*	11	53	78	1
ICR-177*	10	51	70	11
DEP	10	52	66	7
DEO	7	55	67	42

* Data from DE SERRES *et al.* (1971), MALLING (personal communication), ONG (1970), and ONG and DE SERRES (1972).

in inducing reversions when the frequencies are based on survival. From their studies they assumed that EO and DEB induce mutations and cause lethality by similar mechanisms. In *Drosophila melanogaster*, NAKAO and AUERBACH (1961) showed that the ratio of sex-linked recessive lethal mutants to translocations induced by EO is similar to that induced by DEB. Their results led them to suggest that EO and DEB induce mutations and chromosome breaks *via* similar mechanisms.

The frequency of multilocus deletions among DEO-induced *ad-3* mutants is significantly different from that of DEP-induced mutants. It is interesting to note that the frequency of multilocus deletions induced by DEO is higher than that induced by any other physical and chemical agent tested in this system. The survival of conidia after treatment with DEO is lower than that of those treated with comparable concentrations of DEP. A plausible interpretation of these differences is that DEO may cause inter-strand cross-linkage of the DNA molecule that leads to the deletion of small chromosome segments and to higher lethality. It seems, therefore, that the mutation induction by DEO in *N. crassa* can be attributed in part to mechanisms similar to those of monofunctional alkylating agents, but also to the formation of cross-linkages within the DNA molecule.

It is generally known that the N7 of guanine is the most reactive site for alkylation. Therefore, interstrand cross-linkage resulting from the reaction of DNA with difunctional alkylating agents could be due to alkylations at the N7 of a guanine by a functional group and at the N7 of a guanine in the opposite strand by another functional group. Based on the Watson-Crick model of DNA structure, the distance from the N7 position of a guanine to the N7 position of the nearest guanine in the opposite strand is about 7.1 A°. According to Drieding Stereomodels, the distances from the first carbon to the last carbon of DEO can be extended to 8.9 A°, whereas DEP can only be extended to 5.2 A°. With this distance, it is unlikely that DEP can form cross-linkage of guanine residues in opposite strands of the DNA molecule. Such cross-linkage, however, could be

formed by DEO. The data presented here are in agreement with these molecular distances and suggest that the length of the carbon chain between the two functional groups of di- or polyfunctional alkylating agents is important in the formation of cross-linkage which in the case of DEO lead to the production of a high frequency of multilocus deletions.

We wish to thank DR. J. D. MCKINNEY, Environmental Biology and Chemistry Branch, NIEHS, for his help in measuring the molecular distances of DEP and DEO.

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Corresponding editor: D. R. STADLER