CELLULAR PROCESSES AND MUTATIONAL RESPONSE

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ABSTRACT

The collection of papers presented provides evidence which supports the contention that cellular processes play an important role in the determination of the observable mutational response of an organism. They also illustrate the importance of even a partial comprehension of the modes of action of such processes in developing an understanding of mutagen specificity, in linking together the diverse aspects of a chemical's mutagenicity and in probing the mechanism of action of particular mutagenic agents.

The papers forming the first half of the collection deal with ultraviolet mutagenesis and describe several instances in which cellular processes appear to be important in controlling its mutagenic specificity in Neurospora; enhancing, abolishing or even reversing what has come to be regarded as the basic specificity pattern in the strain used. The remaining papers in this section show how even partial knowledge of the mechanism of a particular cellular process (photorepair in this case) can furnish the investigator with a powerful tool for studying certain aspects of the mechanism of $U.V.$ mutagenesis.

The papers of the second half of the collection deal with epoxide mutagenesis and illustrate the developments which have taken place in our understanding of the role played by mutagen-sensitive, mutation-limiting systems in producing the varied aspects of diepoxybutane mutagenesis in Neurospora. The final papers in this section describe attempts to obtain evidence of analagous effects in a different organism, Saccharomyces cerevisiae. At first, in the absence of a suitable mutational system, survival was used as the biological end point studied. Later, a mutational system was discovered and preliminary studies with it have suggested that mutation-limiting systems with high DEB-sensitivity may exist in yeast also.

Index of Papers Submitted

1. The influence of temperature on the ultraviolet induced revertant frequencies of two auxotraphs of Neurospora crassa. B.J. Kilbey Zeitschrift für Vererbungslehre 94, 385-391 (1963).

2. Specificity in the photoreactivation of premutational damage induced in Neurospora crassa by ultraviolet. B.J. Kilbey: Molecular and General Genetics 100, 159-165 (1967).

3. Allele-specific responses to factors that modify UV mutagenesis. B.J. Kilbey: Ciba Foundation Symposium on Mutation as Cellular Process 1969, 50-62. Ed. GEW Wolstenholme and Maeve O'Connor.

4. The modifying effects of strain and age on the mutagenic specificity of ultraviolet light in Neurospora crassa. B.J. Kilbey and Sheena Purdom: Molecular and General Genetics 135, 295-307 (1975).

5. Identification of the genetic alterations at the molecular level of ultraviolet light-induced ad-3B mutants in Neurospora crassa. B.J. Kilbey, F.J. de Serres and H.V. Malling. Mutation Research 12, 47-56 (1971).

6. The nature of photoreactivation in Neurospora crassa. Claude E. Terry and B.J. Kilbey and H. Branch-Howe Jnr. Radiation Research 30, 739-747 (1967).

7. Loss of photorepair ability in conidia of Neurospora crassa. B.J. Kilbey. Nature 214, 1016-1017 (1967).

8. Quantitative and qualitative aspects of photoreactivation of premutation ultraviolet damage at the ad-3 loci of Neurospora crassa. B.J. Kilbey and F.J. de Serres. Mutation Research 4, 21-29 (1967).

9. Differential photoreversibility of ultraviolet-induced premutational lesions in Neurospora. F.J. de Serres and B.J. Kilbey Mutation Research 12, 221-234 (1971).

10. An investigation into the mutagenic after-effect of butadiene diepoxide using Neurospora crassa. H.G. Kølmark & B.J. Kilbey. Zeitschrift für Vererbungslehre 93, 356-365 (1962).

11. A mutagenic after-effect associated with ethylene oxide in Neurospora crassa. B.J. Kilbey and H.G. Kølmark. Molecular and General Genetics 101, 185-188 (1968).

12. Kinetic studies of mutation induction by epoxides in Neurospora crassa. H.G. Kølmark and B.J. Kilbey. Molecular and General Genetics 101, 89-98 (1968).

13. The stabilisation of a transient mutagen sensitive state in Neurospora by the protein synthesis inhibitor actidione. B.J. Kilbey. Molecular and General Genetics 123, 67-72 (1973).

14. The manipulation of mutation-induction kinetics in Neurospora crassa. B.J. Kilbey. Molecular and General Genetics 123, 73-76 (1973).

15. The analysis of a dose-rate effect found with a mutagenic chemical. B.J. Kilbey. Mutation Research 26, 249-256. (1974).

16. Diepoxybutane pretreatment effects on UV-inactivation and photo-reactivation of Saccharomyces cerevisiae. B.J. Kilbey. Mutation Research 8, 73-78 (1969).

17. The interaction between diepoxybutane and ultraviolet light in yeast. II. Experiment with UV-sensitive and resistant strains. B.J. Kilbey. Mutation Research 10, 525-531 (1970).

18. Similarities between a UV-sensitive mutant of yeast and bacterial mutants lacking excision-repair ability. B.J. Kilbey and Sheena M. Smith. Molecular and General Genetics 104, 253-257 (1969).

19. The mutagenic activity of diepoxybutane in yeast. Ewa Olszeneska and B.J. Kilbey.

20. Hyper-UV-sensitive mutants of yeast. I. Isolation and properties of two such mutants. A.M. Brown and B.J. Kilbey. Molecular and General Genetics 108, 258-265 (1970).

<u>Summary of Material Submitted</u>

These papers are concerned, directly or indirectly, with the study of factors involved in the phenotypic realisation of mutagenic damage. They fall into two main groups; those which are concerned with UV mutagenesis and its analysis in <u>Neurospora crassa</u>, and those which are concerned with epoxide mutagenesis and lethality in both Neurospora and <u>Saccharomyces cerevisiae</u>.

1. <u>Papers concerned with UV-induced mutation in Neurospora</u>:

   <u>(a) Reversion Studies (papers 1-4)</u>.

Papers 1-3 are reports of studies made in the original MRC Mutagenesis Unit and are concerned with data which substantiate ideas first formulated by its director, Charlotte Auerbach. It was her contention that while the interaction between a mutagenic agent and the genetic material is an indispensible event in mutagenesis, its occurrence is not a guarantee that genotypic alterations will follow or gain expression. Before phenotypic expression can occur, a sequence of events must take place including fixation, expression and growth. Mutants of different types and in different locations may surmount these obstacles with unequal facility and thus the initial specificity at the DNA level is likely to be distorted by the time the phenotypic level is reached. A further expectation is that the prevailing experimental conditions may be important in modifying the speed and efficiency with which the various cellular events can be completed and may thus, indirectly, modify the observed specificity. It is also worth noting, since the epoxides (which form the subject material of the second group of papers) are a good example of it, that the mutagen may itself interfere with the execution of the cellular events required for the expression of genetic damage.

Papers 1-4 describe several instances in which the mutagenic specificity of UV can be modified in the diauxotroph K3/17 ad⁻ inos⁻.

Paper 1 is concerned with the effects on UV specificity of temperature differences in the irradiated material. When the temperature of the conidial suspension is close to 0°C during exposure to UV, the reversional response of the adenine allele 3A 38701 is elevated relative to the response of the inositol allele 37401 and the specificity normally encountered at room temperature is enhanced.

Paper 2 describes the specific effects of photoreactivation in the same mutational system and shows that the UV-induced events which are responsible for inositol and adenine reversions are differentially photorepairable. Photoreactivation exerts its effect in Neurospora within the first three hours after irradiation (see paper 6) and, therefore, probably acts before the first post-irradiational DNA synthesis. The reasons for differential repairability of these two reversional types are still uncertain and may be related either to the photochemical nature of the lesions themselves or to their relative accessibility to the repair enzymes. The finding that the inositol allele 37401 is unique among those sixteen tested in its failure to respond readily to photoreactivation may suggest the former explanation but does not exclude the latter.

Paper 3 summarises the position in this field as it stood in 1968. Not only does it contain the material referred to in papers 1 and 2 but information is also included on the effects of UV irradiation in buffer versus water (the work of Dr. Allison) and of strain differences (the work of Auerbach and Ramsay) on the development of mutagen specificity in this mutational system.

Paper 4 presents our most recent findings concerning the cellular

control of UV-mutagen specificity in this system. Two derivatives
of the initial K3/17 strain, which possess the same two auxotrophic
mutations referred to earlier, were found to respond quite
differently to the mutagenic effects of ultraviolet. In one strain,
a complete reversal of the normal mutational specificity was
demonstrated but the reversal only developed when the strains were
suitably aged. A single gene difference apparently exists between the
two derivatives.

These experiments have amply verified the expectation that
mutagen specificity can be modified, even to the extent of reversal,
by applying the appropriate conditions. It must be admitted,
however, that the point is only just being reached at which an
investigation of the biochemical aspects of the problem is likely to
prove useful. The results of the most recent experiments certainly
offer the best chances of success in this direction.

1b.   <u>Photoreactivation and the examination of UV mutagenesis in
       Neurospora.</u> (Papers 5-9).

Photoreactivation is one of the best known means of modifying
the mutational yield from a given UV dose. At the outset of the
studies described in papers 5-9, photoreactivation had been noticed
in a variety of microorganisms including Neurospora although, in the
latter case, its mechanism was uncertain. In those organisms in which
photoreactivation had been studied it was possible to conclude that its
sole activity was the monomerisation of pyrimidine dimers produced by
UV. Much of this work involved experiments in which reactivation of
UV-inactivated transforming DNA was studied <u>in vitro</u> using cell-free
enzyme preparations and light of wavelengths around 400 nm.   I

action of a light dependent enzyme or enzymes and that these also probably function by splitting pyrimidine dimers.

Paper 7 is concerned with one aspect of photoreactivation noted in 6 but not studied in detail - the post-irradiational decline in photoreactivation ability. The loss cannot be attributed to a decline in the enzyme activity but rather to a change in state of the photoproducts which renders them resistant to photorepair.

Armed with a suitable genetic system and satisfactory indications concerning the mechanism of photoreactivation in Neurospora, experiments were performed to study the role of pyrimidine dimers in mutagenesis.

Paper 8 describes the results of an examination of point mutants induced at these two loci by UV with and without subsequent photoreactivation. It could be shown that all types of mutational change are equally susceptible to repair and, therefore, it could be concluded that pyrimidine dimers can generate all classes of point mutant. This was the first time such an observation had been attempted in an eukaryote although a little earlier and quite unknown to us, Drake had performed similar experiments and reached similar conclusions for bacteriophage T4. The data also show that photoreactivable and non-photoreactivable damage are equivalent in terms of the mutations each can produce. Whether they are intrinsically different or whether they simply differ in accessibility to repair enzymes can again not be answered.

Paper 9 extends the analysis from point mutations to include "irreparable" mutants at the ad-3A and ad-3B loci. Other investigations have shown that these are often deletions involving several genes. In all, 1239 mutants were analysed - a laborious process - and

useful information was obtained concerning the ratio of presumptive deletions to point mutations following UV, their relative susceptabilities to photoreactivation and their kinetics of induction. Both types of mutant display induction kinetics which could be described as "two-hit". Deletions are photoreactivated to a greater extent than point mutations.

The greater susceptibility of deletions to photoreactivation could be explained if the simple view is accepted that they require two photoproducts for their formation whereas point mutations require only one. The non-linear kinetics of point mutation must then be explained in ways which do not rely upon the interaction of DNA "hits". Repair inactivation (see also in next section) and events linked with the supposed recombinational origin of UV-induced mutants were suggested as possibilities. In the light of bacterial results we might now suggest that the induction of an error-prone repair is also a possibility but at present we are unable to take the analysis in Neurospora further.

2. Epoxide induced lethality and mutations in Neurospora and Yeast (Saccharomyces cerevisiae). (a) Mutagenesis in Neurospora (papers 10-15).

The papers which comprise this group are concerned with the mutagenic action of diepoxybutane (DEB) and ethyleneoxide (EO) in Neurospora. The main object has been to analyse the part played by cellular factors in determining the mutagenic yield and the effect of DEB on these factors. The study started from two different points; an analysis of the mutagenic after-effect of DEB and EO and an investigation into the kinetics of mutant induction by the two agents. As will be seen these two approaches converged in papers 13 and 14. In all the experiments ad-3A 38701 reversion is the endpoint studied.

Paper 10. The mutagenic after-effect associated with DEB

had been noted and described by Auerbach and Kølmark in 1959 but they
had not attempted its analysis. In this paper we were able to show
that sedimentation of the treated conidia is necessary for the
mutagenic after-effect to occur. It was also shown that the after-
effect is reduced when periods of suspension are interpolated
between washing and sedimentation. Although these findings suggest
that suspension facilitates loss of residual mutagen from cells which
retain it after normal washing, no evidence was obtained for any
extracellular mutagen in the supernatant of cells which had been
maintained in suspension for long periods after treatment. The
significance of this observation was only appreciated later on.

Paper 11 describes similar experiments using EO. These were
undertaken to answer a specific question posed by Loveless concerning
the importance of bifunctionality in promoting the after-effect. He
suggested that the after-effect occurred possibly because one of the
functional groups of DEB anchored the molecule in the cell making it
possible for the second functional group to react later with the
genetic material. EO is the monofunctional equivalent of DEB and, if
Loveless had been correct, should not have shown an after-effect. In
fact, E.O. promoted an after-effect which is just as marked as that
found with DEB.

Paper 12 represents the other starting point in the study of
DEB and EO mutagenesis and deals with the kinetics of mutation
induction. Both DEB and EO behave similarly: When mutant yield is
plotted against total dose (exposure time x molarity), both epoxides
generate upward-bending mutation induction curves which are
superimposible over a wide range of molarities. At the lowest
molarities used, however, both agents exhibit linear or near-linear

induction kinetics. The data for EO are less satisfactory than those
for DEB for technical reasons. In this paper I introduced the idea,
first proposed by Haynes for lethal events produced in bacteria and
yeast, that these data could be accounted for by the progressive
inactivation of a repair system and drew attention to the possible
effects of resynthesis and replacement of the damaged repair
components during the extended treatment times at low molarities.
At the time, Kølmark was more inclined to interpret the induction
kinetics in terms of a requirement for several independently occurring
events or hits for the induction of each mutant.

Paper 13 is a central one in this study since it marks the
bringing together of the kinetic information and the after-effect
studies. If DEB inactivates a repair system as I suggested in 12
and the cells can slowly replace this damaged system by resynthesising
its components, three expectations follow: (1) Cells treated with
DEB should possess enhanced sensitivity towards further treatments with
DEB and possibly other mutagens (eg UV). (2) Replacement of the
damaged components should lead to a steady decline in enhanced
sensitivity. (3) Prevention of resynthesis should perpetuate
enhanced sensitivity. Paper 13 concentrates on the last of these and
shows that actidione, an effective protein synthesis inhibitor in
Neurospora, can perpetuate the sensitive state. This information was
then used to show that sensitisation and traces of retained chemical
are required for the production of an after effect but that
sensitisation could be maintained long after the traces of mutagen
were removed by washing. Sensitisation was, therefore, in no way
dependent on the traces of mutagen being present in the cells.

Paper 14 is the logical extension of these results. If the

sensitive state created by DEB is maintained by preventing the resynthesis of components of the repair system, it should be possible to convert the linear dose-effect curve found at low dose-rates of DEB into the upward bending curves characteristic of high dose-rates by simply treating the cells with DEB and actidione together. Paper 14 shows that this can indeed be accomplished and, at the same time, it was shown that, as expected from the proposed mechanism, low dose-rates of DEB with actidione result in the enhancement of sensitivity to DEB and UV, while without actidione the sensitivity increases only slightly.

Paper 15 summarises these results and puts them into the context of other dose-rate studies.

(b) Experiments with Saccharomyces cerevisiae.

Our interpretation of the kinetic data obtained with DEB and EO with Neurospora was greatly facilitated by adopting the ideas of Haynes concerning repair inactivation or saturation in bacteria and yeast. It was therefore of some interest to see whether DEB exhibited the expected properties associated with repair inactivation in the same system used by Haynes for other alkylating agents. If DEB simply inactivates dark repair systems as suggested, the lesions produced by UV in the sensitised cells should be photoreactivable to at least the same degree as similarly produced lesions in cells which had not been exposed to DEB. Photoreactivation was therefore included to verify this. It should be pointed out that these studies are all concerned with lethal damage only; no suitable mutational system was available at this time. It is not surprising, therefore, that the results differ somewhat from the picture already presented. Not only are the lethal events in the cell probably different and more varied compared

with mutagenic lesions, the doses of DEB were also far higher and the survival levels much lower than in the Neurospora experiments. Recently we have been able to study DEB mutagenesis in yeast also and have found striking similarities to Neurospora in its mutational response.

Paper 16 describes the effects of DEB pretreatment on UV-inactivation and photoreactivation in haploid yeast. The situation is clearly more complex than the simple theory predicts although this would have been missed had not photoreactivation been measured. The results appear to be consistent with the conclusion that, although repair inactivation resulting in shoulder-elimination may occur,it is effectively masked by a second series of events. These appear to involve a rapid reaction between retained traces of DEB and UV-irradiated DNA resulting in an initial decline in viability without the accompanying photoreactivation. Once this stage is completed UV inactivation and photoreactivation proceed normally.

Paper 17 provides some further support for this interpretation. Reversal of the treatments abolishes the interaction; photo-reactivation is completely normal. This suggests that the reactive condition induced in the DNA by UV must be very short lived. Loss of this interaction response by reversing the treatments made it possible to demonstrate the elimination of the shouldered part of the UV-inactivation curve by DEB without modification of photoreactivation. In the uvs-1 strain (now known to be excision deficient, see paper 18) lower doses of both DEB and UV are necessary to maintain the survival values at the wild-type levels. In spite of this, pretreatment with DEB produces the same complex interaction as it does in wild-type yeast. This result emphasises to my mind, that the events concerned

with the interaction occur at the DNA level and are subject to excision. Excision is in no way required for the interaction, however. Reversal of the treatment in the uvs-1 strain promotes no interaction at all suggesting that the doses of DEB which can be used are insufficient to inactivate the system responsible for the shouldered UV-inactivation curve.

Paper 18 gives the results of experiments Miss Smith and I performed with uvs-1 (now rad-1). By noting the similarities between its response to photoreactivation and chemical mutagens and its ability to retain photoreactivability following UV treatment, we concluded that the mutant probably lacks the excision functions. This was later confirmed biochemically by Cox and his colleagues.

Paper 19   This material has only recently been obtained and has not yet been published. It refers to experiments in which the mutagenic action of DEB and the interaction between DEB and UV were studied using reversions at the isoleucine valine locus. Although these experiments were complicated by the effects of liquid holding, they present a picture for yeast which resembles that described for Neurospora in several ways: The kinetics of induction are similar in the two organisms, DEB treated yeast cells display enhanced DEB and UV sensitivity, the extra mutants produced by the interaction between DEB and UV are UV-like in their properties and there is evidence of a decline in enhanced sensitivity with storage of DEB-treated cells although the storage effects on mutation complicate the observations. The pattern of events described for Neurospora may, therefore, not be unique to this organism.

Paper 20 arose from an attempt to produce yeast mutants damaged in repair pathways other than excision. Apart from their intrinsic

interest as repair deficient mutants, it was thought that some of them might be useful in studies of the mutagenic activity of UV in yeast.   Although these first results were interesting, the study was not pursued by the author beyond this paper.   A study of two such mutants has since been completed by Dr. A.M. Brown.
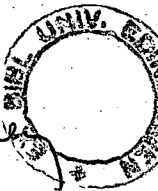
Statement Concerning Author's Role in Presented Papers.

Papers 1, 2, 3, 7, 13, 14, 15, 16 and 17 are entirely the work of

the author.   Papers 4 and 18 and paper 19 were also work which I

initiated and in which I participated.   However, my technicians,

Miss Smith (now Mrs. Purdom) and Miss Olszewska played an important role

in the laboratory work and I wanted them to be co-authors with me.

Papers 5, 8 and 9 were based on work done by the author in the laboratory

of F.J. de Serres.   In fact, the idea of using photoreactivation with

this genetic system was my own and most of the experimental work was also

done by me.   F.J. de Serres, however, provided the facilities and the

technical assistants - particularly for the completion of the mutant

analysis described in paper 9 which was finished after my departure.

H.V. Malling was included in the authorship of paper 5, not because of a

direct involvement, but largely because he had been involved in an

earlier standardisation of the methods used to treat the conidia with the

chemical mutagens and his advice was most valuable when the same

treatments were used in the study of the UV-induced mutants.   Paper 6

was published with Terry and Branch-Howe.   The latter was only very

indirectly involved having been Terry's Ph.D. supervisor when he started

studying photoreactivation a year earlier in Georgia.   Terry and I shared

the work for this paper and the others helping us were Amir Muhammed and

Jane Setlow.   The latter two workers actually performed the transformation

assays, Terry and I were concerned with obtaining the cell-free extracts

of Neurospora, exposing the transforming DNA to UV and to the enzyme in

the presence of monochromatic light and performing the in vivo experiments

with Neurospora.   Papers 10, 11 and 12 were based on work done in

collaboration with H.G. Kølmark.   It is difficult to make a division of

tasks in these cases. Planning experiments and writing were combined activities, although, as indicated in the summary of paper 12, differences of interpretation sometimes developed. Paper 20 is based, in part, on the work done by the author and A.M. Brown while the latter was an undergraduate student working under the author's supervision. Some of the results were included in the undergraduate thesis presented by A.M. Brown for the degree of B.Sc. in 1970. Approximately 80% of the experimental work was done by Brown. The paper is simply included as an illustration of ways in which mutants affecting the expression of induced mutations might be obtained at some future date but it is not essential to the present study.

It is relatively easy in retrospect to remember fairly accurately the practical roles of one's collaborators in each part of the project. However, it is far more difficult to trace the origins of ideas. This is particularly true when the investigator is part of an active research group in which ideas are constantly discussed. Therefore, while I feel safe, for instance, in claiming a large share of the credit for the initiation of the papers from 1 - 9 and from 13 onwards, I would hesitate to make such a claim for paper 12 particularly with respect to the repair inactivation hypothesis. This was borrowed from Haynes as indicated and was widely discussed by the group as a means of explaining DEB mutagenesis. I think, however, the consequences of resynthesis which led to papers 13 and 14 were elaborated by myself. It is pertinent here to acknowledge gratefully the tremendous debt I owe to Charlotte Auerbach in particular, but also to H.G. Kølmark, C.H. Clarke and John Corran with whom it was always possible to discuss the work and receive a sympathetic and constructive interaction.

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THE INFLUENCE OF TEMPERATURE ON THE ULTRAVIOLET INDUCED REVERTANT FREQUENCIES OF TWO AUXOTROPHS OF *NEUROSPORA CRASSA*

By

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With 2 Figures in the Text

Introduction

It is now generally accepted that the mutagenic action of ultraviolet light is, at least in part, an indirect process. Evidence for this belief has come from the finding that certain postirradiation conditions can modify the yield of mutations arising from a given dose of radiation. For example; photoreactivation, protein synthesis and ribonucleic acid synthesis have all been shown to be important in determining the yield of prototrophs when a tryptophan-requiring strain of *Escherichia coli* is treated with ultraviolet light (DOUDNEY and HAAS, 1959; 1960; WITKIN, 1956).

When the responses of two genes to a particular mutagen are compared, a marked mutagen specificity is sometimes encountered. It is often tempting to ascribe this specificity to the ability of the mutagen to act on one gene rather than the other. This may, indeed, be true, but it is also possible that the specificity has arisen at a later level. For example, two types of revertant may differ in that the first requires protein synthesis in order to become established while the other does not. Any experimental condition preventing protein synthesis will lower the frequency of the first type of revertant while not affecting the frequency of the second. Although the frequencies of potential revertants may be the same for the two types, the observed frequencies would indicate a mutagen specificity in favour of the second gene. The mutagenic treatment itself might interfere with a later stage in the mutagenic pathway, or the non-mutagenic conditions chosen for the experiment might have this effect. By changing the experimental conditions slightly, an alteration in the specificity might be found.

A situation has been discovered in the case of two *Neurospora* mutants which could be interpreted in either of these ways. The two mutants were caused to revert with ultraviolet light and it was found that an alteration in the temperature of the conidial suspension brought about a change in the proportions of the two types of revertant scored. The present report deals with these preliminary findings and discusses some possible explanations for the results.

Material and Methods

a) **The strain.** The strain used in these experiments was the di-auxotroph *K 3/17, ad-3A (38701); inos (37401)*. A description of the characteristics and history of this strain may be found in an earlier publication (KOLMARK and KILBEY 1962).

· **b) The media.** The media used all had as their basic component the minimal salts medium of Fries (BEADLE and TATUM 1945). For conidial production each litre of minimal medium was supplemented with 2.5 g Difco powdered yeast extract, 1.0 g casein hydrolysate (British Drug Houses Ltd.), 5.0 g malt extract, 15.0 g glycerol, 4 μg biotin, 100 mg adenine sulphate and 8 mg meso inositol.

Survival was scored on minimal medium containing 10.0 g glucose and 4 μg biotin per litre. This was supplemented with 40 mg adenine sulphate and 20 mg meso-inositol Adenine reversions were scored on the same medium without adenine sulphate and inositol reversions on survival medium without inositol.

All media were solidified by the addition of 15.0 g per litre of Difco Bacto agar.

c) Preparation and treatment of the conidia. Conidia were harvested from slants grown for 7—10 days at 25⁰ C. The suspension was filtered through cotton wool to remove fragments of mycelium and washed twice before mutagenic treatment. After washing, a conidial suspension containing 10^7 conidia per ml was prepared and 12 ml samples were exposed to ultraviolet light in small crystallising dishes. The samples were stirred magnetically throughout the exposure. After irradiation the conidia were plated as quickly as possible.

The ultraviolet source was a low pressure mercury vapour lamp (Hanovia No. 772/64). Approximately 85% of the output of this lamp is at the 2537 Å wavelength. No determination of the absolute output of this lamp has yet been made. The doses were varied by altering the time of exposure. In nearly all experiments the distance between the radiation source and the target was 10 cm.

The temperature of the conidial suspension during the experiments was controlled by means of an ice or water bath. Control to within 1⁰ C was easily achieved.

All experiments were carried out in dim yellow light to avoid any risk of photoreactivation.

d) All experiments were incubated at 25⁰ C for seven days in the dark before the colonies were counted.

Results

1. The effect of temperature on the response of the strain to ultraviolet

In all the earlier experiments with this strain, ultraviolet treatments have been given at room temperature (21⁰—23⁰ C). Under these conditions it has generally been found that two to three times as many inositol revertants occur as adenine revertants. At very low doses it has sometimes been found that the reverse is true and at present there is no satisfactory explanation for this. The response of the adenine mutant to differences in irradiation temperature is not affected when this occurs.

In the first experiments to study the effects of varying the irradiation temperature, a wide range of ultraviolet doses was used. These experiments all gave similar results and the results of one these of experiments are reproduced in Table 1 and Fig. 1.

Table 1. *The effect of temperature on the response of the strain K3/17 ad-3A 38701; inos 37401 to ultraviolet light*

Temper- ature	mins U.V.	0	1	2	4
2⁰ C	% survival . . .	100	97	78	47
	ad+ counted . . .	—	489	711	507
	ad+/10⁶ survivors		12.7	23.1	27.3
	inos+ counted . .	—	267	606	698
	inos+/10⁶ survivors		6.9	19.7	37.5
30⁰ C	% survival . . .	100	91	77	32
	ad+ counted . . .	—	357	338	181
	ad+/10⁶ survivors		10.0	11.0	16.4
	inos+ counted . .	—	294	625	457
	inos+/10⁶ survivors	—	8.2	20.4	41.0

The same dose of ultraviolet light administered at 2⁰ C produces more adenine revertants than when it is administered at 30⁰ C. In contrast, the frequency of

inositol reversions and the percentage of conidia surviving are not greatly influenced by these differences in the irradiation temperatures.

The inability of temperature to influence the frequency of inositol revertants to any great extent makes it possible to use the inositol revertant frequency as a standard with which to compare the response of the adenine mutant. The value of the ratio, $inos^+/ad^+$, is usually calculated. Because, the adenine mutant behaves as it does to changes in the irradiation temperature, the value of this ratio should increase as the temperatures rises.

The effects of intermediate temperatures in this range have also been ascertained. A dose of ultraviolet light giving about 70% survival was administered at the following temperatures: 0⁰, 10⁰, 15⁰, 25⁰, and 35⁰ C. The results of one such experiment are given in Fig. 2.

The slight variations in the frequency of inositol revertants do not represent a trend common to all experiments and they probably reflect slight variations in the dose of ultraviolet. These can be ignored when the values of the $inos^+/ad^+$ ratio for the different temperatures are compared. There is little change in the relative frequencies of inositol and adenine revertants at temperatures below 15⁰ C. Above this temperature, however, a sharp drop in adenine revertants relative to inositol revertants occurs as the temperature is raised.

Fig. 1. Treatment of the strain *K3/17 ad-3A 38701*; inos *37401* with ultraviolet light at 2⁰ C and 30⁰ C. 12 ml samples of conidial suspension were irradiated separately. Samples were allowed five minutes to attain the temperature required for irradiation. After exposure to ultraviolet plating was completed as quickly as possible
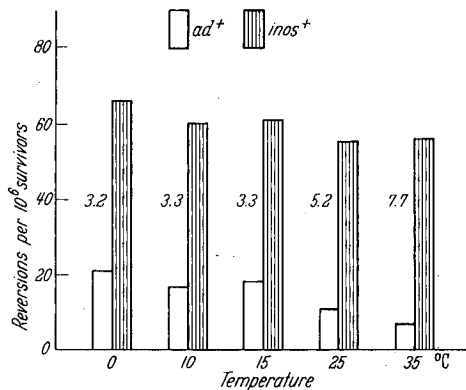
Fig. 2. The effect of various temperatures during irradiation on the frequency of adenine reversions relative to inositol reversions. The figures beside each column are the calculated values of the inos⁺/ad⁺ ratio at each temperature. The average survival in this experiment was 70%

The results presented thus far had all been obtained from experiments in which the temperature difference was applied only during the exposure of the conidia to ultraviolet light. Before and after exposure all the spores were kept at the same temperature. It was therefore important to determine whether the time at which the temperature difference is applied is critical and
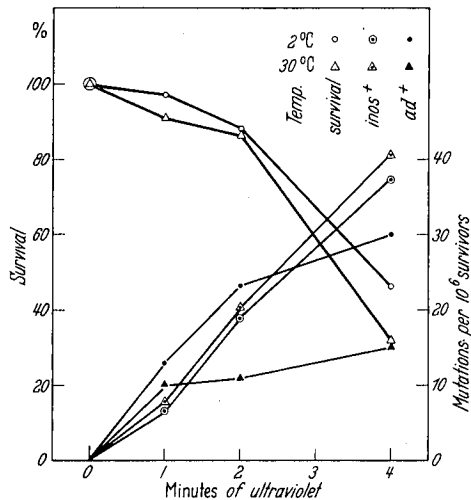
in the next section experiments will be described in which the temperature differences were applied before and after irradiation.

2. Pre- and post-Ultraviolet application of temperature differences

a) Pretreatment experiments. For these experiments the conidial suspension was divided into two parts. One of these was incubated at 30^0 C before exposure to ultraviolet, the other was kept at 0^0 C. Conidia from both samples were then irradiated at either 0^0 or 30^0 C before plating. The data from a typical experiment are given in Table 2. These results clearly demonstrate that pre-irradiation temperature differences do not influence the relative reversion frequencies of the adenine and inositol mutants.

b) Posttreatment experiments. A short intense ultra-violet dose of 30 seconds was used in these experiments. It was argued that if the loss of adenine reversions at higher temperatures is a result of temperature sensitive recovery or decay processes, these conditions would probably be the best for their detection: the proportion of the total possible recovery or decay occuring during irradiation would thereby be minimised.

Table 2. *The effect of incubation at high and low temperatures before ultraviolet exposure on the inos+/ad+ ratio*

Temperature of treatment ^{0} C		Colonies counted (Revertant type)		Ratio $\dfrac{\text{inositol}}{\text{adenine}}$
pre-U.V.	U.V.	adenine	inositol	
0	0	318	508	1.6
0	30	148	476	3.2
30	30	209	610	3.0
30	0	279	522	1.9

Ultraviolet dose = 2 minutes; Period of pretreatment = 45 minutes

The conidia were irradiated at either 0^0 C or 25^0 C and after irradiation samples irradiated at each temperature were quickly transferred to 0^0 C or 25^0 C and maintained at these temperatures for 30 minutes. Data from duplicate experiments are presented in Table 3.

Both the pre- and post-irradiation temperature experiments indicate that a difference in temperature can only influence the relative frequencies of the two types of revertant when applied during the exposure to U. V.

Table 3. *The effect of incubation at high and low temperatures after ultraviolet exposure on the inos+/ad+ ratio*

Experiment	U.V. temp. ^{0}C	Post-U.V. temp. ^{0}C	Reversions counted		inositol adenine
			adenine	inositol	
A B	0	0	109 132 } 120.5	184 188 } 186	1.5
A B	0	25	109 142 } 125.5	193 210 } 201.5	1.6
A B	25	0	78 70	— 236	3.4
A B	25	25	92 78 } 85	363 210 } 286.5	3.4

A and B are replicate experiments. Ultraviolet dose = 30 seconds; Period of posttreatment = 30 minutes.

Discussion

The foregoing results demonstrate the modification of a pattern of mutagen specificity by a non-mutagenic environmental factor. Temperature differences during irradiation specifically affect the frequency of adenine revertants; at the higher temperatures the yield of adenine revertants is reduced. As a result, the

value of the $inos^+/ad^+$ ratio increases with temperature. From the point of view that temperature does not affect all mutants similarly, this result resembles the inhibition of protein synthesis in *Escherichia coli* (WITKIN and THEIL, 1960). However, temperature differs from protein synthesis and some other factors capable of modifying the yield of ultraviolet-induced mutations in that it is not effective as a posttreatment. Alterations in conidial temperature are apparently only effective when applied during irradiation.

The reduction in the frequency of adenine revertants does not appear to be the result of killing revertants which are already established. Several ultraviolet-induced adenine revertants have been tested in reconstruction experiments and, although in a few cases there was a slightly greater killing at the higher temperatures, this cannot account quantitatively for the effect of temperature described here.

Any interpretations of these results must, at present, be speculative. They must take into account, first, the fact that temperature alterations are effective only during irradiation and, second, that the effect of temperature is specific for adenine reversions.

In the first place, the finding that temperature alterations are only effective during ultraviolet treatment might suggest that an initial step in ultraviolet mutagenesis is sensitive to temperature. It is possible, for instance, that fewer adenine reversions are induced at the higher temperature. The particular ultra-violet chromophore concerned with adenine reversion may behave differently at higher temperatures so that less of the absorbed energy is devoted to mutagenic processes.

Although fewer adenine reversions may be induced, it is also possible that, of those induced, some may be repaired before they can become established as revertants. This could result from the operation of a temperature sensitive, adenine reversion-specific repair process which depended in some way on the irradiation for its function. Photoreactivation can be discounted, for, although 11% of the output from the ultraviolet source is visible light, only very limited photoreactivation has been found in this strain (AUERBACH: Personal Communication). There is a possibility that ultraviolet light itself might bring about the reversal of certain types of lesion. It has been shown, for example, that trans-forming principle of *Haemophilus influenzae*, inactivated by large doses of ultra-violet at 2800 Å, can be reactivated by further irradiation at 2390 Å (SETLOW and SETLOW, 1962). This result is interpreted as a change in the position of the equili-brium between thymine monomers and dimers in the DNA molecules. A somewhat similar explanation might apply to *Neurospora* during treatment with ultraviolet light if an equilibrium were to exist between mutant and revertant forms of the *ad-3A* gene. Low temperature during irradiation may then change the position of the equilibrium in favour of the revertant form. A mechanism of this type could presumably, be specific to certain nucleotide configurations. If this were the basis for the specificity of the temperature effect, it should be found that other adenine-3A and inositol alleles exist, which behave in the opposite way from the two representatives studied here.

Dark repair processes, loss of mutagenic intermediates as a result of decay, and the rate at which potential revertants are fixed may also influence the ultimate

frequency of adenine revertants. If the operation of one or other of these processes was influenced by ultraviolet light to different extents at different temperatures, a change in the pattern of mutagen specificity, such as that described, might be observed. Although no evidence for dark repair or decay specific for adenine reversions has been obtained so far, these processes may take place too rapidly, or too slowly to be observed under the experimental conditions used.

Cells carrying a reversion must be able to form a visible clone of cells before the existence of the reversion can be recognised. The effect of ultraviolet on the synthetic capacities of the cell cannot be ignored. It is known, for instance, that cells of *Echerichia coli* treated with ultraviolet light are limited in their abilities to synthesise certain enzymes (BOWNE and ROGERS 1962). A similar limitation may be imposed on adenine revertants by irradiation at high temperature leading to a reduction in the frequency of revertants which can grow to a scorable size. It should be possible to detect sensitivity to the irradiation temperature occurring at a late stage in the mutagenic process: The conidial temperature during a preliminary dose of ultraviolet should influence the yield of adenine revertants from a second dose always given at the same temperature. These experiments are in progress.

Finally, the possibility remains that adenine revertants arise by more than one mutagenic pathway. Some of these pathways may be unable to function after irradiation at high temperatures. As a result, a different spectrum of revertant types might be expected at the different temperatures of irradiation. The genetical analyses necessary to demonstrate this have yet to be carried out.

These results suggest that the adenine and inositol mutants studied revert in different ways. Of the two, the process by which the adenine revertants appear would seem the more susceptable to environmental influence.

Summary

The frequency of ultraviolet-induced adenine reversions relative to inositol reversions in the strain *K3/17 ad-3A 38701: inos 37401* is influenced by temperature. Fewer adenine revertants are obtained from a given ultraviolet dose adminstered at 30^0 C than from the same dose administered at 2^0 C. The inositol reversion frequency and the percentage survival are not greatly affected by these differences in temperature. Temperature differences are only effective if a) applied during irradiation b) higher temperatures above 15^0 C are used. Possible explanations for these results are considered.

Literature

BEADLE, G. W., and E. L. TATUM: Methods of producing and detecting mutations concerned with nutritional requirements. Amer. J. Bot. **32**, 678—686 (1945).

BOWNE, S. W., and P. ROGERS: Ultraviolet light and enzyme synthesis. J. molec. Biol. **5**, 90—96 (1962).

DOUDNEY, C. O., and F. L. HAAS: Mutation induction and macromolecular synthesis in bacteria. Proc. nat. Acad. Sci. (Wash.) **45**, 709—722 (1959).

— — Some biochemical aspects of the postirradiation modification of ultraviolet-induced mutation frequency in bacteria. Genetics **45**, 1481—1502 (1960).

KØLMARK, H. G., and B. J. KILBEY: An investigation into the mutagenic after-effect of Butadiene diepoxide using *Neurospora crassa*. Z. Vererb.-Lehre **93**, 356—365 (1962).

SETLOW, R. B., and J. K. SETLOW: Evidence that ultraviolet induced thymine dimers cause biological damage. Proc. nat. Acad. Sci. (Wash.) **48**, 1250 (1962).

WITKIN, E. M.: Time, temperature and protein synthesis: A study of ultraviolet-induced mutation in bacteria. Cold Spr. Harb. Symp. quant. Biol. **21**, 123—140 (1956).

—, and E. C. THEIL: The effect of posttreatments with chloramphenicol on various ultra-violet-induced mutations in *Escherichia coli*. Proc. nat. Acad. Sci. (Wash.) **46**, 226—232 (1960).

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Specificity in the Photoreactivation
of Premutational Damage Induced
in *Neurospora crassa* by Ultraviolet

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Summary. Photoreactivation of the events which lead to ultraviolet-induced reversion of sixteen mutants has been studied. Reversion of one mutant, the inositol allele *37401*, is photoreactivated far less than lethal damage. Reversion of the remaining 15 mutants, alleles at three different loci, is photoreactivated to the same extent as lethal damage. Various explanations of this finding are considered and the implications of repair specificity in modifying mutagen specificity are discussed briefly.

Introduction

In an earlier report (KILBEY and DE SERRES, 1967) data were presented which showed that all classes of *ad–3 B* mutant induced in Neurospora were photoreactivated to a similar extent. Although these results point to a lack of specificity on the part of photoreactivation for the different classes of *ad–3 B* mutants, two important reservations must be made. First, the data relate to one locus only, and there is no reason for assuming that the damage at other loci is reactivated to the same degree. Second, the design of the experiments and the scale on which they were conducted could not exclude the possibility that a minority of sites within this locus mutate primarily in response to non-photoreactivable photoproducts.

The experiments which are described in the present paper were designed to investigate the possibility that some mutations result mainly from nonphotoreactivable damage. Sixteen mutants at three different genetic loci were induced to revert with ultraviolet and the photoreactivability of the damage causing the reversion was tested. Since the number of different events causing a particular allele to revert is probably very small, this approach was considered more likely to be successful than the extension of forward mutation experiments.

Material and Methods

Mutants tested. Details of the mutants used in these experiments, their mutagenic origin and source are given in Table 1. All these mutants revert in response to ultraviolet but the response of some, notably *5–8–70*, *5–8–76*, and *5–8–430* is rather low ($1–5 \times 10^{-6}$ survivors).

Media. All the media used were based on Fries Minimal Salts Solution (BEADLE and TATUM, 1945). For conidial production single colony isolates were grown in flasks containing Fries minimal salts solution supplemented with 1.5% agar, 1% glucose, 1.5% glycerol, adenine sulphate (100 µg/ml) and inositol (20 µg/ml). For Kølmark's strain, *K3/17*, the

Table 1. *Mutants used and their derivation*

Allele No.	Locus	Induced by	Origin
2–017–0052	ad-3A	NA[a]	DE SERRES
2–017–0077	ad-3A	NA	DE SERRES
38701	ad–3A	UV	Fungal Genetics Stock Center
5–8–4	ad-3B	UV	KILBEY
5–8–13	ad-3B	UV	KILBEY
5–8–70	ad-3B	UV	KILBEY
5–8–76	ad-3B	UV	KILBEY
5–8–113	ad-3B	UV	KILBEY
5–8–115	ad-3B	UV	KILBEY
5–8–167	ad-3B	UV	KILBEY
5–8–342	ad-3B	UV	KILBEY
5–8–410	ad-3B	UV	KILBEY
5–8–430	ad-3B	UV	KILBEY
37401	inos	UV	Fungal Genetics Stock Center
37102	inos	UV	
JH 2626	inos	mustard	

Combined alleles tested

inos 37401 ad-3A 38701 cot (R 155)	ALLISON
inos 37401 ad-3A 38701 (K 3/17)	KØLMARK
inos JH 2626 ad-3A 38701 cot (R 155)	ALLISON

[a] NA = Nitrous acid

methods described earlier were followed (KØLMARK and KILBEY, 1962). Cultures were in all cases incubated at 25° C for 7—10 days before harvesting the conidia.

Plating media were of necessity varied depending on the strain under test. Fries minimal medium was appropriately supplemented with adenine sulphate (40 μg/ml) and/or inositol (20 μg/ml), the carbon source used also depended upon the strain being tested. Colonial mutants (*cot* and *K 3/17*) were tested on medium containing 1% glucose. Non-colonial strains were tested on medium containing 0.05% glucose 0.05% fructose and 1.0% sorbose.

Preparation of conidial suspensions and their treatments

Conidia were harvested by shaking them off into about 30 ml distilled water. The suspension was filtered twice through cotton and washed twice with centrifugation before resuspending the pellet in 20 ml sterile water. The number of conidia per ml was adjusted to 2×10^7/ml and 30 ml aliquots were exposed to ultraviolet in a petri dish. During irradiation the suspension was stirred continuously. The ultraviolet source used was a Phillips T.U.V. 15 watt, mercury vapour lamp, the major output of which is at 253 mμ. The incident energy was monitored using a "Jagger"-type dose rate meter (JAGGER, 1961). The temperature during irradiation was approximately 23° C. Following exposure to ultraviolet, each sample was transferred to an ice bath until plating or photoreactivation. Photoreactivating light was provided by a medium pressure mercury vapour arc enclosed in a glass envelope. Samples were exposed to this source for 30 minutes at 30° C; double the time required for maximum photoreactivation. No inactivation of the conidia was observed under these conditions. During the whole experiment room-light was supplied from Osram "Yellow" fluorescent tubes, thus avoiding uncontrolled photoreactivation.

The treated and control samples of conidia were implated and incubated for seven days at 25° C before survivors and mutations were scored.

Results

1. Response of the alleles ad–3A 38701 and inos 37401 to UV and photoreactivation. The first experiment to be carried out used the strain *K 3/17* which requires both adenine and inositol for growth. Similar tests were also made on a *cot* derivative kindly provided by Mr. Michael Allison. This latter strain was synthesised
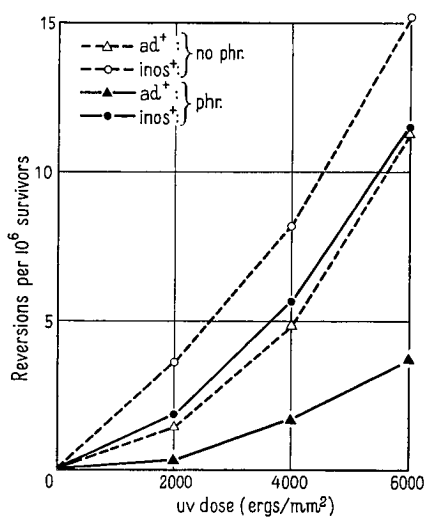
Table 2. *Response of ad-3A 38701 inos 37401 cot to U.V. and photoreactivation*

Conditions (U.V. dose in ergs/mm²)	Survival		ad^+		$inos^+$		$inos^+/ad^+$
	Counts	%	Counts	m/10⁶ surv.	Counts	m/10⁶ surv.	
Control	1,830	100	3	0.08	6	0.016	—
2,000 light	1,564	87.5	7	0.22	56	1.8	8.0
2,000 dark	1,749	96.4	51	1.45	125	3.6	2.4
4,000 light	1,629	90.0	54	1.65	175	5.6	3.2
4,000 dark	1,699	96.5	168	4.80	288	8.2	1.7
6,000 light	1,732	95.0	129	3.7	403	11.5	3.1
6,000 dark	1,453	79.0	323	11.3	428	15.0	1.3

completely independently of *K3/17* and, therefore, the same two alleles were tested against a totally different genetic background. Seven experiments were performed, three with *K3/17* and four with the *cot* derivative. In spite of their different histories the two strains behaved in a similar way. Because of the good agreement between the results in these experiments, the results of one typical case are shown in Table 2, Fig. 1.

In all experiments the value of the ratio of inositol/adenine reversions rose following photoreactivation. Compared to the corresponding values without photoreactivation the increase was generally twofold although sometimes in the lower U.V.-dose ranges it was higher than this. The change in the value of the ratio occurs because photoreactivation

Fig. 1. Response of inos 37401 and ad 38701 in the *cot* derivative to U.V. and photoreactivation

of adenine reversions is marked whereas photoreactivation of inositol reversions is only weak.

Photoreactivation of mutational and lethal U.V. damage can be conveniently compared by plotting mutation frequency against survival. When repair of mutational and lethal damage occurs to the same extent the points obtained with maximum photoreactivation fall on the same curve as the points obtained with no photoreactivation. If repair of mutational and lethal damage does not occur to the same degree the curves obtained with and without photoreactivation

diverge. Data from both *K 3/17* and the *cot* derivative have been plotted in this way and an example of the results is shown in Fig. 2. The data clearly show that whereas adenine reversions are photoreactivated to the same extent as lethal damage, inositol reversions are repaired far less than lethal damage. In fact in all these experiments the photoreactivable sector[1] for survival and adenine reversions is approximately 0.5 while that for inositol reversions is about 0.4 at mutation frequencies of about 2×10^{-6} survivors but falls rapidly to <0.2 at mutation frequencies of 8×10^{-6} survivors and above.
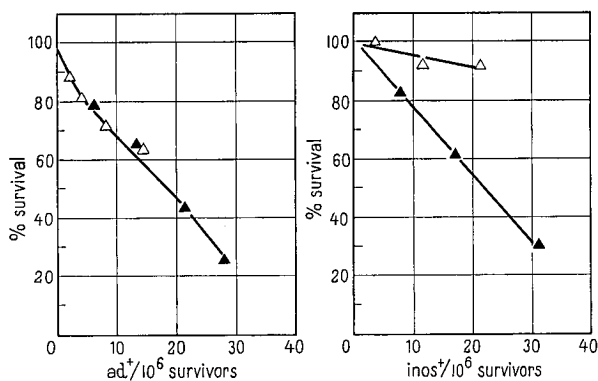

Fig. 2. Comparison between the photoreactivation of lethal damage and mutational damage causing the reversion of the alleles inos 37401 and ad 38701. Open triangles: with photoreactivation. Filled trangles: no photoreactivation

In addition to tests with these mutants in the diauxotrophic strains they were also tested individually in separate monoauxotrophic strains. In both cases the responses were the same as in the diauxotrophic strains. The possibility is thus excluded that the earlier results were somehow dependent on the use of the diauxotrophic strains.

2. *Tests with other alleles.* Once the pattern of response of the *ad–3A 38701* and *inos 37401* alleles had been established it became necessary to determine how typical their responses are of other alleles at these loci. Two other inositol alleles were tested together with two other *ad–3A* mutants. In addition a series of ten U.V.-revertible *ad–3B* alleles were also tested. In every instance, in spite of wide variations in U.V. response, each mutant was able to photoreactivate mutational damage leading to reversion to a similar extent as lethal damage. In one other case two mutants were tested in combination, *ad–3A 38701* and *inos J.H.2626.* Here, too, the response of the alleles did not change in the diauxotrophic strain.

Discussion

Sixteen mutants at three different loci have been induced to revert using U.V. In each case the photoreactivability of the events leading to reversion has

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[1] The photoreactivable sector, or the photoreactivability of the conidia, is given by the term (1-Dose Reduction Factor). The dose reduction factor is expressed by the ratio of

$$\frac{\text{dose required for given biological effect in absence of repair}}{\text{dose required to give the same effect with repair operating.}}$$

For discussion of the photoreactivable sector see DULBECCO (1955).

been tested. Fifteen of the sixteen are able to photoreactivate mutational damage to the same extent as lethal damage. One mutant at the inositol locus (*37401*) shows considerably less photoreactivation of mutational damage than lethal damage. This behaviour is not typical of mutants at this locus since two other inositol alleles tested show normal photoreactivation of premutational damage.

It is possible to exclude several alternative explanations of these results: It is well-known that inositol-requiring mutants are unable to survive for many hours on medium lacking inositol (STRAUSS, 1958). For this reason is it impossible to determine accurately the survival levels on the plates used for scoring inositol reversions simply by plating diluted conidial suspensions on medium containing inositol. The possibility existed that, on the mutation plates which lack inositol, more photoreactivation of lethality occured than was estimated from the survival plates. An under-estimate of survival would lead to an over-estimate of inositol reversion frequency thus minimising the apparent effects of photoreactivation on inositol reversions. This explanation is already made unlikely by the observation that other inositol alleles, while still subject to inositolless death, never-the-less respond normally to photoreactivation. Further evidence against it was obtained from direct determinations of the survival of conidia under the conditions used for scoring inositol reversions. The mutation experiment was carried out using agar soft enough to permit the removal of 0.1 ml samples from the mutation plates for dilution and plating on medium containing inositol, but hard enough to permit normal scoring of mutations. Experiments of this type showed that photoreactivation of survival on the mutation plates occurred to the extent predicted by the survival plates.

The exceptional allele, *inos 37401*, behaves in this way whether it is tested alone or in combination with *ad–3A 38701*. In two such combinations, made completely independently of each other, similar responses of the two alleles were found. Although these results do not eliminate the possibility that other genetic factors cause the non-photoreactivability of the inositol reversions they make this interpretation unlikely.

A further explanation arises from the work of WITKIN who has studied U.V. induced reversion in bacteria. She found that mutations of the suppressor type induced by U.V. in strains possessing host-cell reactivation ability were not photoreactivable by the direct, enzymatic photoreactivating mechanism (WITKIN, 1966).

An interpretation of this type could also apply here if it was found that the majority of inositol reversions result from suppressor mutations. The published results of other investigators (GILES, 1951) indicate that suppressors of *inos 37401* are too infrequent to account for the present result whether they are photoreactivable or not.

Two further explanations are attractive but neither can be proved. First, the majority of inositol *37401* reversions may result from photoproducts which are intrinsically non-photoreactivable. Second, additional factors may determine whether or not normally photoreactivable lesions are available for photoreactivation. The data referred to in the Introduction relating to the forward mutation experiments at the *ad–3B* locus appear to favour the second of these possibilities: Since the photoreactivable and non-photoreactivable damage generate identical

spectra of mutant-types it is simplest to suppose that the photoproducts themselves are of the same type in both cases. Non-photoreactivability may then be determined, for example, by the accessibility of the damage to the photoreactivating enzyme. However, again it must be pointed out that if a few sites exist in the *ad–3 B* locus which mutate by non-photoreactivable lesions they could have escaped notice in the earlier experiments. It is possibly of significance that in the present sample only one allele out of sixteen tested failed to exhibit the usual level of photoreactivation. One possible approach to this problem is to study the action spectra for the induction of *ad–38701* and *inos 37401* reversions. If different photoproducts are involved the action spectra might provide some evidence of this.

These experiments deal with a specific, controllable repair system which is known to act most effectively immediately after irradiation. The experiments have shown that different types of premutational damage can be repaired to different extents thus causing an alteration in the apparent mutagen specificity. From a quantitative point of view, photorepair is not an ideal system with which to demonstrate this: the percentage of photoreactivation of adenine reversions, except at very low U.V. doses, is only 60%—70%. As a result the ratio of $inos^+/ad^+$ reversions can only change by a factor 2.5 to 3 times. In practice the limited photoreactivation of inositol reversions reduced this still further. With specific repair of a more efficient type greater changes in mutagen specificity can be expected.

The results presented here have a bearing on some earlier findings reported in 1963 (KILBEY, 1963). It was found that temperature differences during U.V. irradiation altered the proportion of inositol to adenine reversions in the K3/17 strain. The effect was virtually restricted to an influence on the adenine allele. One possibility, dismissed at the time, is that photoreactivation promoted by visible light from the U.V. source itself is responsible. Lower temperatures during irradiation would inhibit this repair resulting in higher mutation frequencies; higher temperatures would enhance repair lowering mutation frequency. With the finding that specificity of photorepair existed in this strain interest in the temperature effect was renewed. However, it was soon demonstrated that the light from the U.V. tube was unable to produce the photoreactivation necessary to account for the temperature effect. The mechanism of this effect therefore remains unelucidated.

References

BEADLE, G. W., and E. L. TATUM: Methods of producing and detecting mutations concerned with nutritional requirements. Amer. J. Bot. **32**, 678—686 (1945).

DULBECCO, R.: Photoreactivation. In: Radiation biology, p. 455 et seq. vol. II (ed. A. HOLLAENDER). New York: McGraw Hill Book Co. 1955.

GILES, N. H.: Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. Cold. Spr. Harb. Symp. quant. Biol. **16**, 283 (1951).

JAGGER, J.: A small and inexpensive U.V. dose-rate meter useful in biological experiments. Radiat. Res. **14**, 394—403 (1961).

KILBEY, B. J.: The influence of temperature on the ultraviolet induced revertant frequencies of two auxotrophs of *Neurospora crassa*. Z. Vererbungsl. **94**, 385—391 (1963).

—, and F. J. DE SERRES: Quantitative and qualitative aspects of photoreactivation of premutational ultraviolet damage at the *ad–3* loci of *Neurospora crassa*. Mutat. Res. **4**, 21—29 (1967).

KØLMARK, H. G., and B. J. KILBEY: An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa*. Z. Vererbungsl. **93**, 356—365 (1962).

STRAUSS, B.: Cell death and "unbalanced growth" in *Neurospora crassa*. J. gen. Microbiol. **18**, 658—669 (1958).

WITKIN, E.: Radiation-induced mutations and their repair. Science **152**, 1345—1353 (1966).

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ALLELE-SPECIFIC RESPONSES TO FACTORS THAT MODIFY U.V. MUTAGENESIS

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We may define mutagen specificity as either the differential response of two or more genes to a particular mutagen, or the array of responses of a single gene to a number of different mutagens. The phenomenon may be illustrated by reference to some of the early results of Demerec (1953) with *Escherichia coli*. Table I shows the response of a diauxotrophic strain, leucine-less and phenylalanine-less, to three mutagens: manganous chloride, u.v., and β-propiolactone. The two mutants reverted at very different frequencies with each of the mutagens. Manganous chloride and β-propiolactone produced more leucine reversions than phenylalanine reversions; u.v. produced more phenylalanine reversions than leucine reversions. Comparison of the mutagens in terms of their effectiveness in producing a given mutational change is of limited value since nothing is known of the dose actually reaching the genes.

TABLE I

FREQUENCIES OF REVERSE BIOCHEMICAL MUTATIONS IN A DOUBLY MUTANT STRAIN OF *Escherichia coli* AFTER TREATMENT WITH THREE DIFFERENT MUTAGENS (from Demerec, 1953)

	Mutations per 10^8 induced by		
	$MnCl_2$	U.v.	Lactone
Leucine-less	594	57	28
Phenylalanine-less	11	100	3·3

From the example cited one might form the impression that the response of a particular mutant to a particular mutagen is invariable and depends solely on the mutant and mutagen concerned. This idea has been adopted as the basis for using specific reversibility tests in the analysis of the molecular basis of mutation. From their response to a number of selected mutagens, a high proportion of the *rII* mutant sites in bacteriophage T4 have been unambiguously assigned base pairs (Champe and Benzer, 1962). In cellular organisms, unambiguous assignment is more difficult but it is still often

possible to place a mutant in a particular class of genetic alteration, e.g. frame shift or base substitution (de Serres, 1964). This difficulty arises, without doubt, from the modification of the primary mutational response by other cellular events. It has become very clear during the last few years that the response of a particular mutant to a mutagen can be drastically modified by ancillary treatments. Most of this work has been done with bacteria, using u.v., but examples are also on record for chemically induced mutations (Corran, 1968). The molecular basis for these phenomena has still to be elucidated in most cases. Repair activity is often assumed to be responsible, but it must be admitted that independent evidence for the involvement of repair is often lacking. However, whatever cellular events are responsible, the fact remains that the response of a gene to a mutagen is not fixed but is subject to a number of modifying factors.

Several cases are now known in which modifying factors are specific for a particular mutation. This is of great importance for the practical aspects of mutagen specificity, since it opens up the way for a certain amount of directed mutation treatment. The purpose of this contribution is to present an instance in which the response of two mutants to a particular mutagen can be modified by factors which are mutant specific. By means of these factors, which are themselves non-mutagenic, the specificity of the mutagen, u.v., can be considerably increased.

EXPERIMENTAL MATERIAL

Since the same mutational system forms the basis both for this discussion and the next paper, by Professor Auerbach, I shall describe it in detail here. Most of the experiments have been performed on the strain of *Neurospora* first isolated by Kølmark and given the isolation number K3/17. This strain carries the two mutants *ad-3A* 38701, *inos* 37401 which determine requirements for adenine and inositol, respectively. In addition, several colonial mutations have been accumulated which produce a small compact colony in the absence of those growth-limiting substances that are usually obligatory for the colonial growth of wild-type *Neurospora*. The details of the culture conditions have been described elsewhere (Kølmark and Kilbey, 1962). In addition to the K3/17 strain, another, synthesized independently by M. Allison, has also been used. It carries the same two auxotrophic mutations together with the mutation *cot* (colonial temperature-sensitive). At temperatures below 29°C this strain grows as freely as wild-type. Above 29° growth is restricted and small colonies are formed.

Conidia were harvested, washed and suspended in water for mutagenic treatment. The cell density normally used was 1 to 3×10^7 cells/ml. The

suspension was either irradiated (for details see Kilbey, 1967) or treated with a chemical. In the latter case, treatment was terminated by centrifugation and washing, or by membrane filtration and washing.

By using suitable media, both types of reversion can be scored in the same experiment; adenine reversions may be selected on minimal medium plus inositol, inositol reversions on minimal medium plus adenine. Survival is scored on minimal medium plus both supplements. The plates were incubated for seven to ten days at 30° before scoring.

Mutagen specificity is conveniently expressed as the ratio of inositol to adenine reversions (i/a). Since the survival estimate at each dose is the same for both types of revertant, it can be ignored and the ratio can be calculated from the absolute numbers of revertants scored.

EFFECTS OF DOSE AND GENETIC BACKGROUND

In a paper dealing with the effects of several mutagens in K3/17, Wester-gaard (1957) reported that u.v. induces twice as many inositol as adenine reversions. The data in Fig. 1 show that this is only partly true. When
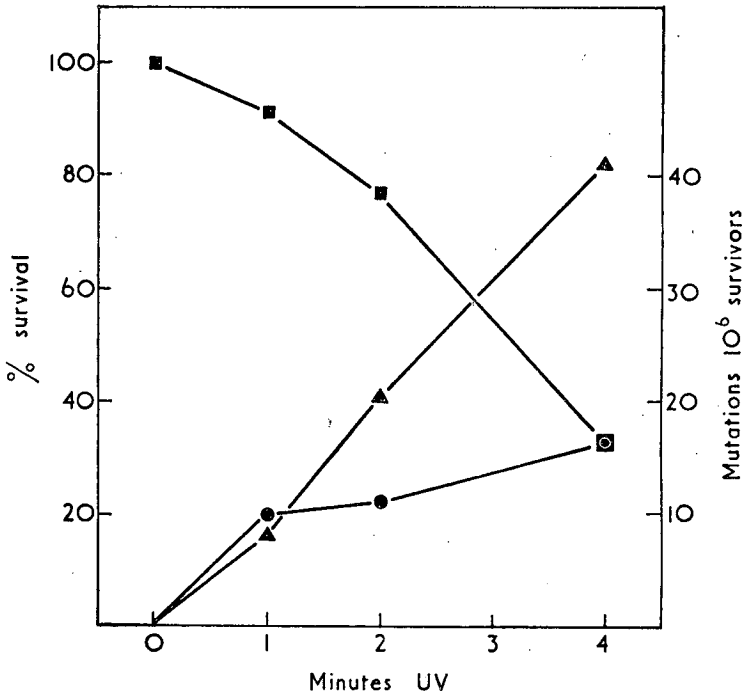

FIG. 1. The response of K3/17 to u.v. Squares: survival; triangles: inositol reversions; circles: adenine reversions.

K3/17 is treated with a series of u.v. doses the i/a ratio changes with dose. At low doses it is one or less than one, as in the experiment shown here. At higher doses it increases to between two and three. The increase in the i/a ratio with dose leads to divergence of the two dose–effect curves. This has also been observed by other workers with this strain (Kølmark, 1953; Auerbach and Ramsay, 1968).

Several explanations may be put forward to account for the increase in i/a ratio with dose. One is that inositol revertants might be selected because they are more resistant to u.v. than adenine revertants. Auerbach and
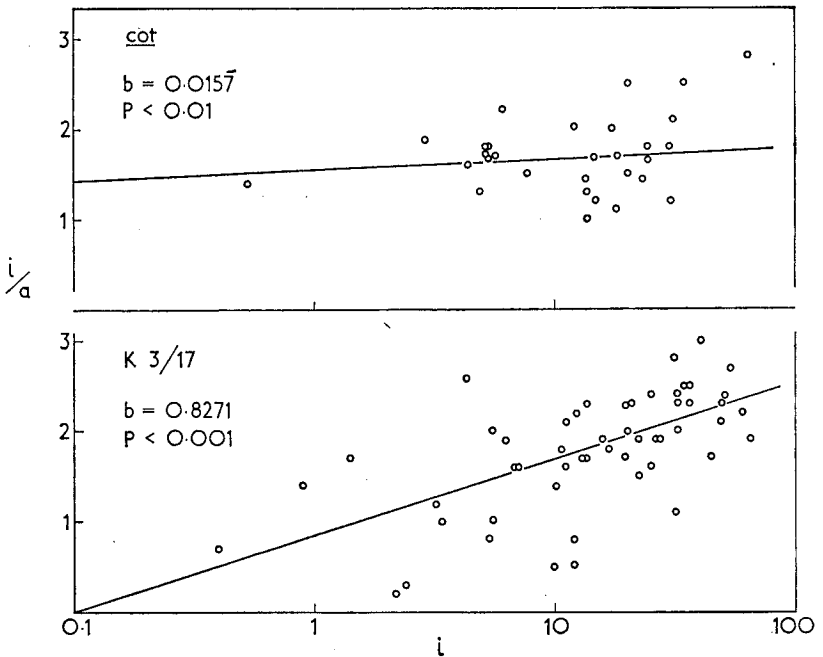
FIG. 2. The effect of genetic background on the variation of i/a with dose of u.v. measured in terms of inositol reversions (i). b: regression coefficient.

Ramsay (1968) ruled out this possibility by using mixtures of revertant and non-revertant cells in reconstruction experiments. If anything, inositol revertants proved to be slightly less resistant to u.v. than adenine revertants. Second, inositol revertants might be more sensitive than adenine revertants to the suppressive effects of the background of non-revertant cells on the mutation plates. The effect should be most noticeable at low doses and should decrease as more of the background cells are killed. This was excluded by plating u.v.–treated suspensions at different dilutions. Dilution

did not alter the frequency of revertants scored. The final possibility considered by Auerbach and Ramsay was that, in the multinucleate conidia of strain K3/17, inositol reversions were less dominant than adenine reversions. This effect should be more acute at low than at high u.v. doses, when a reduction in the number of viable nuclei per conidium should occur. In fact, most of the reversions tested from low doses were heterokaryotic. Trivial explanations of this type do not appear to be responsible for the divergent mutation induction curves.

Divergence of the mutation induction curves, and the consequent rise in i/a ratio with dose, is an expression of the fact that the two curves differ in their kinetics. The genetic differences between the K3/17 and *cot* strains have been found to be important in influencing the kinetics of mutation induction. Although the mutational responses of the strains are superficially similar, the *cot* strain differs from K3/17 in that the i/a ratio shows little change with dose, i.e. the kinetics of mutation induction are rather similar for the two mutants in the *cot* background. When the results from a number of experiments are pooled for each strain and compared, the change in behaviour becomes obvious (Fig. 2). In K3/17 there is a clear positive regression of i/a upon dose as measured in inositol reversions. The regression coefficient is 0.8271, the standard error being 0.1504 ($t_{51} = 5.501$, $P < 0.001$). In contrast, the results from the *cot* derivative show only slight positive regression, the regression coefficient being 0.0157 with S.E. 0.0055 ($t_{28} = 2.833$, $P < 0.01$).

With the K3/17 strain, divergence was invariably found in our experiments; in the *cot* strain, however, some experiments showed slight convergence. Malling and co-workers (1959) presented data for the K3/17 strain which are at variance with the pattern of response described above. In their experiments convergence was noted. It is hard to give reasons for this discrepancy since nothing is known of differences in genetic background between the strains, or of slight variations in technique.

These results show clearly that the response of the two mutants is not fixed but can be modified, albeit only slightly, by altering either the u.v. dose or the genetic environment in which they are treated.

THE EFFECT OF TEMPERATURE

I would now like to turn to the effect of temperature on the response of the adenine and inositol mutants to u.v. In 1962, while working with the K3/17 strain, I discovered that the temperature of the conidial suspension during irradiation played an important role in determining the u.v.

response of the two mutants (Kilbey, 1963). Fig. 3 shows the result of a typical experiment which demonstrates this effect. At 30°C the response of the strain is as described earlier. The i/a ratios gradually increase with dose from 0·82 to 2·5. At 2° the ratios still increase with dose but they are all approximately half the corresponding values at 30°. This happens because, at the lower temperature, the frequency of adenine reversions is
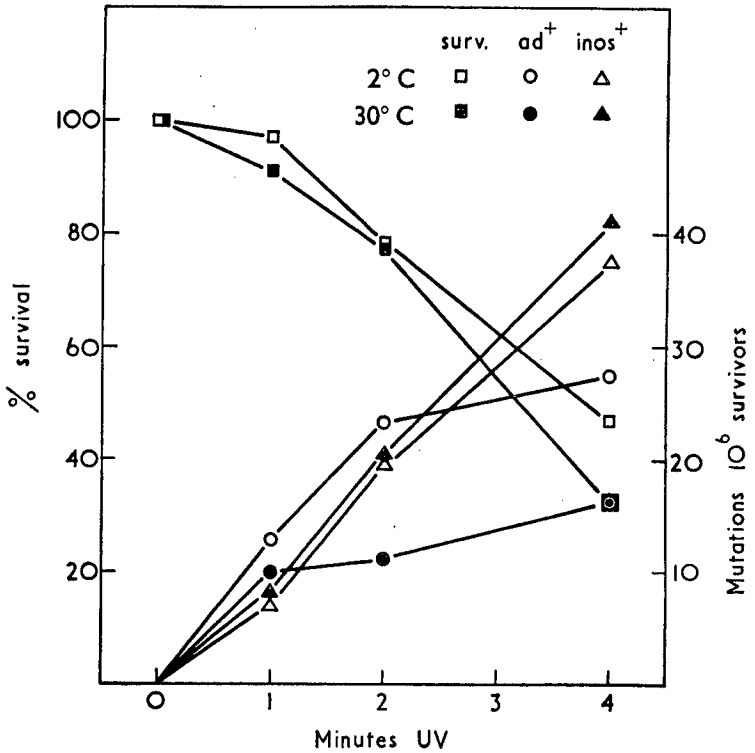
FIG. 3. The effect of temperature on the response of K3/17 to u.v.

increased specifically. There is little or no effect on either survival or inositol reversions. Reconstruction experiments showed that the effect could not be attributed to a greater u.v. sensitivity of adenine reversions at high temperatures.

It seems improbable that the temperature difference used in these experiments would greatly influence the photochemical events concerned in the induction of adenine reversions. In the first place the temperature coefficients for photochemical reactions are small, and secondly the coefficient

would have to be negative. The more probable interpretation seemed to be that, at low temperature, slowing of repair brought about an increase in adenine revertants. If this were so, post-irradiation temperature differences might also be expected to modify mutation frequency. Table II shows the results of one of the experiments in which this was tested. U.v. exposures were given at high intensity for a short time to minimize repair during irradiation. It can be seen that temperature is only effective when applied during the u.v. treatment, making repair retardation at low temperatures an unlikely explanation. It still seemed possible that photoreactivation using visible light from the u.v. tube might be responsible but at that time no evidence had been obtained that it occurred in K3/17 or that it possessed the required specificity for adenine reversions. Subsequently these tests were made and photorepair was shown not to be responsible for the temperature effect.

TABLE II

THE EFFECT OF INCUBATION AT HIGH AND LOW TEMPERATURES AFTER ULTRAVIOLET EXPOSURE ON THE RATIO OF INOSITOL TO ADENINE REVERSIONS

Expt	U.v. temp. (°C)	Post-u.v. temp. (°C)	Reversions counted Adenine	Reversions counted Inositol	Inositol/ adenine
A B	0	0	109 132 } 120·5	184 188 } 186	1·5
A B	0	25	109 142 } 125·5	193 210 } 201·5	1·6
A B	25	0	78 70	— 236	3·4
A B	25	25	92 78 } 85	363 210 } 286·5	3·4

A and B are replicate experiments. Ultraviolet dose: 30 seconds; period of post-treatment: 30 minutes.

An alternative explanation for these results is that u.v., at the lower temperature, damages a cellular process, e.g. repair, which only becomes involved in the mutational pathway at a later stage. This is difficult to test at present.

For another mutagen it was clear that temperature acted at a later stage. Auerbach and Ramsay (1967) showed that temperature is important in determining the mutagen specificity of nitrous acid in K3/17. Here the temperature at which plated conidia were incubated was important. This post-treatment effect has two basic similarities to the u.v. experiments in that (a) the main effect was on the adenine reversions, (b) the higher temperature (32°) produced fewer reversions than the lower one (25°).

PHOTOREPAIR

Photorepair is the only repair system in fungi for which definite bio-chemical evidence exists at present. The enzyme has been extracted from *Neurospora* conidia and can reactivate u.v.-inactivated transforming principle *in vitro* (Terry, Kilbey and Branch-Howe, 1967). In earlier experiments Kilbey and de Serres (1967) were able to show that photorepair is apparently unspecific: all classes of u.v.-induced *ad-3B* mutants were diminished when photorepair was allowed to function. In view of this it was all the more surprising to find that in both the K3/17 and the *cot* strains, photorepair is specific for adenine reversions (Kilbey, 1967). Fig. 4 shows
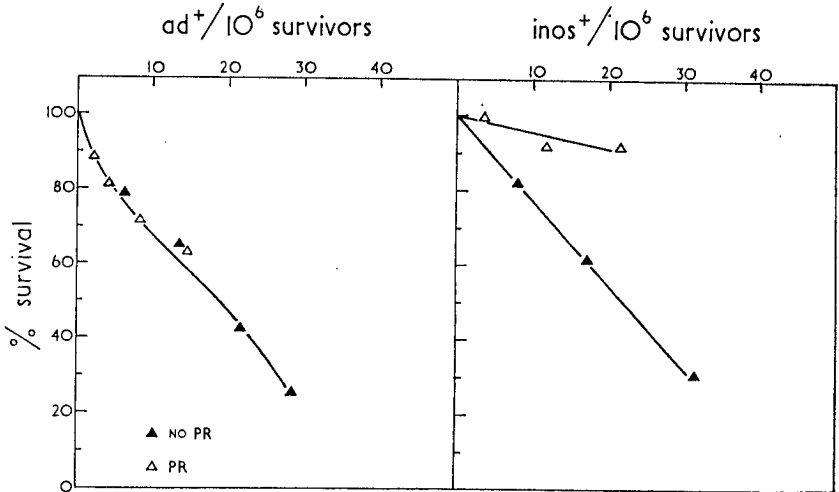
FIG. 4. Differential photoreactivation of mutation and survival in the *cot* derivative. PR: photorepair.

the two types of mutation plotted versus survival, with and without photorepair. The same dose reduction factor applies to both adenine reversions and survival; thus the points fall on one curve whether they come from photoreactivated samples or not. Inositol reversions are only slightly responsive to photorepair and, when plotted against survival, points from the light and dark samples fall on different curves.

The behaviour of the inositol mutant appears to be unique. Two other *inos* alleles were tested together with two *ad-3A* alleles and ten *ad-3B* alleles. All of these were photoreactivated to the same extent as *ad-3A* 38701 although their u.v. responses differed considerably.

The finding that photorepair operated specifically upon adenine rever-sions in this system led to a re-examination of the possibility that photo-reactivation, caused by the visible light emitted by our u.v. source, was

responsible for the temperature effect. Accordingly attempts were made to use the u.v. lamp as a photoreactivating source after first filtering the radiation through window glass to remove u.v. No photoreactivation was observed and it seems, therefore, that this cannot be the explanation for the temperature effect.

BUFFER DURING IRRADIATION

During experiments in which the pH dependence of a number of mutagenic treatments was being studied, Dr Allison in our laboratory found that the ratio i/a in the *cot* derivative could be enhanced by irradiating the conidia in phosphate buffer instead of water. In contrast to temperature and photoreactivation, which modify adenine reversion frequency, buffer acts by increasing inositol reversions.

Allison has shown (unpublished) that buffer acts only during irradiation and that the effect is the same at pH 6 and pH 7. The relative proportions of the sodium and potassium ions do not seem to be important and, at present, he favours the view that the phosphate ions are responsible for the effect.

Fig. 5 shows the effects of buffer and photorepair on the i/a ratio in the *cot* derivative. The line marked "no specificity" corresponds to an i/a ratio of unity; departures from this line indicate specificity. It is clear that photorepair and buffer both displace the curves towards the inositol revertant axis. In other words, both treatments enhance the specificity of u.v. The two treatments are approximately additive in their effects. This is to be expected since one enhances the i/a ratio by acting on adenine reversions and the other does it by enhancing inositol reversions.

Irradiation in buffer also modifies the kinetics of mutant induction in the *cot* strain. As shown earlier, treatment in water produces little or no divergence in this strain. In buffer there is a slight convergence. Evidence for this is apparent from the bends in the buffer curves in Fig. 5 and a more precise estimate of the effect can be obtained if data from several experiments are combined and plotted in the same way as in Fig. 2. A slight negative regression is obtained.

DISCUSSION

The effects of dose, genetic background, temperature, photorepair and buffer demonstrate the ability of experimental conditions to modify the mutational specificity of a particular mutagen in a particular mutational

Adenine revertants $/ 10^6$ survivors
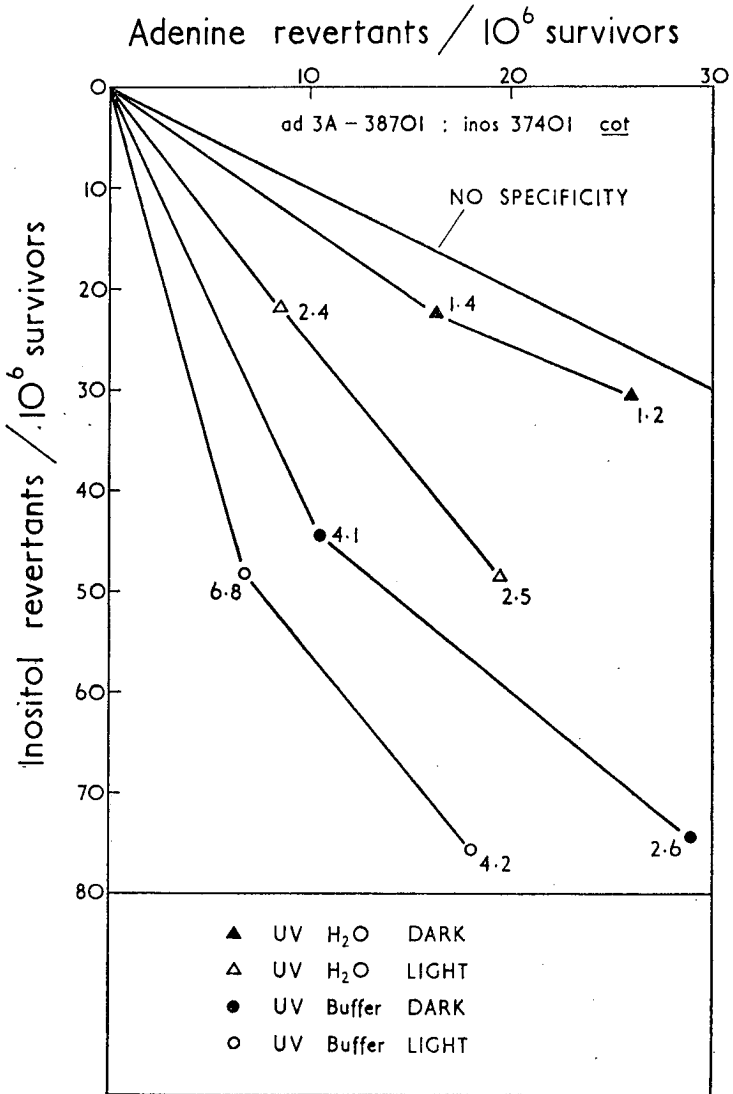
FIG. 5. The effects of photoreactivation and irradiation in buffer on the mutagen specificity of u.v. in the *cot* derivative.

system. Other instances have been recorded in which modifications have occurred. These are usually concerned with the effects of genetic background on mutagen specificity (Glover, 1956; Chang, Lennox and Tuveson, 1968; Witkin and Theil, 1960) but examples have also been reported in which a class of mutant has responded specifically to an ancillary treatment,

e.g. plating medium (Bridges, Dennis and Munson, 1967; Zetterberg, 1962).

Very little is so far known of the mechanisms which underly such effects. The kinetic differences between the two mutation induction curves in K3/17 do not appear to be the result of trivial causes, and we are left with the idea, stressed by Auerbach and Ramsay (1968), that the curves include components which represent treatment effects on cellular processes which are important in mutation. The altered response in the *cot* strain demonstrates that these cellular events may be influenced by the genetic background.

Since low temperature and buffer act only during irradiation it is tempting to suggest that they act by permitting the induction of more of one type of reversion by u.v. However, the possibility should not be discounted that they may permit the differential damage of some other cellular components which impinge on the mutational pathway at a later stage.

We can be more precise for photorepair. Here the specificity arises from events which occur after u.v. treatment is complete. The obvious explanation for this specificity is that the photoproducts giving inositol revertants are mainly non-photoreactivable. Although this may be true, there is no reason to believe that photoreactivable and non-photoreactivable damages are different photochemically. They are both equivalent in the types of mutant they generate in *Neurospora* (Kilbey and de Serres, 1967), and bacteriophage (Drake, 1966). However, rare instances of mutants produced by intrinsically non-photoreactivable damage would possibly have been missed in these experiments. The *inos* 37401 allele was, surprisingly, the first used to demonstrate that premutational damage is photoreparable in *Neurospora* (Brown, 1951). Although killing was high in these experiments and selective killing cannot be excluded, photoreactivation of mutation does seem to have occurred. Since the strain used was microconidiating and genetically quite different from those used in the present studies, the possibility remains that the photoreactivability of this allele may have been influenced by the genetic background. Photoreactivation may have been prevented in the *cot* and K3/17 strains by inaccessibility of the damage to the repair enzyme rather than the intrinsic non-reparability of the lesions concerned.

These results and those of other workers deal principally with reversion. It may legitimately be asked to what extent they will help us towards the control of mutagen specificity in practice, where we are concerned mainly with forward mutation systems. Mutagens which are specific in reversion tests by virtue of their ability to react with specific damage in DNA often

fail to show specificity in forward mutation tests. This is not unexpected since the chemical similarity between genes is such that it is unlikely that one would be singled out by a mutagen. In the same way it could be argued that conditions which modify mutational specificities in reversion experiments are also specific because they recognize specific types of DNA damage. If this is true it may also be unlikely that they would act specifically in forward mutation experiments. In spite of this, however, modification of forward mutational specificities does occur. Zetterberg (1961, 1962) for instance has shown that the plating medium and the mutagen interact to determine the spectrum of amino-acid-requiring mutants in *Ophiostoma*. Amino-acid-requiring mutants are obtained on complete medium after treatment with both nitrosomethylurethane and u.v. However, after u.v., no histidine-less mutants were found although nitrosomethylurethane induced them. If, instead of complete medium, minimal medium plus histidine was used to plate treated cells, histidine-less mutants were obtained after both treatments. It would appear that a constituent of complete medium suppressed the growth of u.v.-induced histidine-less mutants while not inhibiting histidine-less mutants induced by nitrosomethylure-thane.

There seem to be two main priorities for mutation research at present: (*a*) the elucidation of the mechanisms by which specificity is modified in reversion experiments, and (*b*) the biochemical investigation of instances, such as the one described by Zetterberg (1961, 1962), in which specificity has been observed in a forward mutational system.

SUMMARY

The u.v.-induced revertibility of two mutants of *Neurospora*, *ad-3A* 38701 and *inos* 37401, is described. The mutational specificity of u.v., expressed as the ratio between inositol and adenine reversions, can be modified by a variety of experimental conditions. U.v. is more specific for inositol reversions at high than at low doses in the K3/17 strain although this effect is dependent on the residual genetic background. Temperature during irradiation, the suspending fluid for the cells and photorepair all act to enhance the i/a ratio. The relationship of these results to the general problem of mutagen specificity is briefly discussed.

REFERENCES

AUERBACH, C., and RAMSAY, D. P. (1967). *Mutation Res.*, **4**, 508–510.

AUERBACH, C., and RAMSAY, D. P. (1968). *Molec. gen. Genet.*, **103**, 72–104.

BRIDGES, B., DENNIS, R., and MUNSON, R. J. (1967). *Genetics, Princeton*, **57**, 897–908.

BROWN, J. S. (1951). *J. Bact.*, **62**, 163–167.

CHAMPE, S. P., and BENZER, S. (1962). *Proc. natn. Acad. Sci. U.S.A.*, **48**, 532–546.

CHANG, L. T., LENNOX, J. E., and TUVESON, R. W. (1968). *Mutation Res.*, **5**, 217–224.

CORRAN, J. (1968). *Molec. gen. Genet.*, **103**, 42–57.

DEMEREC, M. (1953). *Symp. Soc. exp. Biol.*, **7**, 43–54.

DE SERRES, F. J. (1964). *J. cell. comp. Physiol.*, **64**, Suppl. 1, 33–42.

DRAKE, J. W. (1966). *J. Bact.*, **92**, 144–147.

GLOVER, S. W. (1956). In *Genetic Studies with Bacteria*, pp. 121–136. Washington, D.C.: Carnegie Institution of Washington, Publication No. 612.

KILBEY, B. J. (1963). *Z. VererbLehre*, **94**, 385–391.

KILBEY, B. J. (1967). *Molec. gen. Genet.*, **100**, 159–165.

KILBEY, B. J., and DE SERRES, F. J. (1967). *Mutation Res.*, **4**, 21–29.

KØLMARK, H. G. (1953). *Hereditas*, **39**, 270–276.

KØLMARK, H. G., and KILBEY, B. J. (1962). *Z. VererbLehre*, **93**, 356–365.

MALLING, H., MILTENBURGER, H., WESTERGAARD, M., and ZIMMER, K. G. (1959). *Int. J. Radiat. Biol.*, **1**, 328–343.

TERRY, C. E., KILBEY, B. J., and BRANCH-HOWE, H. (1967). *Radiat. Res.*, **30**, 739–747.

WESTERGAARD, M. (1957). *Experientia*, **13**, 224–243.

WITKIN, E., and THEIL, E. C. (1960). *Proc. natn. Acad. Sci. U.S.A.*, **46**, 226–231.

ZETTERBERG, G. (1961). *Hereditas*, **47**, 295–303.

ZETTERBERG, G. (1962). *Hereditas*, **48**, 371–389.

SB

4.

The Modifying Effects of Strain and Age
on the Mutagenic Specificity of Ultraviolet Light
in *Neurospora crassa*

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Summary. Two derivatives of K3/17 *ad-3A 38701; inos 37401* of *Neurospora crassa* are described which show opposite specific reversional responses to UV. Both derivatives carry the same two auxotrophic alleles and appear to differ only in a single gene which influences the pattern of mutagen specificity. The differences between the derivatives only develop after the cultures have been aged for two to four weeks. Various possible explanations are considered.

Introduction

Of the various aspects of mutagen specificity which may be considered, much interest has been devoted to the study of the relative responses of different alleles to specific mutagenic agents. The main aim of these studies has been to elucidate the nature of the genetic alterations which underlie observable mutations and considerable success has been achieved in this, principally with bacteriophage. In eukaryotic microorganisms the picture is much less clear. In the diauxotrophic strain of *Neurospora* used in this study, for example, it has been possible to demonstrate that the two auxotrophic mutations it carries, *ad-3A 38701* and *inos 37401*, revert at very different frequencies with a variety of mutagens, revealing marked mutagenic specificities. It is of course to be expected that the molecular conditions at the site of each mutant will be important in influencing the probability that each will respond to a particular mutagenic agent: Unless a mutagen is capable of inducing the required alteration in DNA structure, no further development towards an observable mutation can take place. But, as has been pointed out elsewhere (Allison, 1969), the patterns of specificity observed with this system as well as the changes in specificity which occur if the mutagenic dose is altered (Auerbach and Ramsay, 1968) have destroyed any lingering hopes that mutagen specificity can be attributed exclusively to the nature of the DNA at the mutant site. Other factors are clearly involved in its determination and they must include differential repair, transcription, translation, and a variety of other cellular functions and events which are necessary for the establishment of a new phenotype. These may themselves be subject to temporary disruption by mutagenic agents. Again, it seems a reasonable expectation that manifestations of these secondary factors might be more obvious when reversion is studied rather than forward mutation; revertant expression necessitates the synthesis of new RNA species and new enzymes and does not simply involve the disruption of already existing functions. However, there is sufficient information available to

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suggest that forward mutation may also be subject to at least some of the same cellular factors which appear to be important in determining reversional specificities. This is probably particularly true of many mutations which are industrially important and which, for example, improve the yields of antibiotics or specific enzymes. Thus, it is important not only from the theoretical but also from a practical standpoint that the role of cellular factors in mutagenesis be studied and, as far as possible, understood.

Several ancillary factors have been described which modify the mutagenic action of UV in *Neurospora* and promote changes in its mutagenic specificity (Kilbey, 1963; Allison, 1972). The best understood of these is undoubtedly photorepair (Kilbey and de Serres, 1967; Kilbey, 1967). Here we know that the repaired and unrepaired samples start with identical amounts of DNA damage. Any modification of specificity must take place after the induction of the damage and probably before the first DNA replication to follow exposure to UV. Examples of specific photorepair are available at the allelic level and between different types of genetic end-point (de Serres and Kilbey, 1971). Other cases are less well understood and an important step which must now be taken is the biochemical analysis of some of the better documented ones. A start in this direction has been made with the analysis of the dose-rate effect which is characteristic of diepoxybutane and possibly other epoxides (Kølmark and Kilbey, 1968; Kilbey, 1973a; Kilbey, 1973b; Kilbey, 1974). In this case the effect can be shown to result, in all probability, from the temporary destruction of a repair enzyme by the mutagen. Provided conditions for resynthesis of the enzyme are prevailing in the conidia, treatment with the agent over extended times produces a linear relationship between mutations induced and dose administered. If resynthesis of the damaged components is prevented or the time for resynthesis curtailed, upward-bending curves are obtained instead. This study is currently being extended to include other organisms and other types of damage, but, in the meantime, further examples of the activity of cellular effects are being collected and studied with a view to subjecting them to a deeper analysis. The present communication describes a situation which should prove of particular interest for subsequent study. It involves the specificity of UV in the K3/17 system and the effects of strain differences and aging on specificity.

Materials and Methods

Media. All media have been described previously (Kølmark and Kilbey, 1962). They are based on Fries Minimal Salts Medium.

Methods. As in previous work cultures were grown on the surface of solid complete medium at room temperature for various known times before harvesting the conidia, washing them twice with centrifugation and preparing suspensions at concentrations of $1-2 \times 10^7$ conidia per ml. Sterile distilled water was used throughout to wash and suspend the conidia for treatment. UV was provided by an Hanovia mercury vapour lamp. The dose rate was not determined in these experiments but care was taken to keep the conditions constant throughout the experiments. 15 ml aliquots were exposed to UV for periods of time up to 50 seconds at a distance of 10 cm. After exposure each suspension was transferred to an ice bath until it could be diluted and plated. All operations were performed under yellow light to avoid uncontrolled photoreactivation. The samples were diluted and plated on the appropriate media for survival and for both adenine and inositol reversions. The incubation temperature was 25° C. The plates were scored after three to four days for survival and seven to ten days for the reversions.

Strains. The original K3/17 strain was isolated by Kølmark and described in detail in 1962 (Kølmark and Kilbey, 1962). The strain here designated EDI is derived from an isolate of that original strain which was kept on silica gel at 0° C for eight years before being reisolated. Its response after reisolation was unchanged from the original. The strain designated K3/17 UPP also originated as a subculture of the original material but was maintained by Kølmark in Uppsala from 1962 onwards by serial isolation. This isolate was kindly given us by Dr. Kølmark in 1972. During the course of the work a number of strains were used in outcrossing experiments with the K3/17 strains these will be referred to where appropriate.

Results

1. The Response of K3/17 EDI and UPP to UV

Fig. 1 shows the result of a typical experiment in which the UV responses of the two derivatives of the original K3/17 strain were compared. Survival data are not included here in order to simplify the comparison. In fact, both strains exhibit a similar inactivation kinetics and are equally sensitive to UV. In the experiments shown, survival fell to between 30 and 40% at the highest dose in both strains. In contrast to the survival characteristics, the mutational response of the two strains is markedly different. In the EDI isolate the same pattern of specificity is observed as has always been found with these two mutants whether in the original K3/17 strain or in different genetic backgrounds entirely. Apart from the suggestion of a lack of specificity at the lowest UV dose there is a preferential induction of inositol revertants compared with adenine reversions. The UPP derivative on the other hand displays completely the reverse behaviour. Here, adenine reversions are predominant; the ratio of adenine/inositol revertants rising rapidly to ten or more and then falling to about two as the dose increases.

Although in the dose ranges over which the differences between the strains is most marked (10–30 seconds) killing is not high, it was thought advisable to test the relative viabilities of established revertants of both types from both strains in reconstruction experiments using backgrounds of treated and untreated cells of both origins. In these experiments the numbers of revertants added to the background cell populations were of the same order as those expected from induction. In none of the combinations used; (1) EDI *ad*-revertant with EDI background; EDI *inos*-revertant with EDI background; (2) EDI *ad*-revertant with UPP background; EDI *inos*-revertant with UPP background, (3) UPP *ad*-revertant with UPP background; UPP *inos*-revertant with UPP background, (4) UPP *ad*-revertant with EDI background; UPP *inos*-revertant with EDI background, was any evidence found for the selective elimination of one or other of the two revertant types. This was also true even after the cultures were suitably aged (see below section 3). Thus, it appears unlikely that differential killing of completed revertants is responsible for the pattern of UV specificity shown by the EDI strain nor for its reversal in the UPP derivative. This agrees with the findings of earlier workers (Auerbach and Ramsay, 1968).

2. Evidence for the Identity of the Auxotrophic Mutations in the Two K3/17 Derivatives

The obvious question to arise in a circumstance such as this is whether the auxotrophic mutations in the new derivative are altered in any way themselves so that their UV responses are different from those of the mutations in the original material. Two pieces of evidence point clearly to the identity of the auxotrophs
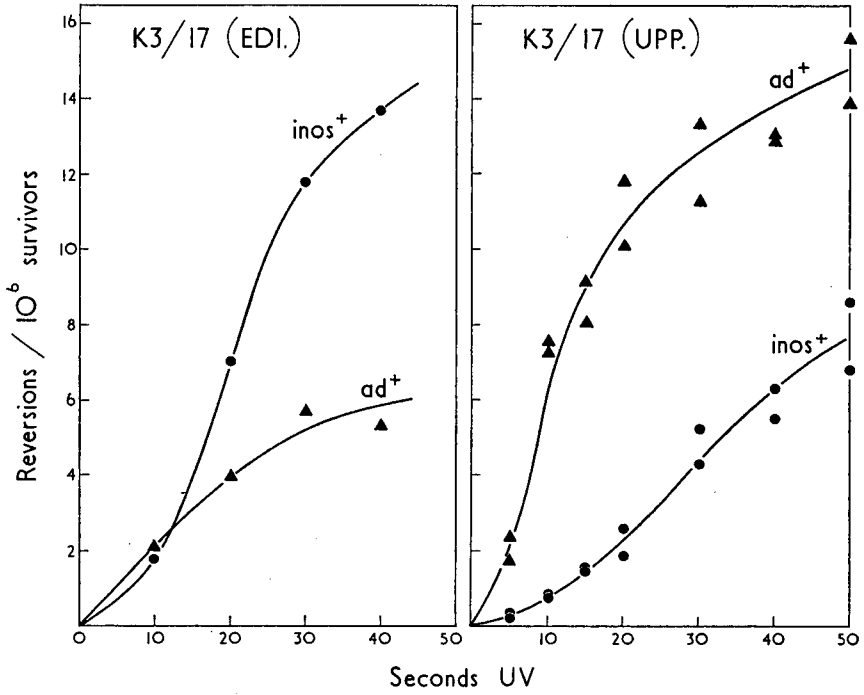
Fig. 1. The response of K3/17 derivatives EDI and UPP to UV. The initial suspension density was 2×10^7 viable conidia per ml. Survival at the highest UV doses used was between 30% and 40%
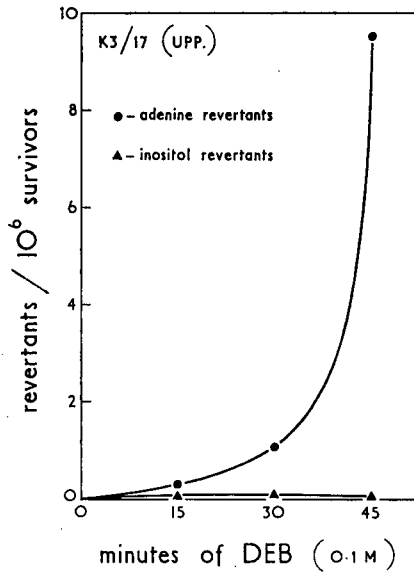

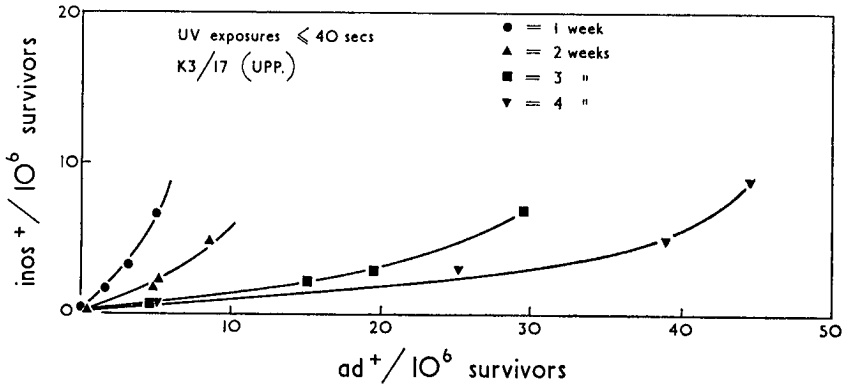Fig. 2. The response of K3/17 UPP to diepoxybutane

Fig. 3. Changes in the mutagenic specificity of UV in K3/17 UPP with age of culture. The UV doses used were 10, 20, 30 and 40 seconds. The sensitivity of the aging cultures to UV did not change and survival was reduced to between 30–40 % at the highest doses of UV used

in the two strains. In the first place, the response of the UPP strain to diepoxy-butane, an alkylating agent with a very characteristic pattern of specificity, is the same as reported earlier for the original K3/17, the derived EDI strain and the same two auxotrophic mutants in totally different backgrounds. Fig. 2 gives a typical experimental result of treating K3/17 UPP with DEB. This is a mean-ingful observation since it shows that the adenine alleles are unlikely to be diffe-rent in the two strains. ad-3A 38701 is the only mutant allele to be found in Neurospora so far which has such a high responsiveness to DEB and it is unlikely, therefore, that the adenine allele in UPP is different from that in EDI. Un-fortunately, DEB does not discriminate between different inositol alleles—all of those so far tested are equally refractory towards it.

The second and more decisive evidence that the alleles are the same in the two strains comes from outcrossing K3/17 UPP to the strain cot al-2 pan-2 and isolating progeny which possessed none of the colonial determinants of K3/17 but which had received the two auxotrophic mutations ad-3A 38701 and inos 37401. Two of these were found and both showed the UV response for the two alleles which had been described in the earlier literature. There is, therefore, no good reason to doubt that the two auxotrophic mutants present in K3/17 UPP are ad-3A 38701 and inos 37401. The genetic basis for the difference in the UV response of the two alleles to UV in the EDI and UPP strains is examined in section 4.

3. The Effects of Culture Age on Specificity

The altered behaviour of the UPP strain was first noticed during experiments designed to investigate the effects of monochromatic light of different wave-lengths and dose-rate on the mutability of these two mutants in the K3/17 strain. It quickly became apparent that the new response had no connection with the altered conditions of irradiation used in these experiments but represented an intrinsic difference between the two isolates UPP and EDI. One variable which was found to be of major importance, however, was the culture age at the time of UV exposure. Fig. 3 summarises the data from one group of experiments in
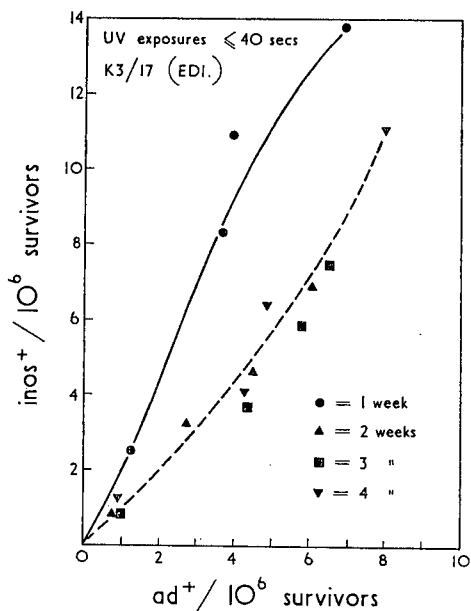
Fig. 4. Changes in the mutagenic specificity of UV in K3/17 EDI with age of the culture. The conditions for the experiments were the same as outlined for K3/17 UPP. The dotted line indicates a trend which applies to the data obtained from cultures aged 2, 3 and 4 weeks

which cultures of K3/17 UPP were allowed to age for up to four weeks before exposure to UV. Four experiments of this type have now been made with UPP and they are in complete agreement. The data have been plotted in a way which clearly shows the change in specificity with culture age. A line drawn at 45 degrees to the abscissa and passing through the origin would represent no specificity on this graph. It will be seen that in young cultures (1 week) the specificity encountered in the UPP strain is indistinguishable from that normally observed for the K3/17 strain EDI. However, as the culture gets older, the pattern of specificity rapidly changes: Specificity is first lost while later it is reversed so that adenine revertants far outnumber inositol revertants. The change in specificity with age appears to result less from changes in the frequency of inositol revertants than from a marked increase in the response of the adenine mutant to UV. In the early experiments we were rather fortunate to have used, unknowingly, conditions which allowed the UPP response to be expressed.

The EDI strain was also tested for the age effect. Three experiments were performed in the same way as those described for the UPP strain. All of them gave similar results and a typical set of data have been presented as a graph in Fig. 4. In contrast to UPP, EDI fails to show a marked change in specificity of UV response with age. If there is a change at all it only proceeds as far as the loss of specificity, but never beyond this to the reversal of specificity. There is thus a real difference between the two derivatives both in their response to aging of the culture and consequently, in their response to UV. In these experiments

survival measurements were also made and, although they are omitted here, they show that no difference in UV sensitivity exists with difference in age of the cultures of either strain.

4. The Genetic Basis for the Difference in Behaviour of K3/17 EDI and UPP

Attempts were next made to establish the genetic basis of the difference between the two strains. Unfortunately, it is impossible to cross them to each other since they are of the same mating type and, furthermore, they both carry the same adenine allele. Crosses between strains which are both adenine auxotrophs are often sterile in *Neurospora*. Instead, the UPP strain was crossed to a strain carrying the markers *cot*, *pan-2* and *al-2*. In these crosses the protoperithecial parent was of necessity the *cot* strain since K3/17 UPP and EDI do not produce functional protoperithecia. From this cross progeny were isolated which have the mutations *ad-3A 38701* and *inos 37401*. Fourteen of these were obtained and each was tested for its response to UV. In every case the response was similar to the EDI strain which suggested that either a cytoplasmic difference exists between the two K3/17 strains which could be cancelled by cytoplasm from the *cot* parent or, alternatively, because of the low ascospore viability in this cross, UPP progeny were selected against during the maturation of the ascospores or during the heat activation procedure. When these strains were first tested, the significance of culture age had not been realised. With this knowledge, the tests were repeated in duplicate with aged cultures of each of the fourteen segregants. The data are presented in an abbreviated form in Table 1. In order to reach a conclusion concerning the type of response exhibited by each of the segregants two criteria were applied:

(a) Segregants exhibiting an $inos^+/ad^+$ ratio which is consistently greater than one, especially at the higher UV doses, or which show little or no specificity towards UV after aging are classified as of the EDI type. This is based on the known behaviour of the EDI strain which, as we have seen, shows little change in specificity with culture age and which gives an excess of inositol revertants except possibly at very low UV doses in some experments.

(b) Segregants which display an excess of adenine revertants in response to UV are classified as UPP-like. Although there are quantitative variations between the response to UV of different segregants as might be expected in view of the probable existence and segregation of other genetic factors which affect the UV responses of the two strains, it is possible to apply these rules to achieve a remarkably clear differentiation into EDI-like and UPP-like segregants. As can be seen from the table, 6/14 have been classified as UPP-like and 8/14 as EDI-like. In only one case, that of *73-002a-007*, is the outcome of the test in any doubt because the specificity is less marked. It has been classified as UPP-like since in most of the determinations of the ratio $inos^+/ad^+$, the values obtained are less than unity. Even if it is classified as EDI-like the basic conclusion from the results remains the same; that is, that they support the view that a single gene difference is responsible for the difference in behaviour of UPP and EDI. The data also indicate that there is no marked selection against the UPP-like ascospores during the maturation of the meiotic products or their activation.

Table 1. *The UV responses of 14 ad-3A 38701; inos 37401—bearing segregants from out crossing K3/17 UPP to cot al-2 pan-2. Mutations are expressed per 10^6 survivors*

Strain tested	Parameter	UV dose				Conclusion
		10''	20''	30''	40''	
73-002a-001	% surv.	86.80	81.12	58.87	34.40	
	inos+	2.86	6.53	12.25	9.50	
	ad+	3.89	6.18	8.14	4.91	
	inos+/ad+	0.73	1.05	1.51	1.93	EDI
73-002a-001	% surv.	80.50	66.78	54.87	37.18	
	inos+	2.33	13.19	23.68	36.31	
	ad+	1.97	7.46	8.68	9.13	
	inos+/ad+	1.18	1.76	2.73	3.97	
73-002a-002	% surv.	79.93	65.12	65.12	29.62	
	inos+	7.64	10.62	12.42	19.79	
	ad+	10.96	18.58	17.44	27.29	
	inos+/ad+	0.69	0.57	0.71	0.72	UPP
73-002a-002	% surv.	73.18	54.41	29.65	23.34	
	inos+	2.97	7.48	13.51	19.46	
	ad+	14.39	20.93	36.17	24.86	
	inos+/ad+	0.20	0.35	0.37	0.68	
73-002a-005	% surv.	81.20	75.91	66.78	50.36	
	inos+	4.49	9.90	15.36	—	
	ad+	2.88	5.29	6.77	—	
	inos+/ad+	1.55	1.87	2.30	—	EDI
73-002a-005	% surv.	75.91	44.52	18.24	9.48	
	inos+	3.85	21.97	40.80	43.08	
	ad+	2.31	6.88	13.60	9.23	
	inos+/ad+	1.66	3.19	3.00	4.66	
73-002a-006	% surv.	93.8	97.09	91.00	74.90	
	inos+	4.49	10.41	14.92	20.34	
	ad+	3.72	6.29	8.55	12.08	
	inos+/ad+	1.20	1.65	1.74	1.68	EDI
73-002a-006	% surv.	105.71	73.57	73.14	54.85	
	inos+	1.94	15.57	21.76	30.94	
	ad+	2.94	17.59	20.78	28.64	
	inos+/ad+	0.65	0.88	1.04	1.08	
73-002a-007	% surv.	97.54	95.32	88.36	67.15	
	inos+	1.07	3.81	4.19	5.61	
	ad+	2.78	8.67	8.64	7.57	
	inos+/ad+	0.38	0.43	0.48	0.74	UPP
73-002a-007	% surv.	68.81	66.66	51.93	22.17	
	inos+	3.78	9.66	11.68	18.58	
	ad+	3.37	10.04	12.73	22.84	
	inos+/ad+	1.12	0.96	0.91	0.81	

Table 1 (continued)

Strain tested	Parameter	UV dose				Conclusion
		10″	20″	30″	40″	
73-002a-009	% surv.	84.10	40.60	35.30	27.14	
	$inos^+$	3.27	15.10	16.34	19.48	
	ad^+	6.90	16.80	15.90	12.10	
	$inos^+/ad^+$	0.48	0.89	1.02	1.60	EDI
73-002a-009	% surv.	87.30	56.07	31.51	12.51	
	$inos^+$	2.84	9.56	14.80	34.28	
	ad^+	4.98	8.69	10.10	13.68	
	$inos^+/ad^+$	0.57	1.1	1.47	2.50	
73-002a-010	% surv.	82.81	73.28	94.67	79.63	
	$inos^+$	2.19	8.32	10.27	19.78	
	ad^+	1.53	7.13	5.66	10.79	
	$inos^+/ad^+$	1.43	1.16	1.81	1.83	EDI
73-002a-010	% surv.	95.71	104.28	89.40	71.75	
	$inos^+$	2.53	9.21	15.43	28.86	
	ad^+	1.53	3.97	5.92	10.23	
	$inos^+/ad^+$	1.65	2.43	2.60	2.82	
73-002a-012	% surv.	86.15	78.63	71.36	48.56	
	$inos^+$	2.53	7.71	10.30	17.69	
	ad^+	1.53	16.24	20.10	22.16	
	$inos^+/ad^+$	1.65	0.47	0.51	0.79	UPP
73-002a-012	% surv.	89.35	49.04	30.98	14.06	
	$inos^+$	1.87	6.98	10.43	15.40	
	ad^+	22.30	45.27	54.72	63.51	
	$inos^+/ad^+$	0.08	0.15	0.19	0.24	
73-002a-014	% surv.	111.99	119.44	81.02	69.03	
	$inos^+$	1.25	4.19	6.72	8.80	
	ad^+	3.74	6.84	8.85	9.07	
	$inos^+/ad^+$	0.33	0.61	0.75	0.97	UPP
73-002a-014	% surv.	96.58	65.64	51.43	39.02	
	$inos^+$	2.53	10.52	17.48	18.80	
	ad^+	6.29	18.68	25.31	32.72	
	$inos^+/ad^+$	0.40	0.56	0.69	0.57	
73-002a-016	% surv.	81.90	54.18	30.83	12.90	
	$inos^+$	4.50	17.30	32.42	67.80	
	ad^+	22.38	26.90	33.30	43.20	
	$inos^+/ad^+$	0.20	0.64	0.97	1.57	UPP
73-002a-016	% surv.	75.40	40.50	26.30	15.10	
	$inos^+$	2.93	13.13	20.26	92.30	
	ad^+	38.14	50.40	53.44	103.70	
	$inos^+/ad^+$	0.08	0.30	0.40	0.88	
73-002a-018	% surv.	85.35	68.39	55.94	26.66	
	$inos^+$	3.74	8.82	11.65	24.28	
	ad^+	3.77	5.93	8.82	12.05	
	$inos^+/ad^+$	0.99	1.48	1.32	2.01	EDI

Table 1 (continued)

Strain tested	Parameter	UV dose				Conclusion
		10″	20″	30″	40″	
73-002a-018	% surv.	80.56	70.74	36.07	18.83	
	inos⁺	2.79	6.06	32.44	15.74	
	ad⁺	6.39	7.42	10.33	10.64	
	inos⁺/ad⁺	0.43	0.81	3.14	1.47	
73-002a-019	% surv.	95.69	109.67	108.60	82.25	
	inos⁺	2.92	3.72	7.62	19.15	
	ad⁺	1.79	2.84	2.47	3.01	
	inos⁺/ad⁺	1.51	1.30	3.08	6.36	EDI
73-002a-019	% surv.	116.66	90.00	110.00	50.00	
	inos⁺	0.57	—	4.24	14.67	
	ad⁺	1.14	—	2.42	5.33	
	inos⁺/ad⁺	0.50	—	1.75	2.75	
73-002a-020	% surv.	100.58	90.81	67.20	45.91	
	inos⁺	3.10	7.54	10.54	11.23	
	ad⁺	1.62	3.53	5.81	6.09	
	inos⁺/ad⁺	1.91	2.13	1.81	1.84	EDI
73-002a-020	% sun.	100.85	66.02	78.41	30.76	
	inos⁺	1.27	7.64	8.12	12.64	
	ad⁺	2.20	4.79	4.47	6.53	
	inos⁺/ad⁺	0.57	1.59	1.81	1.90	
73-002a-021	% surv.	68.44	59.82	42.96	31.93	
	inos⁺	1.26	7.12	12.27	23.89	
	ad⁺	6.26	15.93	21.53	29.76	
	inos⁺/ad⁺	0.20	0.44	0.56	0.80	UPP
73-002a-021	% surv.	82.92	76.91	60.18	46.99	
	inos⁺	1.82	4.56	10.92	22.76	
	ad⁺	7.09	18.10	27.51	50.73	
	inos⁺/ad⁺	0.25	0.25	0.39	0.44	

Discussion

These results may be summarised as follows: A new derivative of the original K3/17 ad^- $inos^-$ strain has been isolated which has been designated "UPP". It differs from the original strain (EDI) in its pattern of mutagen specificity with UV which is completely reversed. The adenine and inositol alleles present in UPP appear to be identical with those in the original strain. The reversal of UV specificity noted with UPP only develops when conidial populations from aged cultures are used. The response of EDI is scarcely affected by culture age. The differences between UPP and EDI appear from preliminary data to be determined by a single mutational change at a site unlinked to both ad and $inos$ mutants.

The changes taking place in a maturing culture are compounded of events in the mycelium, the conidia and the medium Thus, although conidia are the units

tested in these experiments, their changing UV response may be the result of changes occurring primarily in either the conidium, the mycelium or both. Furthermore, very little is known at present concerning the composition of the conidial population at various times during the culture maturation. It is possible that the early conidial crop dies and is replaced by successive crops of new conidia, although this seems improbable since the proportion of inviable conidia in older samples does not rise appreciably over this time period. Another possibility is that conidia are not replaced but are added to slowly and that, as the culture ages, conidia produced later have an even greater specificity towards UV than the overall population. A direct test of these possibilities and a third, *vis* that the changes occur entirely in a conidial population which does not recieve significant additions or replacements, may be possible by testing the UV response of conidia produced on old cultures once the main crop has been harvested. Unfortunately, this second crop is very sparse but it would provide new conidia grown on old mycelium, a useful combination in this context.

Whether the changes responsible are initiated in the conidium or in the mycelium, the conidial population obtained from old and young cultures of UPP clearly differ in their response to UV and the nature of the cellular changes responsible must be explained. It is also important when considering possible explanations to take into account the fact that the differences occurring with age are apparently determined by a single gene difference between UPP and EDI. In the first place it appears unlikely that the difference in mutational response between the strains after aging results from parallel variations in the extent of UV damage inflicted on the DNA. Considerable care was taken to eliminate variations in the conditions of radiation and the similarities between the survival estimates, in spite of age and strain differences, support the expectation that similar degrees of UV damage were inflicted by the same UV dose from experiment to experiment. It is also possible to exclude mutant selection as a cause of the switch in specificity. Extensive reconstruction studies were made and in no case was any differential viability of either revertant type detectable.

If these conclusions are correct, the more satisfactory explanations would seem to be those which are based on changes occurring in the aging material which affect either the processing of the initial lesions (repairing them or converting them into mutational changes) or on the metabolic events which influence the likelihood that the new mutations will be expressed.

One suggestion which can immediately be excluded is that in the aged conidia there is a greater susceptibility to the conditions which promote inositolless death. These exist on the plates used for the selection of inositol revertants and it has always seemed possible that some revertants might be lost because they die before full expression of the reversion takes place. Direct tests have, however, shown that the rate of inositolless death is the same for both aged and unaged conidia of UPP. Furthermore, since the reversal of specificity with age is apparently mainly the consequence of changes in adenine reversion frequency, an explanation which requires changes in the inositol reversion frequencies must be considered unlikely.

Variations in the activity of cellular dark repair systems could certainly account for these observations. Such a repair system would be required to (a) be specific in its action for damage which produces adenine reversions and (b) to vary in activity with age. Precedent for the first of these requirements already exists. Photo-repair has been shown to act more strongly on UV damage producing adenine reversions than on the damage producing inositol reversion (Kilbey, 1967). There is, however, little direct evidence that aging cells in general display variation in repair activity (e.g. Painter *et al.*, 1973). However, in one study (Gampel and Toha, 1969) using Neurospora conidia aged under circumstances similar to those used in the present study, a change in radiation sensitivity was noted. This was manifest as a reduction in the shouldered part of the survival curve at low doses of UV. The authors interpret it as a fall in the number of "independent hereditary units" or targets during aging, but the results are just as consistent with the diminished activity of a repair process responsible for the shoulder on the UV survival curve. No evidence was found for variations in the activity of photorepair in these experiments. An explanation based on variations in the activity of a specific repair process can also be linked to the single gene difference which apparently exists between UPP and EDI if it is assumed that in the UPP strain the mutation has produced an altered enzyme with a lower stability under conditions of storage. The failure to observe any marked change in sensitivity with age towards the lethal effects of UV in the UPP strain need not invalidate this conclusion.

It is clear, however, that an explanation for these data may lie in metabolic changes which occur in aging conidia and which have no direct relationship with repair. Here we are without much information to guide us. Stine (1967) has shown that several enzyme activities decline when Neurospora conidia are allowed to age and, although his conditions of storage were different from ours, they may point to similar events in our experiments. We are currently following several different lines in our attempts to reach a solution of this problem. In the preliminary experiments it has been possible to show that the response of the adenine mutant and the rate at which this changes with age is affected by both the temperature of storage and the level of adenine present in the growth medium. The second of these observations may be indicative of a role for the regulation of adenine biosynthesis in determining the expression of adenine revertants. We are also attempting to obtain information concerning the effects of age on the responses of these two isolates to mutagenic chemicals. The rationale for this approach is that if agents which have very different primary actions all show an age effect, it would be more reasonable to suppose that the effect of age is mediated through changes in the later parts of the mutation pathway than in its early stages. So far, only nitrosomethylurethane has been tested, and from the preliminary data it would appear that an age effect exists for this mutagen also. These data will be presented in more detail elsewhere.

The evidence at present available on UPP indicates more clearly than any we have presented so far with this system that the observable mutagen specificity is not an inviolable characteristic of the two alleles in question. Provided suitable conditions are applied the specificity can be removed and even reversed. Observa-

tions of this type obviously bear considerable relevance to problems encountered when mutation yields and the kinetics of mutant production are being considered and it is now important to extend this type of study into the field of forward mutations as well as to understand the biochemical basis of these phenomena.

References

Allison, M. J.: Mutagen specificity at the *ad-3A* and *inositol* loci in *Neurospora crassa*. Mutation Res. **7**, 141–151 (1969)

Allison, M. J.: The effect of phosphate buffer on the differential response of two genes in *Neurospora crassa* to UV. Mutation Res. **16**, 243–248 (1972)

Auerbach, C., Ramsay, D. P.: Analysis of a case of mutagen specificity in *Neurospora crassa*. I. Dose response curves. Molec. gen. Genet. **103**, 72–104 (1968)

Auerbach, C., Ramsay, D. P.: Analysis of a case of mutagen specificity in *Neurospora crassa*. Molec. gen. Genet. **109**, 1–17 (1970)

De Serres, F. J., Kilbey, B. J.: Differential photoreversibility of ultraviolet-induced premutational lesions in *Neurospora*. Mutation Res. **12**, 221–234 (1971)

Gampel, Z., Toha, J. C.: Aging and repair in *Neurospora crassa* studied by ultraviolet irradiation. Radiat. Res. **40**, 525–533 (1969)

Kilbey, B. J.: The influence of temperature on the ultraviolet induced revertant frequencies of two auxotrophs of *Neurospora crassa*. Z. Vererbungsl. **94**, 385–391 (1963)

Kilbey, B. J.: Specificity in the photoreactivation of premutational damage induced in *Neurospora* by ultraviolet. Molec. gen. Genet. **100**, 159–165 (1967)

Kilbey, B. J.: The stabilisation of a transient mutagen-sensitive state in *Neurospora* by the protein synthesis inhibitor Actidione. Molec. gen. Genet. **123**, 67–72 (1973a)

Kilbey, B. J.: The manipulation of mutation-induction kinetics in *Neurospora crassa*. Molec. gen. Genet. **123**, 73–76 (1973b)

Kilbey, B. J.: The analysis of a dose-rate effect found with a mutagenic chemical. Mutation Res. **26**, 249–256 (1974)

Kilbey, B. J., De Serres, F. J.: Quantitative and qualitative aspects of photoreactivation of premutational ultraviolet damage at the *ad-3* loci of *Neurospora crassa*. Mutation Res. **4**, 21–29 (1967)

Kølmark, H. G., Kilbey, B. J.: An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa*. Z. Vererbungsl. **93**, 356–365 (1962)

Kølmark, H. G., Kilbey, B. J.: Kinetic studies of mutation induction by epoxides in *Neurospora crassa*. Molec. gen. Genet. **101**, 89–98 (1968)

Painter, R. B., Clarkson, J. M., Young, B.: Ultraviolet-induced repair replication in aging diploid human cells W1–38. Radiation **56**, 560–564 (1973)

Stine, G. J.: Germination and enzyme activities affected by the aging of *Neurospora crassa* conidia in water. Neurospora Newsl. **11**, 7–8 (1967)

Worthy, T. E., Epler, J. L.: Biochemical basis of radiation-sensitivity in mutants of *Neurospora crassa*. Mutation Res. **19**, 167–173 (1973)

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IDENTIFICATION OF THE GENETIC ALTERATION AT THE MOLECULAR LEVEL OF ULTRAVIOLET LIGHT-INDUCED *ad-3B* MUTANTS IN *NEUROSPORA CRASSA**

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SUMMARY

The genetic alterations in UV-induced *ad-3B* mutants of *Neurospora crassa* have been identified by tests for specific revertibility after treatment with nitrous acid (NA), ethyl methanesulfonate (EMS), hydroxylamine (HA), and a monofunctional nitrogen mustard derivative of acridine, ICR-170. On the basis of their reactions with these chemicals, mutants were classified as base-pair transitions, base-pair transversions, base-pair insertions or deletions, or nonrevertible.

A random sample of 151 UV-induced *ad-3B* mutants were analyzed: 46 with nonpolarized and 49 with polarized complementation patterns and 56 that are non-complementing. The mutants were characterized as to reversion mechanism as follows: (*1*) base-pair transitions AT $\rightarrow$ GC, 27.7%; GC $\rightarrow$ AT, 8.0%; (*2*) base-pair transversions, 6.6%; (*3*) base-pair insertions or deletions, 27.0%; (*4*) nonrevertible, 8.6%; (*5*) mutants which revert only spontaneously, 22.1%.

The correlation between complementation pattern and genetic alteration at the molecular level found previously by MALLING AND DE SERRES among NA- and EMS-induced *ad-3B* mutants, was also found among UV-induced mutants. Mutants with nonpolarized patterns resulted mainly from base-pair substitutions, whereas mutants with polarized complementation patterns and noncomplementing mutants are derived from a variety of genetic alterations.

INTRODUCTION

UV light is an important mutagen. Several attempts have been made to identify the genetic alteration in UV-induced mutants at the molecular level. The most extensive of these, a study by DRAKE[7] of UV-induced rII mutants in bacteriophage T4, led to the conclusion that about one-half of these mutants resulted from base-pair

transitions, the remainder from base-pair addition or deletion. The majority of the mutants resulting from base-pair transition appear to revert by means of an AT → GC transition. It was therefore inferred that induction of the original mutants involved a GC → AT base-pair transition. Similar conclusions were reached by HOWARD AND TESSMAN[9] in studies using phages with single-stranded DNA. In this case the cytosine residue was implicated as the main UV target.

In the present report similar data will be presented, which were obtained for UV-induced mutants at the *ad-3B* locus in *Neurospora crassa*. The main value of these data lies in the fact that they enable a comparison to be drawn between the effects of UV in bacteriophage, a prokaryote, and its effects in Neurospora, a eukaryote, in which the chromosomal organization appears to have the same complexity as found in higher plants.

As in the bacteriophage studies, the main part of the data was obtained from tests of revertibility using specific chemical mutagens. An additional parameter was used to describe each mutant studied, that of its characteristics in heterokaryon tests for allelic complementation.

MATERIALS AND METHODS

(A) Strains

The adenine-3 (*ad-3*) mutants studied were induced in 2 forward mutation experiments in which the wild-type strain 74-OR23-1A was used. Conidial suspensions were prepared in the following manner: single-colony isolates from silica gel stocks of the wild-type strain were grown for 7 days on glycerol complete medium[8] in the dark (to prevent carotinoid accumulation) at 25°. Conidia were harvested from these cultures and filtered twice through cotton pads before they were washed twice with water. The washed suspension was then adjusted to give a conidial density of approx. $1.2 \cdot 10^6$ conidia per ml.

(B) Induction of ad-3 mutants

30 ml of this suspension was irradiated with the use of a G.E. germicidal lamp (15 W No. G15-T8) as described previously[11]. During the exposure the conidial suspension was contained in a quartz flask and stirred magnetically. The temperature during irradiation was approx. 25°. Throughout the treatment and posttreatment period, illumination was supplied from a G.E. (15 W F15T8 60) "Gold" lamp to prevent photoreactivation. The UV dose was determined from readings taken at the surface of the irradiated suspension with the use of a Jagger-type dose-rate meter[10]. The treated suspensions and untreated control suspensions were then inoculated into 12-l flasks for the determination of survival and mutation frequency with the use of the forward-mutation technique described by DE SERRES AND KØLMARK[3].

(C) Isolation and characterization

Purple colonies were isolated from the flasks and each mutant was made homokaryotic and tested for its genotype (*ad-3A* or *ad-3B*). The *ad-3B* mutants were each tested for their ability to complement with a set of tester mutants. These testers define a map of 17 complementation units or complons[5], and from the response obtained with each mutant it was assigned to a particular class: nonpolarized, polar-

ized, or noncomplementing. The methods used in the heterokaryon tests for genotype and allelic complementation have been described in detail elsewhere[2].

(D) Reversion tests

The mutagens used for the tests of specific revertibility were NA, HA, EMS and ICR-170. The sample of ICR-170 was obtained from Dr. H. J. CREECH, Institute for Cancer Research, Philadelphia.

(1) General treatment conditions. Preparation of the conidial culture and media was described previously[4]. A detailed description of the treatment of the conidia with NA, EMS, HA and ICR-170 was given earlier[14-16]. In brief, the conditions were as follows. NA: 40 min treatment with 0.005 M $NaNO_2$ in a 0.05 M sodium acetate buffer adjusted to pH 4.5; EMS: 300 min treatment with 0.1 ml EMS dissolved in 9.7 ml potassium sodium phosphate buffer (0.067 M) adjusted to pH 7.0 (the final concentration of EMS was 0.1 M); ICR-170: 130 min treatment in the same buffer as described under the EMS treatment at a final concentration of 10.58 μM; HA: suspension in 3 M NaCl and then dilution 5 times with the reaction mixture described by STRACK *et al.*[19], which is composed of 2.6 g NH_2OHCl, 10 ml H_2O and 2.5 ml of 10 M NaOH. The final pH of the solution was 6.2, and in this solution the concentration of the HA was 1 M. The treatment time was 5 h. A 3-M solution of sodium chloride was used to terminate the mutagenic treatment. There was no mutagenic effect associated with control treatments with 3 M NaCl alone, for up to 12 h (ref. 18). This 3-M solution of sodium chloride also constituted the washing solution[13].

(2) Plating procedure and media. These have been described earlier[14]. In the case of HA-treated conidia, the density of cells on the reversion plates was lowered to 2.5 · 10^5/ml medium, the total volume of medium used being 400 ml. Revertants were scored after 9 days' incubation at 30°.

(3) Statistical analysis. These were done according to BIRNBAUM[1] and were described previously[12].

RESULTS

Mutation induction

2 forward-mutation experiments (5-8 and 5-9) were conducted to obtain the adenine mutants used in the subsequent analysis. In both the dose of UV was approx. 2500 erg/mm². The experiments gave closely agreeing survival values, the mean survival being 70% and the mean forward-mutation frequency for purple adenine mutants being 108 · 10^{-6} survivors.

Mutants were used from both experiments in the subsequent analysis. Functional allelism tests were used to assign each mutant to the *ad-3A* or the *ad-3B* class. Only mutants of the latter type were studied further. By means of the series of allelic tester mutants described earlier, each of the *ad-3B* mutants was assigned unambiguously into noncomplementing (NC), nonpolarized (NP), and polarized (P) complementing classes. The numbers of mutants in each class in the 2 experiments are given in Table I. These data do not differ significantly ($\chi^2_{4\ d.f.} = 5.02$; $0.25 < P < 0.50$) from those obtained in a similar experiment (5-6) of DE SERRES *et al.*[6], in which a wide range of doses was used. The data from the 3 experiments have been combined to obtain the following average percentages: NP = 25.7%, P = 12.0%, and NC = 62.3%.

TABLE I

COMPARISON OF TESTS FOR ALLELIC COMPLEMENTATION OF UV-INDUCED *ad-3B* MUTANTS FROM 3
DIFFERENT EXPERIMENTS ON WILD-TYPE STRAIN 74-OR23-1A

Experiment number	Type of complementation pattern						Total sample
	Nonpolarized (NP)		Polarized (P)		Noncomplementing (NC)		
	Number	%	Number	%	Number	%	
5-6[a]	37	33.3	13	11.7	61	55.0	111
5-8	62	22.7	36	13.2	175	64.1	273
5-9	145	25.7	65	11.5	354	62.8	564
Total	244	25.7	114	12.0	590	62.3	948

[a] Taken from DE SERRES *et al.*, in the press.

Mutants were drawn at random from each of these 3 classes, from experiments 5-8 and 5-9. Each of the mutants was tested for its ability to revert with NA, EMS, HA and ICR-170. The results of these tests are given in Tables II–IV. The mutants in Tables II and III were grouped according to their complon coverage[5]. Within each group of complementation patterns the mutants were grouped according to their tentative reversion mechanism or type of unidentified genetic alteration. The criteria for making these assignments are given in the DISCUSSION. The noncomplementing mutants in Table IV were grouped according to their assigned reversion mechanism or as to whether they reverted spontaneously. (An asterisk above a number indicates a significant increase in the reversion frequency after a particular mutagenic treatment as compared with the control values.) Significance has been judged at the 1% level of probability. The levels of survival[14] in the tests with EMS, NA and ICR-170 approached 100%. In the case of HA the treatment resulted in a survival[14] of 50%. Very little variation in survival was noted from mutant to mutant.

DISCUSSION

Identification of the genetic alteration at the molecular level by a specific revertibility test has 2 inborn uncertainties: *(1)* the specificity of the mutagens used for this characterization and *(2)* the mechanism of reversion. As a result of these uncertainties the assignment of a particular genetic alteration to a mutation can only be of tentative nature. These 2 points have been discussed in earlier papers[14–16]. The rules which have been followed in the identification of the genetic alterations are as follows:

(1) Mutants which revert with NA, EMS, HA, or any combination of these mutagens, whether they revert with ICR-170 or not, are classified as base-pair substitution mutants. Within this class, mutants which reverted with NA but not after treatment with HA, were classified as reverting by an AT → GC transition. Mutants which were reverted by NA and HA were assumed to revert by a GC → AT transition. Some mutants revert only after EMS treatment and they may be transversions, classified as TV. Some of these same mutants also revert with ICR-170 and this reversion is believed to result from the alkylating activity of this compound.

(2) Mutants which revert only with ICR-170 are classified as reverting by base-pair insertion or deletion (+/−). On a few occasions it has been noted that a mutant behaving in this way will also revert at a very low frequency with EMS. In general,

TABLE II

REVERTIBILITY OF UV-INDUCED *ad-3B* MUTANTS WITH NONPOLARIZED COMPLEMENTATION PATTERN IN *Neurospora crassa* AFTER TREATMENT WITH NA, EMS, ICR-170 AND HA

Mutant	Complon coverage	Number of revertants per 10^8 conidia untreated or after treatment with						Reverse mutation mechanism or unidentified genetic alteration[c]
		Control	NA	EMS	ICR-170	Control[a]	HA	
5-8-230	2	0	1	3	0	6	27*[b]	GC → AT
5-9-76	2	2	25*	4	34*	1	1	AT → GC
5-8-23	2-4	8	805*	245*	31*	0	7	AT → GC
5-9-183	2-4	0	57*	90*	20*	0	5	AT → GC
5-9-377	2-4	1	51*	103*	8	0	5	AT → GC
5-8-59	2-4	0	19*	70*	181*	0	3	AT → GC
5-8-238	2-4	0	7*	101*	13*	0	0	AT → GC
5-9-411	2-4	1	0	82*	1	0	0	TV
5-8-75	2-4	0	2	16*	14*	0	0	TV
5-9-355	2-4	0	0	8	0	0	0	SP
5-9-216	2-5	4	7	3	2	5	6	SP
5-8-332	2-6	0	54*	341*	4	0	18*	GC → AT
5-9-457	2-8	3	81*	305*	21*	1	4	AT → GC
5-8-246	2-11	1	9	2	4	0	1	SP
5-9-147	2-11	0	0	1	0	1	0	SP
5-9-499	6-10	12	554*	0	5	0	2	AT → GC
5-9-73	6-10	4	54*	2	8	1	1	AT → GC
5-9-175	6-10	0	50*	40*	8*	0	3	AT → GC
5-8-106	6-10	3	21*	1	2	1	4	AT → GC
5-9-106	6-10	1	4	2	5	0	0	SP
5-8-201	6-12	11	46*	47*	10	2	1	AT → GC
5-9-78	6-12	0	0	7*	17*	0	0	TV
5-9-53	6-12	5	4	6	35*	0	0	+/−
5-9-307	6-15	3	12*	1	5	3	4	AT → GC
5-9-337	7-15	1	12*	32*	25*	0	1	AT → GC
5-8-95	8-12	1	0	25*	0	0	2	TV
5-8-249	8-12	0	0	1	1	0	0	SP
5-9-145	8-14	0	2	7*	29*	0	0	TV
5-8-430	8-15	0	1	1	15*	1	0	+/−
5-9-249	8-15	0	1	0	0	0	0	SP
5-8-397	9-12	6	45*	270*	1004*	4	21*	GC → AT
5-8-166	9-15	0	0	1500*	0	0	0	TV
5-8-105	9-16	0	0	0	161*	0	0	+/−
5-9-116	10-11	1	19*	570*	1	2	111*	GC → AT
5-9-414	10-11	10	60*	177*	4	10	16	AT → GC
5-8-349	10-11	0	15*	14*	6*	4	4	AT → GC
5-8-130	11-12	3	12*	11*	1	10	34*	GC → AT
5-8-57	11-12	0	0	6*	1071*	0	1	AT → GC
5-9-464	11-13	1	29*	28*	2	17	15	AT → GC
5-8-13	11-16	0	14*	2	0	0	1900*	GC → AT
5-8-354	11-16	2	15*	1	0	0	0	AT → GC
5-8-113	12-13	0	0	2	0	1	1132*	GC → AT
5-9-433	12-13	1	73*	593*	5	3	13*	GC → AT
5-8-410	12-13	6	10	7	76*	4	7	+/−
5-9-257	12-13	0	0	1	0	2	0	SP
5-8-7	15	0	0	1	0	2	0	SP

[a] Control for HA treatment; performed in separate experiments.
[b] An asterisk above a number indicates a significant increase in the reversion frequency after a particular mutagenic treatment as compared with the control values.
[c] TV, transversion; SP, spontaneous revertibility; +/−, base-pair insertion or deletion; non, no revertibility.

TABLE III

REVERTIBILITY OF UV-INDUCED *ad-3B* MUTANTS WITH POLARIZED COMPLEMENTATION PATTERN IN *Neurospora crassa* AFTER TREATMENT WITH NA, EMS, ICR-170 AND HA

Mutant	Complon coverage	Number of revertants per 10^8 conidia untreated or after treatment with						Reverse mutation mechanism or unidentified genetic alteration[c]
		Control	NA	EMS	ICR-170	Control[a]	HA	
5-8-417	1-7	0	0	0	0	0	0	non
5-9-155	1-10	0	0	0	0	0	1	non
5-8-278	1-10	1	0	21*[b]	0	0	0	TV
5-8-380	1-12	6	100*	16*	17*	25	44*	GC → AT
5-9-594	1-12	0	147*	25*	5	9	9	AT → GC
5-8-76	1-12	3	11*	104*	110*	10	12	AT → GC
5-8-152	1-12	0	7*	1	83*	0	0	AT → GC
5-8-41	1-12	0	0	1	337*	1	6	+/−
5-9-382	1-12	0	0	0	147*	0	0	+/−
5-9-33	1-12	0	0	1	49*	0	1	+/−
5-9-352	1-12	0	3	2	0	0	2	SP
5-9-335	1-12	0	0	0	0	1	0	non
5-9-591	1-12	0	1	0	0	0	0	non
5-9-407	1-13	0	139*	27*	140*	8	0	AT → GC
5-9-124	1-13	1	59*	1	1	6	9	AT → GC
5-9-21	1-13	0	8*	17*	9*	1	2	AT → GC
5-9-190	1-13	0	0	7*	947*	0	0	+/−
5-8-92	1-13	0	0	0	335*	0	1	+/−
5-8-335	1-13	0	0	0	63*	0	0	+/−
5-8-169	1-13	0	1	0	46*	0	1	+/−
5-9-181	1-13	0	2	0	2	0	0	SP
5-9-305	1-13	0	0	0	0	0	0	non
5-8-70	1-14	5	134*	33*	58*	12	11	AT → GC
5-8-185	1-14	9	104*	14	2355*	5	9	AT → GC
5-9-327	1-14	2	20*	4	2	4	7	AT → GC
5-8-167	1-14	0	4	2	0	1	1	SP
5-9-478	1-14	0	0	0	0	0	1	non
5-8-134	1-15	2	42*	56*	3	7	19	AT → GC
5-8-252	1-15	0	21*	3	19*	0	0	AT → GC
5-9-227	1-15	2	15*	3	4677*	3	0	AT → GC
5-8-133	1-15	0	10*	96*	2	1	0	AT → GC
5-8-337	1-15	0	0	2	109*	0	0	+/−
5-9-304	1-15	0	1	1	39*	0	0	+/−
5-8-213	1-15	0	1	1	7*	1	3	+/−
5-8-135	1-15	0	4	106*	3	0	0	TV
5-8-307	1-15	2	5	98*	5	2	0	TV
5-8-352	1-15	0	5	89*	1	11	7	TV
5-8-229	1-15	0	0	0	0	0	0	non
5-9-452	1-16	0	16*	41*	11*	1	0	AT → GC
5-9-568	1-16	1	12*	19*	23*	0	3	AT → GC
5-8-241	1-16	0	1	5	1297*	0	0	+/−
5-9-110	1-16	2	1	6	940*	2	2	+/−
5-8-266	1-16	0	0	7*	383*	0	0	+/−
5-9-527	1-16	0	0	4	313*	1	0	+/−
5-8-123	1-16	0	1	0	11*	0	0	+/−
5-9-55	1-16	1	0	4	0	1	0	SP
5-8-314	1-16	0	0	4	0	0	0	SP
5-9-521	1-16	0	2	0	0	0	1	SP

[a], [b], [c] See footnotes Table II.

REVERTIBILITY OF NONCOMPLEMENTING UV-INDUCED *ad-3B* MUTANTS IN *Neurospora crassa* AFTER TREATMENT WITH NA, EMS, ICR-170 AND HA

Mutant	Number of revertants per 10^8 conidia untreated or after treatment with						Reverse mutation mechanism or unidentified genetic alteration[c]
	Control	NA	EMS	ICR-170	Control[a]	HA	
5-8-22	7	109*[b]	2	4	3	14*	GC → AT
5-8-63	0	37*	19*	1	1	12*	GC → AT
5-8-66	0	0	0	0	0	9*	GC → AT
5-9-149	2	424*	2	0	4	1	AT → GC
5-8-40	6	162*	21*	55*	15	10	AT → GC
5-9-133	0	79*	6*	6*	4	9	AT → GC
5-8-27	2	75*	13*	12*	6	17	AT → GC
5-8-53	3	74*	3	1	10	4	AT → GC
5-8-24	1	47*	3	19*	3	3	AT → GC
5-9-154	0	36*	190*	13*	1	0	AT → GC
5-8-72	0	35*	144*	6*	0	3	AT → GC
5-8-48	3	21*	6	1	0	1	AT → GC
5-8-36	0	14*	9*	1	11	4	AT → GC
5-9-23	2	10*	12*	2	1	1	AT → GC
5-8-42	0	9*	8*	1441*	0	0	AT → GC
5-8-29	2	7	13*	3	2	4	TV
5-9-4	0	0	12*	19*	0	2	TV
5-9-187	1	7	12*	2548*	0	1	+/−
5-8-44	3	4	9	2342*	0	0	+/−
5-9-109	2	2	9	1688*	0	0	+/−
5-9-144	2	3	12*	1614*	3	0	+/−
5-8-14	3	0	3	1500*	3	1	+/−
5-9-58	0	1	1	1158*	3	1	+/−
5-9-43	0	0	0	705*	2	1	+/−
5-9-113	0	1	1	688*	0	0	+/−
5-9-114	0	0	0	602*	0	1	+/−
5-8-8	0	1	6*	483*	0	0	+/−
5-8-38	0	1	5	364*	3	1	+/−
5-9-95	1	2	9*	285*	0	0	+/−
5-8-31	0	1	1	141*	0	0	+/−
5-9-63	0	0	4	111*	0	1	+/−
5-8-71	0	1	1	93*	0	1	+/−
5-9-80	6	6	4	45*	0	0	+/−
5-8-32	1	1	2	43*	0	0	+/−
5-8-60	0	0	0	37*	0	0	+/−
5-9-119	0	0	0	13*	1	0	+/−
5-8-18	4	2	4	1	6	2	SP
5-8-12	1	7	3	6	0	0	SP
5-9-123	0	3	5	1	0	0	SP.
5-9-79	1	6	0	0	1	0	SP
5-8-50	1	3	0	0	2	1	SP
5-9-182	0	1	0	3	0	0	SP
5-8-25	0	1	2	0	0	0	SP
5-8-30	3	0	0	0	0	0	SP
5-8-62	0	0	2	0	0	1	SP
5-8-68	0	0	1	0	2	0	SP
5-8-37	1	1	0	0	0	0	SP
5-8-25	0	0	0	0	2	0	SP
5-9-107	1	0	1	0	0	0	SP
5-9-108	0	2	0	0	0	0	SP
5-9-103	0	0	1	0	0	0	non
5-8-51	0	0	0	0	0	0	non
5-8-68	0	0	0	0	0	0	non
5-9-64	0	0	0	0	0	0	non
5-9-85	0	0	0	0	0	0	non
5-9-143	0	0	0	0	0	0	non

a, b, c See footnotes Table II.

mutation frequencies with EMS are 2% or less of the frequency with ICR-170 at the same survival level before a mutant is placed in this category.

(3) The remaining mutants in Tables II–IV which failed to respond to the mutagens used here, may be subdivided on the basis of their spontaneous revertibility (SP) or by the fact that they do not revert (non). Mutants assigned to the latter class have no more than 1 colony scored in either control or treated series. Those mutants which failed to revert may represent instances of intralocal deletions involving small regions of the *ad-3B* gene.

Using these rules to interpret the revertibility tests, the data on all mutants from experiments 5-8 and 5-9 can be tabulated as a function of complementation pattern (Table V). The percentages of each type of complementation pattern were used to determine the percentages of each class of genetic alteration with that pattern.

TABLE V

THE FREQUENCY OF THE DIFFERENT TYPES OF UV-INDUCED *ad-3B* MUTANTS OF *Neurospora crassa* CHARACTE:
BY THEIR COMPLEMENTATION PATTERN AND TYPE OF GENETIC ALTERATION

Complementation pattern type	Percentage	Total mutants analyzed	Base-pair substitutions			Base-pair insertion or deletion $(+/-)$	Unidentified	
			Transitions		Trans-versions? (TV)		Reverting spontaneously (SP)	Non rever (non
			Mutant → Wild-type					
			$AT \to GC$	$GC \to AT$				
Nonpolarized	25.7	46	10.6 (19)[a]	4.5 (8)	3.4 (6)	2.2 (4)	5.0 (9)	0.0 (
Polarized	12.0	49	3.7 (15)	0.2 (1)	1.0 (4)	3.7 (15)	1.5 (6)	1.9 (
Noncomplementing	62.3	56	13.4 (12)	3.3 (3)	2.2 (2)	21.1 (19)	15.6 (14)	6.7 (
Total	100.0	151	27.7	8.0	6.6	27.0	22.1	8.6

[a] The number in parentheses is the actual number of mutants found.

3 points emerge from these data. The first is that a high percentage of nonpolarized mutants results from base-pair substitution ($33/46 = 71.7\%$) and if those mutants that only revert spontaneously ($9/46 = 19.6\%$) revert by base-pair transversion, then the base-pair substitution percentage may actually be higher ($42/46 = 91.3\%$). In addition, it is possible that 4 of the 5 mutants which have been classified as $+/-$ may be misclassified since they respond to NA and EMS as well as to ICR-170. It is also possible that they are especially sensitive to ICR-170 and reflect its ability to produce base-pair substitutions by alkylation.

The second point is that a mutant which has a polarized complementing pattern or is noncomplementing appears to have an equal chance of arising from base-pair substitution or base-pair addition or deletion. Finally, the third point is that, as might be anticipated, the nonrevertible mutants have polarized patterns or are noncomplementing.

These results agree well with the predictions based on the results of forward-mutation analyses with this system with chemical mutagens[14-18]. Agents, *e.g.* NA, which induce large numbers of nonpolarized mutants are also those which lead to base-pair substitution events in the gene. Such limited changes in the gene should lead to enzyme molecules with localized areas of damage which permit extensive complementation interactions. However, the above results indicate the reverse to be true; nonpolarized mutants induced by UV behave as base-pair substitution events in revertibility tests. ICR-170 appears to have a radically different action from NA

since it has been found that the majority of the mutants it induces are of the polarized and the noncomplementing types. The events it induces are assumed to be predominantly the addition or deletion of base-pairs. ICR-170 used in these reversion tests defines a definite class of mutants which fail to revert with agents causing base-pair substitutions. Very few of these mutants (2.2%) are of the nonpolarized complementing type. The results of the reversion tests indicate that both base-pair substitution and nonbase-pair substitution can give rise to enzyme alterations which either fail to complement or which produce polarized complementation patterns. This may be understood, as has already been pointed out, if these base-pair substitutions lead to nonsense codons and the nonbase-pair substitutions give frame-shift mutants.

Even though 30.7% of the *ad-3B* mutants are unidentified in these experiments (Table V), the result agrees surprisingly well with DRAKE's finding[7] that about half of the UV-induced rII mutants were the result of base-pair substitution and about half of base-pair addition or deletion.

Presently, no evidence has been found for the existence of suppressor mutations among *ad-3B* revertants. However, it is doubtful whether it can ever be determined from a genetic test if the site of a reversion is the same as the site of the original mutational damage. If this is assumed as it was with the rII mutants, a further comparison can be made between the bacteriophage data and those presented here. Only 12/69 or 17.4% of the base-pair substitution mutants of Neurospora revert significantly with HA. In the case of bacteriophage the percentage was even less than this, indicating a similarity in the principal UV target in these organisms. It has been assumed that the main activity of HA is to cause GC → AT transitions. Since the majority of mutants fail to react to HA, it may be suggested that they contain an AT base pair at the mutant site and that the action of UV was initially to cause GC → AT transitions.

It is rather premature to favor a single photochemical mechanism in UV mutagenesis to the exclusion of all others in eukaryotes. The excision of pyrimidine dimers could be an important source of mutation in Neurospora but there are as yet no conclusive data. It is also known that cytosine–cytosine and cytosine–thymine dimers can be found. Deamination of cytosine may then lead to uracil and a base-pair substitution event. Further experiments are required to assess the importance of this sequence of chemical events in the production of specific locus mutations.

REFERENCES

1 BIRNBAUM, A., Statistical methods for poisson processes and exponential populations, *J. Am. Statist. Ass.*, 49 (1954) 254.
2 DE SERRES, F. J., A procedure for making heterokaryon tests in liquid minimal medium, *Neurospora Newsletter*, 1 (1962) 9–10.
3 DE SERRES, F. J., AND H. G. KØLMARK, A direct method for determination of forward-mutation rates in *Neurospora crassa*, *Nature*, 182 (1958) 1249–1250.

4 DE SERRES, F. J., AND H. V. MALLING, Measurement of recessive lethal damage over the entire genome and at two specific loci in the *ad-3* region of *Neurospora crassa* with a two-component heterokaryon, in A. HOLLAENDER (Ed.), *Environmental Chemical Mutagens*, Plenum, New York, 1971.

5 DE SERRES, F. J., H. E. BROCKMAN, W. E. BARNETT AND H. G. KØLMARK, Allelic complementation among nitrous acid-induced *ad-3B* mutants of *Neurospora crassa*, *Mutation Res.*, 4 (1967) 415–424.

6 DE SERRES, F. J., H. E. BROCKMAN, W. E. BARNETT AND H. G. KØLMARK, Mutagen specificity in Neurospora, *Mutation Res.*, in the press.

7 DRAKE, J. W., Properties of ultraviolet-induced rII mutants of bacteriophage T4, *J. Mol. Biol.*, 6 (1963) 268–283.

8 HOROWITZ, N. H., Methionine synthesis in Neurospora, The isolation of cystathionine, *J. Biol. Chem.*, 171 (1947) 255–264.

9 HOWARD, B. D., AND I. TESSMAN, Identification of the altered bases in mutated single-stranded DNA, III. Mutagenesis by ultraviolet light, *J. Mol. Biol.*, 9 (1964) 372–375.

10 JAGGER, J., A small and inexpensive UV dose-rate meter useful in biological experiments, *Radiation Res.*, 14 (1961) 394–403.

11 KILBEY, B. J., AND F. J. DE SERRES, Quantitative and qualitative aspects of photoreactivation of premutational damage at the *ad-3* loci of *Neurospora crassa*, *Mutation Res.*, 4 (1967) 21–29.

12 MALLING, H. V., Hydroxylamine as a mutagenic agent for *Neurospora crassa*, *Mutation Res.*, 3 (1966) 470–476.

13 MALLING, H. V., Mutagenicity of methylhydroxylamines in *Neurospora crassa*, *Mutation Res.*, 4 (1967) 559–565.

14 MALLING, H. V., AND F. J. DE SERRES, Relation between complementation patterns and genetic alterations in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa*, *Mutation Res.*, 4 (1967) 425–440.

15 MALLING, H. V., AND F. J. DE SERRES, Correlation between base-pair transition and complementation pattern in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa*, *Mutation Res.*, 5 (1968) 359–371.

16 MALLING, H. V., AND F. J. DE SERRES, Identification of genetic alterations induced by ethylmethanesulfonate in *Neurospora crassa*, *Mutation Res.*, 6 (1968) 181–193.

17 MALLING, H. V., AND F. J. DE SERRES, Genetic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Neurospora crassa*, *Mol. Gen. Genet.*, 106 (1970) 195–207.

18 MALLING, H. V., AND F. J. DE SERRES, Hydroxylamine-induced purple adenine (*ad-3*) mutants in *Neurospora crassa*, I. Characterization of mutants by genetic tests, *Mutation Res.*, 12 (1971) 35–46.

19 STRACK, H. B., E. B. FREESE AND E. FREESE, Comparison of mutation and inactivation rates induced in bacteriophage and transforming DNA by various mutagens, *Mutation Res.*, 1 (1964) 10–21.

The Nature of Photoactivation in *Neurospora crassa*[1]

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Lethal effects produced by far ultraviolet light (254 nm) in *Neurospora crassa* conidia can be reversed by light of longer wavelengths (310 to 450 nm) administered after UV. Action spectra indicate that the most effective wavelengths for photoreactivation of *N. crassa* are in the 400-nm region, in contrast with *Escherichia coli* and *Streptomyces griseus*, where the peak efficiencies are around 365 nm and 440 nm, respectively. The rate of reactivation is dependent on the temperature during illumination. Extracts of *N. crassa* conidia with light of the proper wavelengths will repair UV-irradiated *Hemophilus influenzae* transforming DNA *in vitro*.

The action spectrum, temperature dependence, and ability of extracts to reverse UV damage *in vitro* suggest that photoreactivation of lethal damage in *N. crassa* is of the direct type, and involves light-dependent repair enzymes.

Neurospora crassa; Photoreactivation; Action Spectra; Extracts, Photoreactivation by

INTRODUCTION

Lethal damage produced in *Neurospora crassa* conidia by ultraviolet (UV) radiation of less than 300 nanometers (nm) can be partially repaired by posttreat-

ment with radiations of longer wavelengths (1, 2). This process, called photoreactivation, has been shown to involve a photoreactivating enzyme in *Escherichia coli* and *Saccharomyces cereviseae* (3, 4). The present paper presents evidence that extracts of *Neurospora* conidia will function in *in vitro* photoreactivation, suggesting that *Neurospora* also contains such an enzyme.

Several previous studies of photoreactivation of lethal damage and mutation have been carried out on *Neurospora*, but not with different wavelengths of monochromatic light. We have determined the action spectrum for photoreactivation in *Neurospora*. It was found to differ from those of *E. coli* (5) and *Streptomyces griseus* (6).

MATERIALS AND METHODS

Six strains of *Neurospora* were tested for photoreactivation. Macroconidial strains tested include *meth-7* (4894)*A*, *inos* (37401)*A*, *arg-5* (27947)*a*, *ad-3B* (5-8-700)*a*, and wild-type 74 Or-23-1a. A microconidial strain, *al-2, cot, pan-2A*, was also tested.

Cultures were grown on Vogel's medium N (7), supplemented where necessary with the required growth factor. The cultures were maintained at 30°C in the dark for 7 to 10 days before harvesting. Conidia were harvested in distilled water and filtered through cotton cheesecloth pads, and the resulting suspension was diluted to give 1 to 2 $\times$ 10^6 conidia per milliliter. Twenty-milliliter aliquots of conidial suspensions were exposed, with stirring, to a General Electric 15-watt germicidal lamp, which emitted $\sim$90% of the total radiant energy as a single line at 2537 A. The dose rate in all experiments was 33 ergs/mm^2/sec. Room illumination for all steps was supplied by General Electric "Gold" fluorescent lamps, with no emission below 500 nm. Two sources of photoreactivating radiation were used. A large quartz prism monochromator was used in experiments where specific wavelengths were desired; band-pass widths of 10 nm or less and dose rates of less than 100 ergs/mm^2/sec were used. Experiments at different dose rates indicated that recipcitory holds up to $\sim$100 ergs/mm^2/sec. The average dose through the sample was calculated according to Morowitz (8). For routine experiments, a battery of four General Electric H-5 medium-pressure mercury vapor lamps surrounding a thermostatically controlled glass water bath was used. In this apparatus, approximately 1800 ergs/mm^2/sec of radiant energy in the range of wavelengths active in photoreactivation reached the irradiated suspension. Maximum photoreactivation was produced by approximately 40,000 ergs/mm^2 at 405 nm, or by approximately 10 minutes of irradiation with the battery of lamps. All experiments were performed at least three times. The data in the figures (except Fig. 3) are from one typical experiment.

Conidial suspensions were always chilled to 1° to 2°C immediately following UV irradiation to prevent a gradual loss of photoreactivability which occurred at higher temperatures. Irradiation and photoreactivation were performed at 25°C unless otherwise indicated.
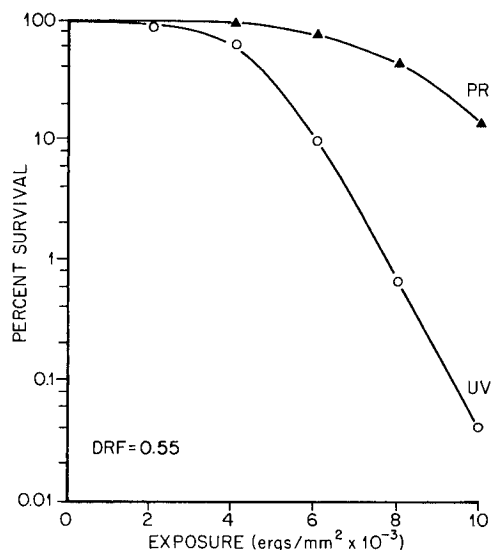
Fɪɢ. 1. Survival of colony-forming ability in *Neurospora crassa* as a function of UV dose, with and without maximum photoreactivation.

Treated conidia were suitably diluted and plated on Vogel's Medium N, supplemented as required, and containing 1 % sorbose according to the method of Brockman and de Serres (9).

Extracts of conidia were prepared by sonication of dense conidial suspensions in $M/15$ PO_4 buffer (pH 6.8) for approximately 5 minutes with a Biosonik probe. The resulting suspension was centrifuged for 15 minutes, the pellet was discarded, and the supernatant was either frozen immediately or filtered and then frozen. This solution was diluted to approximately 1 mg of protein per milliliter and mixed 1:1 with 1 μg of irradiated *Hemophilus influenzae* DNA per milliliter (10).

RESULTS AND DISCUSSION

Prevalence of Photoreactivation among the Strains Tested

All six strains of *Neurospora* tested showed photoreactivation. Consequently, only one of these strains, *meth-7(4894)A*, was selected for further study, and this is the strain used in all subsequent experiments reported here.

Effect of Photoreactivation on Survival

Typical survival curves for UV-irradiated conidia, with and without *maximum* photoreactivation (conditions used: 10 minutes with four mercury vapor lamps at 25°C), are presented in Fig. 1. The shoulder on the survival curves is at least partially due to the multinucleate nature of the conidia (11). Under these conditions, photoreactivation results in a constant dose-reduction factor (DRF) (12) of 0.55 (that is, the photoreactivable sector is 0.45). The constancy of the DRF was re-
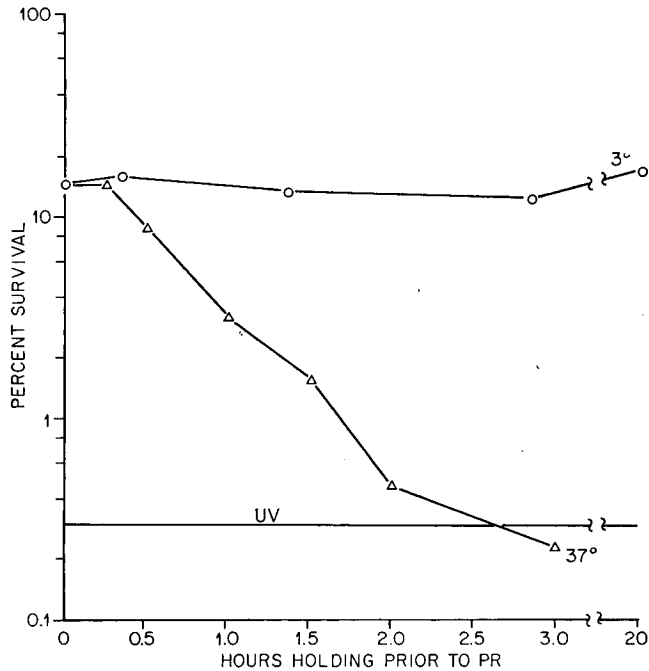
FIG. 2. Loss of photoreactivability following irradiation as a function of holding time in distilled water and temperature prior to photoreactivation.

ported earlier by Goodgal (1), although its value was not presented. This constant DRF indicates that the ratio of reversible to nonreversible damage is the same at all dose levels.

Loss of Photoreactivability in the Dark

Irradiated conidia lose their photoreactivability if held in the dark after irradiation. The rate at which this loss occurs is temperature-dependent. The following procedure was used to study this loss: Immediately after irradiation of an aqueous conidial suspension at 25°C, a sample was withdrawn and maximally photoreactivated. The remaining conidial suspension was halved; one fraction was held at 3°C, and the other was held at 37°C. Samples were withdrawn from these suspensions periodically and maximally photoreactivated. In distilled water at 37°C, the response to photoreactivating light is completely lost in 120 minutes, whereas at 3°C full responsiveness is retained for at least 20 hours (Fig. 2).

The nature of the events that render part of the damage progressively insensitive to photoreactivation is not known. One possibility is that photoreactivable damage in the DNA is excised, loss of photoreactivability in *E. coli* being correlated with excision (13).
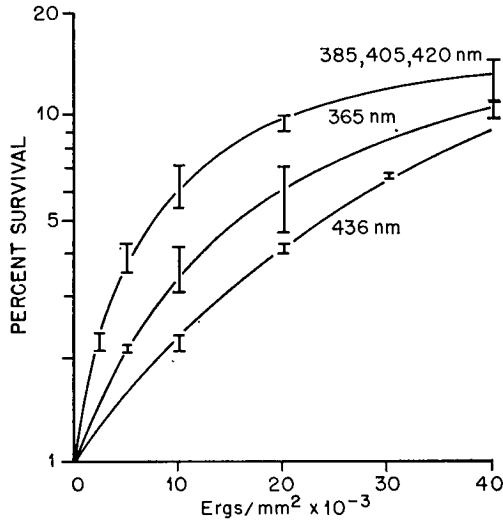
Fɪɢ. 3. Wavelength dependence of photoreactivation in *Neurospora*. The initial UV dose was 8000 ergs/mm², giving survival of approximately 1%. Data are from three experiments.

Action Spectra

When aliquots of a conidial suspension which had been previously irradiated with 254 nm were irradiated with various wavelengths (313 to 450 nm, with the intensity through the sample the same for all wavelengths, 33 ergs/mm²/sec in most experiments), the effectiveness in producing photoreactivation was found to be wavelength-dependent (Fig. 3). By taking a given level of photoreactivation in the lower portion of the curves, plotting the reciprocals of the incident energies necessary to produce that amount of photoreactivation at different wavelengths, and applying quantum corrections, an action spectrum was constructed (Fig. 4). This spectrum does not closely resemble that of either *E. coli* (5) or *S. griseus* (6). Large areas of overlap exist, but the *Neurospora* spectrum shows peak efficiency between the other two from 385 to 405 nm. At least two possible reasons exist for this difference. There may be absorption of some wavelengths by cellular components not involved in photoreactivation, thereby lowering the efficiency of those wavelengths. The possibility also exists that the chromophore for photoreactivation is different in these different systems. The action spectrum of *Neurospora* is difficult to correlate with the absorption spectrum of any single known cellular constituent. The peak efficiency of 405 nm does coincide with the Soret band of cytochrome c, although the shapes of the spectra are different. This similarity is made more interesting by the observations that cytochrome is associated with partially purified yeast photo-reactivating enzyme, although most cytochrome material is lost on further purifica-
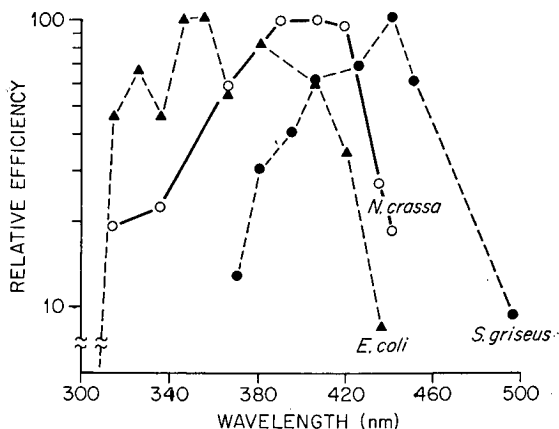
FIG. 4. Action spectrum for photoreactivation in *Neurospora*. Values are the reciprocals of the incident energies required to produce a given amount of photoreactivation at different wavelengths with quantum corrections applied. The action spectrum for *Neurospora* is superimposed on those of *E. coli* (5) and *S. griseus* (6). The wavelength of maximum efficiency for each spectrum is arbitrarily set as 100.

tion (*14*), and that photoreactivation in *Azotobacter* appears to be related to porphyrin content (*15*). Kelner (*6*) has noted that the spectrum of *S. griseus* suggests a porphyrin. The spectrum of *E. coli* does not suggest a porphyrin absorption, and Latarjet and Beljanski (*16*) found no correlation between porphyrin content and photoreactivability in this organism.

The *Neurospora* action spectrum coincides more closely with the spectrum for direct photoreactivation (*17*) than with indirect photoreactivation in *E. coli* B phr⁻, which shows a maximum at 334 nm (*18*). Also, activation energies of about 16 kcal were determined for photoreactivation in *Neurospora* for a 10-degree change between 25°C and 35°C (Fig. 5). These values are in broad agreement with those reported by Rupert *et al.* (*3*). The temperature dependence of photoreactivation in *Neurospora*, which suggests that the primary event is enzymatic, is compatible with direct photoreactivation but not with indirect photoreactivation, which is largely independent of temperature during illumination (*18*).

Photoreactivation of DNA by Extracts

Further evidence that photoreactivation is enzymatic in *Neurospora* comes from *in vitro* photoreactivation of *Hemophilus influenzae* transforming DNA with *Neurospora* extracts. These extracts showed photoreactivating activity (Fig. 6) roughly comparable (on the basis of mg of protein per milliliter) with that of crude yeast extracts (*19*). Setlow *et al.* (*20*) have shown that photoreactivation of irradiated transforming DNA by yeast extracts is at least partially due to photoenzymatic

cleavage of pyrimidine dimers. Since *Neurospora* extracts produce similar recovery, it may be assumed that the same photochemical events are involved in photoreactivation of *Neurospora* cells.
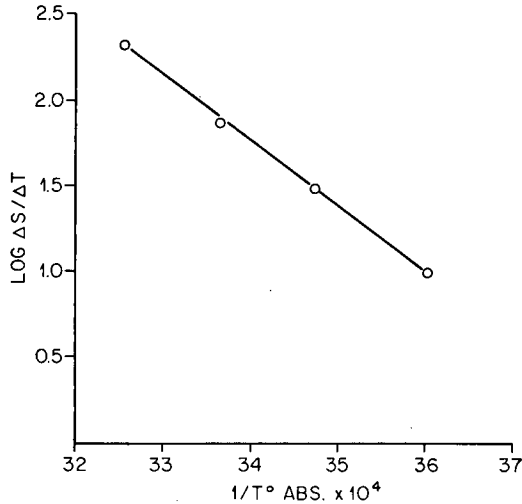

FIG. 5. The effect of temperature on the rate of photoreactivation. Data are plotted as log change in survival versus the reciprocal of the absolute temperature. The apparent activation energy is approximately 16 kcal.
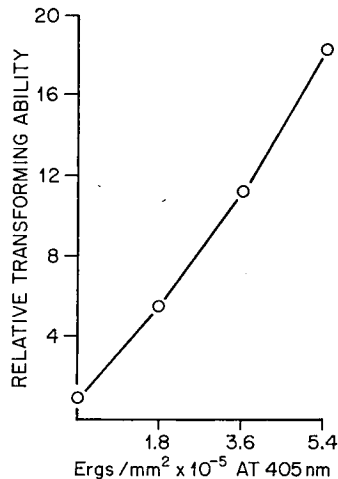

FIG. 6. Photoreactivation of *Hemophilus* transforming DNA inactivated by 2537-A irradiation. The marker transformed was streptomycin resistance. Photoreactivating wavelength was 405 nm (intensity 60 ergs/mm²/sec). Experimental procedure was as in Setlow and Boling (10). Samples contained approximately 0.5 mg of protein and 0.5 μg of DNA per milliliter.

SUMMARY

The action spectrum for photoreactivation in *Neurospora crassa* has been determined and does not coincide with that of either *E. coli* or *S. griseus*, although it overlaps both. No single chromophore is indicated by the spectrum, although the high efficiency of the 405-nm wavelength is in the region of the porphyrin Soret band absorption.

Photoreactivation in *Neurospora* is temperature-dependent and reduces UV lethal damage by a constant factor. Extracts of *Neurospora* cause *in vitro* photoreactivation of UV-irradiated transforming DNA. These observations indicate that there is direct enzymatic photoreactivation in *Neurospora crassa*.

ACKNOWLEDGMENTS

REFERENCES

1. S. H. GOODGAL, The effects of photoreactivation on the frequency of ultraviolet-induced morphological mutations in the macroconidial strain of *Neurospora crassa*. *Genetics* **35**, 667 (1950).

2. J. S. BROWN, The effect of photoreactivation on mutation frequency in *Neurospora*. *J. Bacteriol.* **62**, 163–167 (1951).

3. C. S. RUPERT, S. H. GOODGAL, and R. M. HERRIOTT, Photoreactivation *in vitro* of ultraviolet inactivated *Hemophilus influenzae* transforming factor. *J. Gen Physiol.* **43**, 451–471 (1958).

4. C. S. RUPERT, Photoreactivation of transforming DNA by an enzyme from baker's yeast. *J. Gen. Physiol.* **43**, 573–595 (1960).

5. J. JAGGER and R. LATARJET, Spectres d'action de la photo-restauration chez *E. coli* B/r. *Ann. Inst. Pasteur* **91**, 858–873 (1956).

6. A. KELNER, Action spectra for photoreactivation of ultraviolet-irradiated *Escherichia coli* and *Streptomyces griseus*. *J. Gen. Physiol.* **34**, 835–852 (1951).

7. H. J. VOGEL, A convenient growth medium for *Neurospora* (Medium N). *Microbial Genet. Bull.* **13**, 42–44 (1956).

8. H. J. MOROWITZ, Absorption effects in volume irradiation of microorganisms. *Science* **111**, 229–230 (1950).

9. H. E. BROCKMAN and F. J. DE SERRES, "Sorbose toxicity" in *Neurospora*. *Am. J. Botany* **50**, 709–714 (1964).

10. J. K. SETLOW and M. E. BOLING, The action spectrum of an *in vitro* DNA photoreactivation system. *Photochem. Photobiol.* **2**, 471–477 (1963).

11. K. C. ATWOOD and A. NORMAN, On the interpretation of multi-hit survival curves. *Proc. Natl. Acad. Sci. U.S.* **35**, 696–709 (1949).

12. J. JAGGER, Photoreactivation. *Bacteriol. Rev.* **22**, 99–142 (1958).

13. R. B. SETLOW and W. L. CARRIER, The disappearance of thymine dimers from DNA: an error correcting mechanism. *Proc. Natl. Acad. Sci. U.S.* **51**, 226–231 (1964).

14. A. MUHAMMED, Purification of photoreactivating enzyme. *Biophys. J.* **5**, (Suppl. 1), 112 (1965).

15. C. R. GOUCHER and W. KOCHOLATY, A comparison of the cytochrome structure and radiation effects in *Azotobacter*. *Arch. Biochem. Biophys*. **68**, 30–38 (1957).

16. R. LATARJET and M. BELJANSKI, Photo-restauration de bacteries depourves de porphyrines. *Ann. Inst. Pasteur* **90**, 127–132 (1956).

17. J. K. SETLOW, The molecular basis of biological effects of ultraviolet radiation and photoreactivation. In *Current Topics in Radiation Research*, (M. Ebert and A. Howard, eds.), Vol. 2, pp. 195–248, North-Holland Publishing Co., Amsterdam, 1966.

18. J. JAGGER and R. S. STAFFORD, Evidence for two mechanisms of photoreactivation in *Escherichia coli* B. *Biophys. J*. **5**, 75–88 (1965).

19. A. MUHAMMED, Studies on the yeast photoreactivating enzyme: I. A method for the large-scale purification and some properties of the enzyme. *J. Biol. Chem*. **241**, 516–523 (1966).

20. J. K. SETLOW, M. E. BOLING, and F. J. BOLLUM, The chemical nature of photoreactivable lesions in DNA. *Proc. Natl. Acad. Sci. U.S.* **53**, 1430–1436 (1965).

7

Loss of Photorepair Ability in Conidia of Neurospora crassa

PHOTOREPAIR in *Neurospora* conidia has been shown to be an enzyme process which operates on both lethal and mutagenic damage induced by ultra-violet light[1,2]. When irradiated conidia are incubated in the dark they lose their ability to respond to subsequent exposure to photo-repairing light. This loss is dependent on temperature and can be much reduced at temperatures approaching 0° C. It occurs as well on the surface of growth medium as in water. The main features of the phenomenon are shown in Fig. 1. The results here refer to survival, but essentially the same results were obtained for adenine reversion induced by ultra-violet light (Kilbey, unpublished experiments).

The purpose of this note is to examine various suggestions concerning the mechanism of the loss of photo-repair ability and to indicate the lines along which a solution is more likely to be attained. The two essential components of the system are (*A*) the photorepair mechanism which is made up of the photorepair enzyme and possibly an independent chromophore and (*B*) the
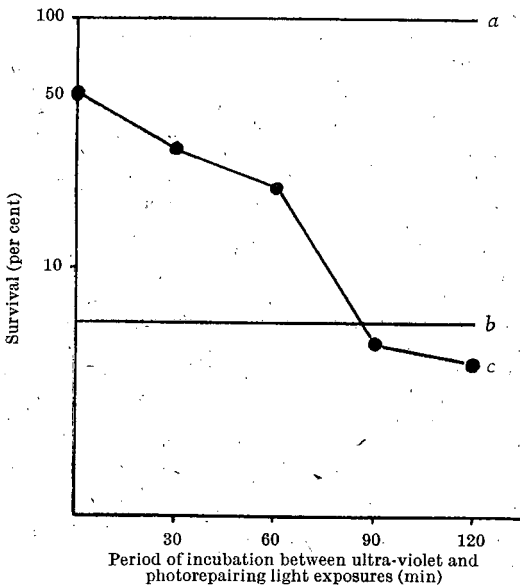

Fig. 1. The loss of photorepair ability with storage. *a,* Control survival; *b,* ultra-violet dark; *c,* ultra-violet + photorepair.
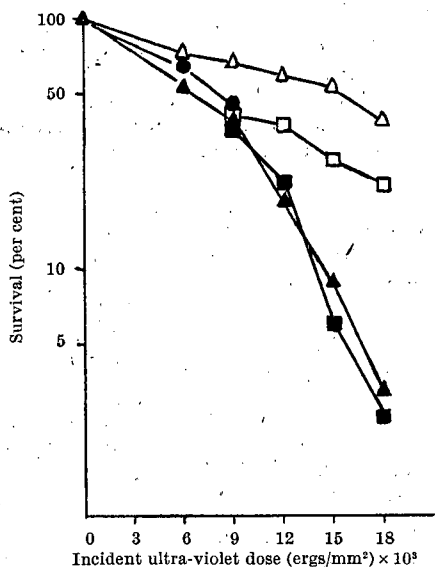
Fig. 2. Inactivation and photorepair in unfractionated and fractionated ultra-violet exposures (see text for explanation). Unfractionated: △, photorepair; ▲, dark. First fraction: ●, dark. Second fraction: □, photorepair; ■, dark.

substrate of the enzyme, the lesions, which are presumed to be pyrimidine dimers. Loss of photorepair ability can reasonably be attributed to a change in either of these components. The photorepair enzyme itself may be inactivated or an alteration may occur in the state of the lesions which prevents their repair by the photorepair enzyme. One can distinguish between these possibilities experimentally: if the photorepair mechanism itself is inactivated during post-irradiation incubation, the cells should be unable to photorepair lesions incurred from a further dose of ultra-violet light. To test this, two suspensions of conidia were prepared, each of 20 ml. and containing 6×10^6 conidia/ml. Both suspensions received a total incident dose of ultra-violet light of 18,000 ergs/mm² (measured directly beneath what was effectively a point source of ultra-violet light). The first suspension received this total dose in an uninterrupted exposure. The second suspension was exposed to two fractions each of 9,000 ergs/mm² separated by an interval of 3 h during which the suspension was incubated in the dark at 35° C. Samples of 0·1 ml. were withdrawn throughout the uninterrupted exposure and during the second half of the fractionated exposure in order to follow the course of survival with and without photorepair. Five experiments were carried out each of which gave similar results. An

Table 1. INACTIVATION AND PHOTOREPAIR IN UNFRACTIONATED AND FRACTIONATED DOSES OF ULTRA-VIOLET LIGHT

Ultra-violet dose (ergs/mm² ×10³)	Unfractionated				Ultra-violet dose (ergs/mm² ×10³)		Fractionated			
	Dark		Light				Dark		Light	
	Cols.	Survival (%)	Cols.	Survival (%)			Cols.	Survival (%)	Cols.	Survival (%)
0	8,470	100·0	—	—	Fraction 1	0	7,050	100·0	—	—
6	4,860	53·3	6,260	74·0		6	4,570	65·0	—	—
9	3,330	39·5	5,620	66·0		9	3,290	46·0	—	—
12	1,555	18·4	5,020	59·0	Fraction 2	9	2,560	36·0	2,920	41·4
15	744	8·8	4,590	53·0		12	1,564	22·0	2,620	37·4
18	269	3·2	3,310	39·0		15	423	6·0	1,950	27·6
						18	189	2·7	1,520	21·4

example of the type of result obtained is given in Table 1 and Fig. 2.

During the interval between the two ultra-violet fractions the ability to photorepair lesions induced by the first fraction is completely lost. In spite of this the lesions induced by the second fraction are photorepaired normally. The photoreactivable sector (1—dose reduction factor) differed slightly from experiment to experiment, but within each experiment the value was the same for the unfractionated dose and for the second half of the fractionated dose. These results are taken to indicate that the photorepair mechanism is not affected by incubation after irradiation and that the loss of photorepairability is the result of modification of the lesions themselves. At present direct evidence concerning the alterations of these lesions is lacking, but two suggested explanations may be considered. The first suggestion is that the activity of a dark repair process modifies the damage induced by ultra-violet light, rendering it inaccessible to photo-repair[2]. This might be the excision-repair type of process similar to that found in some bacteria. Close scrutiny of this suggestion makes this an unlikely explanation. No dark repair processes or any evidence of liquid holding recovery have been satisfactorily shown to exist in *Neurospora* to date. In any case, the activity of a dark repair acting on the same lesions as those which are potentially photoreactivable might be expected to mask photorepair itself. The second suggestion is that ultra-violet damage which leads both to lethality and mutation becomes stabilized towards photorepair when DNA replication takes place. At present independent results concerning the timing of DNA synthesis are difficult to obtain for *Neurospora* conidia for technical reasons. An indirect approach to the problem of DNA replication is possible, however, and this may be able to provide evidence of a correlation between DNA replication and the loss of photorepair ability.

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[1] Kilbey, B. J., and de Serres, F. J., *Mutation Res.*, **4**, 21 (1967).
[2] Terry, C. E., Kilbey, B. J., and Branch-Howe, jun., H., *Radiat. Res.*, **30**, 739 (1967).

QUANTITATIVE AND QUALITATIVE ASPECTS OF PHOTOREACTIVATION OF PREMUTATIONAL ULTRAVIOLET DAMAGE AT THE *ad-3* LOCI OF *NEUROSPORA CRASSA*

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SUMMARY

Experiments have been carried out to determine whether photoreactivation is specific for a particular fraction of the UV-induced premutational damage leading to *ad-3B* mutants in *Neurospora*. Populations of mutants were compared which had been induced by UV in the presence and absence of photoreactivation. In spite of the reduction in mutation frequency brought about by photoreactivation, no evidence was found to suggest that any part of the mutant population was repaired preferentially. The simplest interpretation of this result is that there is but one type of premutational lesion induced by UV in this system. Repair of a fraction of this damage should affect all types of mutants similarly. Alternatively, if more than one type of premutational lesion exist, some of which are nonphotoreactivable, it is necessary to assume that, in terms of the mutants they are capable of producing, the photoreactivable and non-photoreactivable lesions are equivalent.

INTRODUCTION

Photoreactivation of the lethal and mutagenic effects of ultraviolet radiation in bacteria is a well-documented phenomenon [9]. Studies using *Neurospora* also demonstrated the capacity of this organism to exhibit photoreactivation of both lethal and mutagenic effects [4,8]. Recent studies have confirmed the earlier ones and have extended the observations to include several *Neurospora* strains. In addition, details of the action spectrum for photoreactivation in this fungus have been obtained [13].

In all the work preceding this report, attention has been centered almost exclusively on the quantitative aspects of the phenomenon. In all cases studied, it has proved impossible to achieve complete photoreactivation, and a part of the damage remains unaffected. This is true even in the case of experiments with transforming

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principle where the nonphotoreactivable damage is sometimes very small[11]. The impossibility of attaining complete photorepair of lethal and mutagenic damage raises the important question of whether there is a qualitative difference between damage which is photoreactivable and damage which is not. This question is particularly relevant to premutational damage since there is still little known about the induction of mutations by UV, and photoreactivation may provide a useful tool in its further analysis. Besides this, the specificity of photorepair must be put in the wider context of the specificity of other repair systems which may act on premutational damage since this has an important place in discussions of mutagen specificity.

In order to study the qualitative aspects of photoreactivation at the biological level, a way of isolating mutants at known genetic loci was required. In addition, means were required for the detailed analysis of these mutants with respect to the genetic changes induced in them. *Neurospora* offers this facility in the *ad-3* system developed by DE SERRES AND KØLMARK[7]. Mutants at the *ad-3A* and the *ad-3B* loci can be isolated on a basis of their purple phenotype when grown in the presence of limiting adenine. Tests of complementation and specific reversibility can then be applied to characterize these mutants with respect to the genetic alterations that each carries. Tests of this type have been applied to a set of UV-induced *ad-3B* mutants in order to characterize them, and it has been found that UV induces a variety of clearly distinguishable mutant types (unpublished data of B. J. KILBEY, F. J. DE SERRES AND H. V. MALLING.)

In the present study, mutants were isolated after treating conidia of a wild-type strain of *Neurospora* with various doses of UV, each of which was either followed or not followed with a dose of photoreactivating light sufficiently large to give maximum photoreactivation of survival (B. J. KILBEY, unpublished data). The questions for which answers were sought were: *(1)* What effect does photoreactivation have on the frequency of mutation at the *ad-3A* and the *ad-3B* loci? *(2)* Is photoreactivation specific for any particular class or classes of mutants? *(3)* Can any conclusions be drawn concerning the nature of the mutation-induction process by UV in *Neurospora*?

MATERIALS AND METHODS

Strain

The strain used throughout this study was a wild-type macroconidiating strain of *Neurospora* designated by the symbol 74–OR23-1A (see ref. 5). Cultures of this strain were prepared from single-colony isolates grown on Fries' glycerol complete medium[1] in 150-ml Erlenmeyer flasks. The flasks were incubated for 1 day at 30° and subsequently for 6 days at 25°. From the time of inoculation onwards, the cultures were maintained in the dark. Conidial suspensions were prepared by harvesting conidia into distilled sterile water and filtering the resultant suspension twice through a layer of cotton. The filtered suspension was washed twice with centrifugation, and the conidial density adjusted to give $2 \cdot 10^6$ conidia per ml.

Exposure and postirradiation treatment

30-ml aliquots of this suspension were irradiated separately with constant stirring in open petri plates 100 mm in diameter. The irradiation was done using a G.E.

germicidal lamp (15 W No. G15-T8) at a dose rate of 18.5 erg · mm^{-2} · sec^{-1} as measured by a Jagger-type dose rate meter[10]. The temperature during irradiation was 20°. During and after irradiation, light was provided for general illumination from 15-W G.E. "Gold" lamps (F 15T8 Go) which emit light of wavelengths longer than 500 Å.

Following exposure to UV, each suspension was divided into 2 parts. One of these was transferred to a tube completely enclosed in aluminum foil while the other was placed in a similar tube without the foil. Both tubes were placed in a water bath at a distance of 8.5 cm from a 300-W incandescent lamp for a period of 35 min. The temperature of the suspensions during postirradiation treatment was 35°. Under these conditions, it has been shown that maximum photoreactivation of survival is obtained (unpublished data). Presumably photoreactivation of mutation is also maximal.

Mutant isolation

Treated conidia were inoculated and incubated following the method cited earlier[7]. After 7–8 days' incubation, purple colonies were isolated from the contents of the mutation flasks and aliquots were taken in order to estimate the survival after treatment. Each mutant was first made homokaryotic by repeated plating on Fries' minimal salts medium supplemented with 1.0% sorbose, 0.1% sucrose, and 2 μg/ml adenine sulfate. On this medium, adenine-requiring homokaryotic mutants are morphologically distinguishable.

Tests of complementation

Tests of functional allelism were carried out with each mutant in order to determine whether it was a mutant at the *ad-3A* or the *ad-3B* locus. For this purpose, conidia from a culture of each unknown mutant were mixed in turn with conidia from a known *ad-3A* mutant and a known noncomplementing *ad-3B* mutant. The tests were carried out in Fries' liquid minimal medium supplemented with 0.1 μg adenine sulfate per ml. The *ad-3B* mutants were tested further to determine the complementation pattern of each mutant. For this purpose each mutant was tested against a set of mutants which together define a complementation map of 17 complementation units[3]. As a control for leakiness, each mutant was also tested alone for growth in the absence of adenine. The tests were performed in Fries' liquid minimal medium supplemented with 1.5% sucrose and with 2 μg of calcium pantothenate and 0.1 μg of adenine sulfate per ml. Each liter of this medium contained 200 mg casamino acids. Usually conidial suspensions were prepared in this medium at a density of 10^5 conidia per ml, although in a few cases the density was reduced to 10^4 conidia per ml in an attempt to overcome leakiness on the part of some mutants.

RESULTS

(a) Quantitative data

(1) Survival. In these experiments all the UV treatments used to produce adenine mutants resulted in survival values above 35% (Fig. 1a). Because of this, increases in survival brought about by photoreactivation are not so marked as those observed at lower survival levels. Calculations of the dose-reduction factor

$$\frac{\text{dose for a given survival in the dark}}{\text{dose for the same survival in the light}}$$

have been made at various survival levels. At survivals of 84%, 65%, and 50%, the dose-reduction factors were 0.5, 0.6 and 0.7, respectively. These figures agree fairly well with the figure of 0.55 obtained earlier for photoreactivation of survival using other strains of *Neurospora crassa* and alternative sources of photoreactivating light[13].

(2) Mutation. Mutants were isolated following incident UV doses varying from 1000–4800 erg/mm² with photoreactivation and from 1000–3500 erg/mm² without photoreactivation. All of these points fall on the ascending part of the curves plotted of mutation × 10⁻⁶ survivors *versus* dose of UV (Fig. 1b).
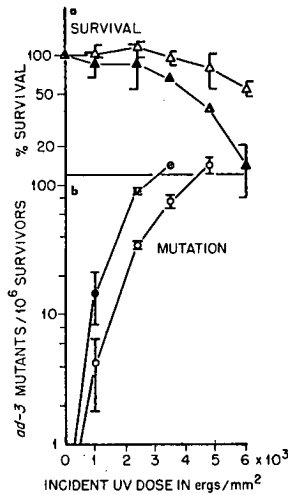

Fig. 1. Survival curves and curves for mutation induction at the *ad-3* loci in strain 74-OR23A1 after treatment with UV with and without photoreactivation. ▲, dark; △, photoreactivated. ●, dark; ○, photoreactivated.

For all the UV doses for which a comparison can be made, the effect of photo-reactivation is to cause a marked reduction in the frequency of mutants in the surviving population. The absolute numbers of mutants were also reduced following photoreactivation, thus ruling out any trivial explanation of the effect based on selection. For forward-mutation frequencies of 35, 75, and 107 · 10⁻⁶ survivors, the dose-reduction factors for mutation

$$\frac{\text{dose required in the dark for a given mutation frequency}}{\text{dose required in the light for the same mutation frequency}}$$

are 0.7, 0.6, and 0.7, respectively. These values are probably not significantly higher than those obtained for survival.

The classification of adenine mutants into the *ad-3A* and *ad-3B* types permits the mutation induction curves of Fig. 1 to be resolved into their component parts. This has been done in Fig. 2. Approximately twice as many *ad-3B* mutants are induced as *ad-3A* mutants but mutation at both loci is similarly affected by photo-reactivation.
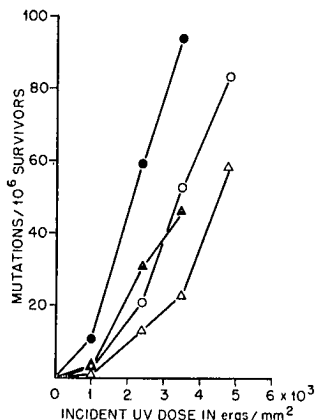
Fig. 2. The contributions from the *ad-3A* and the *ad-3B* loci to the total *ad-3* mutation rate after UV treatment. *ad-3B:* ●, dark; ○, photoreactived. *ad-3A:* ▲, dark, △, photoreactivated.

b) Qualitative data

For the qualitative analysis approximately 250 *ad-3* mutants were isolated from each of the points shown in Fig. 1. At the lowest dose, however, particularly in photoreactivated samples, the frequency of adenine mutants was too small for this, and all the mutants induced were isolated for analysis. In the first place each mutant was classified as either an *ad-3A* or an *ad-3B* mutant as described earlier. Since the *ad-3A* mutants fail to show complementation, only the *ad-3B* mutants were studied further.

It is becoming increasingly evident that the nature of the molecular change which results in a particular mutant is reflected in the type of complementation pattern the mutant displays. Three types of patterns have been described[6]. These are the noncomplementing mutants, the polarized-complementing class, and the nonpolarized-complementing class. The complementing mutants can, of course, be subdivided further on a basis of their detailed complementation behavior. All the data at present available indicate clearly that nonpolarized-complementing mutants arise exclusively from base-pair substitution events. Polarized-complementing mutants and noncomplementing mutants on the other hand may result from base-pair substitution events but in addition may result from nonbase-pair substitution types of change, some or all of which may be addition or deletion mutations. Complementation would appear to be an adequate test for qualitative similarity between two populations of adenine mutants. Whereas it might not detect subtle changes in the frequencies of, say, base-pair substitutions resulting from different mutagenic treatments, it is quite sensitive enough to detect a qualitative change of the extent required to match the quantitative change resulting from photoreactivation. For this reason, and because of the simplicity of the test, complementation was used exclusively to determine whether qualitative differences occurred between the populations of UV-induced adenine mutants as a result of photoreactivation.

In order to facilitate the division of the adenine mutants into their various complementional types, a set of tester mutants was used (see earlier). From the patterns of growth response shown by each unknown with the test mutants, it was pos-

sible to achieve an unambiguous separation of the adenine mutants into noncomplementing mutants and polarized- and nonpolarized-complementing mutants. In most cases, the tests also enabled most of the mutants to be classified further on a basis of the complementation pattern each displayed.

In all, approximately 870 mutants were tested in this way. The results of the division of these mutants into noncomplementing, polarized-, and nonpolarized-complementing classes are shown in Table I. Although slight fluctuations occurred

TABLE I

THE NUMBERS AND PERCENTAGES OF POLARIZED- AND NONPOLARIZED-COMPLEMENTING MUTANTS OBTAINED AFTER EACH UV DOSE WITH AND WITHOUT PHOTOREACTIVATION

Incident energy (erg/mm²)	Total mutants			Complementing mutants		
	Non-complementing	Complementing	Total	Non-polarized	Polarized	Total
1000 D	32 (54%)	27 (46%)	59 (100%)	23 (85%)	4 (15%)	27 (100%)
L	16 (50%)	16 (50%)	32 (100%)	9 (56%)	7 (43%)	16 (100%)
2400 D	88 (56%)	68 (44%)	156 (100%)	43 (64%)	25 (36%)	68 (100%)
L	72 (50%)	73 (50%)	145 (100%)	54 (73%)	19 (26%)	73 (100%)
3500 D	97 (55%)	80 (45%)	177 (100%)	57 (71%)	23 (29%)	80 (100%)
L	99 (53%)	89 (47%)	188 (100%)	65 (74%)	24 (26%)	89 (100%)
4800 D	—	—	—	—	—	—
L	74 (52%)	68 (48%)	142 (100%)	50 (74%)	18 (26%)	68 (100%)

from group to group, no significant difference could be detected between the various mutant populations either as the UV dose increased or as a result of photoreactivation. A more detailed analysis of the patterns obtained among the complementing mutants also failed to reveal any significant differences resulting from photoreactivation (Table II). There was also no change in the average complon coverage of either the polarized or the nonpolarized mutants; neither did the frequency of leaky mutant change, although the data for mutants of this type were rather scanty.

DISCUSSION

The foregoing results can be summarized as follows:

(1) Both the mutagenic effects of UV (as measured by mutation at the ad-3A and ad-3B loci of Neurospora) and its lethal effects are subject to repair from photoreactivation. Mutation at both the ad-3A and the ad-3B loci is similarly affected by photoreactivation.

(2) Reduction of the mutagenic effects of UV by photoreactivation is not accompanied by a detectable change in the composition of the mutant population before and after repair. From the point of view of complementation, at least, photorepair is not specific for a particular class or classes of ad-3B mutant.

(3) There is also no change in the composition of the mutant population as the dose of UV used to induce the mutants is increased within the ranges used.

There is probably no significant difference between the photoreactivable sector for forward-mutation and killing. However, this alone does not permit a decision as

TABLE II

AN ANALYSIS OF THE PATTERNS OF COMPLEMENTATION FOUND AMONG COMPLEMENTING ad-$3B$ MUTANTS INDUCED BY UV TREATMENT

Complemen-ation patterns^a	Mutagenic conditions (erg/mm²)							Total
	1000 dark	1000 light	2400 dark	2400 light	3500 dark	3500 light	4800 light	
:	—	—	—	—	1	—	—	1
-7	1	—	—	—	—	—	—	1
-10	—	—	—	1	—	1	—	2
-11	—	—	1	—	—	—	—	1
-13	—	4	8	7	6	10	2	37
-14	2	1	5	4	4	4	6	36
-15	—	—	7	4	6	4	3	24
-16	1	—	3	2	5	3	4	18
-4	1	—	1	3	3	3	1	12
-5	—	1	—	2	—	—	2	5
-6	—	—	—	1	—	—	—	1
-7	—	—	1	2	—	—	—	3
-8	—	—	2	—	—	1	—	3
-10	—	1	—	—	—	—	—	1
-11	4	—	1	—	4	2	—	11
-15	—	—	—	—	1	—	—	1
-7	1	1	—	1	1	2	—	6
-10	1	1	1	3	2	5	2	15
-14	—	—	—	2	2	2	—	6
-15	1	1	—	2	2	4	3	13
-16	—	—	—	1	—	1	1	3
-17	—	1	—	1	—	—	—	2
-10	—	—	—	—	1	1	—	2
-13	—	—	—	2	—	4	—	6
-14	—	—	1	1	3	—	6	11
-15	—	—	6	3	4	—	—	13
-11	—	—	1	2	—	—	—	3
-14	—	—	—	—	—	—	1	1
-15	—	1	—	—	—	—	—	1
-16	1	—	2	—	1	1	—	5
)	—	—	—	—	—	1	—	1
)-11	3	3	2	3	2	3	4	22
)-13	1	1	2	5	3	4	6	22
)-14	—	—	—	1	—	3	—	4
)-15	—	—	—	3	5	—	1	9
2-13	1	—	4	2	2	6	4	19
		—	—	—	—	—	1	—	1
|-17	1	—	—	—	1	—	—	2
;	—	—	7	6	4	7	5	29
;-17	—	—	4	—	1	6	2	11
)-17	2	—	1	—	—	2	1	6
								368

The complementation map defined by the tester mutants used is divided into 17 units or com-plons. These are numbered sequentially from left to right and the numbers in this column refer to the particular complons each type of mutant covers on this map.

whether mutations and lethality result from different classes of UV damages or not. is hoped that further information on this point will be provided by experiments currently in progress in which a genetically-marked heterokaryon[14] is being used to

study the effectiveness of photoreactivation in repairing UV damage giving rise to *ad-3* mutants either by chromosomal deletion or by point mutation.

In spite of the considerable reduction in mutation frequency which results from photoreactivation following UV, there are no indications that the repair has concerned a particular fraction of the premutational damage. If a particular type of premutational damage is repaired preferentially, the specificity is not reflected in the types of mutants obtained with and without photoreactivation. In earlier experiments, it has been shown that there is a close correlation between the origin of *ad-3B* mutants and the types of complementation patterns they exhibit[6]. In general, mutagens which are believed to act by inducing base-pair substitutions induce a predominance of nonpolarized-complementing mutants. Mutagens which act in other ways, *e.g.*, acridine mustard, have been found to induce very few mutants of this type (ref. 2; personal communication from H. V. MALLING). The converse is also true; mutants with nonpolarized-complementation patterns behave as base-pair substitutions in tests of specific reversibility (unpublished data of B. J. KILBEY, F. J. DE SERRES AND H. V. MALLING). Nonbase-pair substitution mutants appear to be confined to the classes which exhibit polarized-complementing patterns or which fail to complement at all. These considerations point to the conclusion that the marked reduction in mutation frequency which occurs with photoreactivation is not the result of the repair of a specific fraction of the population of mutants induced by UV. The simplest interpretation of this result is that a single type of premutational lesion is induced by UV which is in some way transformed with definite probabilities into one or the other of the mutant types observed. Repair of a fraction of this premutational damage should have no effect on the composition of the mutant population. Alternatively, several premutational lesions might be postulated, some of which are nonphotoreactivable. In this case it is necessary to assume that they are equivalent in terms of the mutations they can give rise to.

Throughout this paper, the assumption has been made that the photoreactivation observed is of the "direct" enzymatic type which is believed to involve the splitting of pyrimidine dimers[12]. This assumption is based on the following evidence: *(a)* An enzyme has been obtained from *Neurospora* which has the ability to repair UV-damaged DNA *in vitro* in the presence of photoreactivating light[13]. *(b)* The action spectrum for *in vitro* photoreactivation (*i.e.*, direct photoreactivation only) is similar to that for *in vivo* photoreactivation (personal communication from J. K. SETLOW AND C. E. TERRY). If photoreactivation acts in *Neurospora* only by splitting pyrimidine dimers enzymatically, it might be concluded that pyrimidine dimerization can cause all the different types of UV-induced mutants observed here. Whether this means that nonphotoreactivable mutations also result from pyrimidine dimers which somehow escape photoreactivation cannot be decided at present.

In conclusion, it can be said that photoreactivation of forward mutation at the *ad-3* loci of *Neurospora* results in a marked reduction in mutation frequency. However, no evidence could be obtained that specific types of mutants were repaired preferentially. By making some reasonable assumptions, it is possible to conclude that pyrimidine dimerization can cause all the mutational *types* observed since mutants of all types are lost on photoreactivation. This does not necessarily mean that pyrimidine dimers are responsible for *all mutations* induced at these loci.

REFERENCES

1 BEADLE, G. W., AND E. L. TATUM, *Neurospora*, II. Methods of producing and detecting mutations concerned with nutritional requirements, *Am. J. Botany*, 32 (1945) 678–686.
2 BROCKMAN, H. E., Mutagenicity of a monofunctional alkylating derivative of acridine in *Neurospora*, *Science*, 147 (1965) 750–751.
3 BROCKMAN, H. E., AND F. J. DE SERRES, Induction of *ad-3* mutants of *Neurospora crassa* by 2-aminopurine, *Genetics*, 48 (1963) 597–604.
4 BROWN, J. S., The effect of photoreactivation on mutation frequency in *Neurospora*, *J. Bacteriol.*, 62 (1951) 163–167.
5 CASE, M. E., H. E. BROCKMAN AND F. J. DE SERRES, Further information on the origin of the Yale and Oak Ridge wild-type strains of *Neurospora crassa*, *Neurospora Newsletter*, 8 (1965) 25–26.
6 DE SERRES, F. J., Mutagenesis and chromosome structure, *J. Cellular Comp. Physiol.*, 64, Suppl. 1 (1964) 33–42.
7 DE SERRES, F. J., AND H. G. KØLMARK, A direct method for determination of forward-mutation rates in *N. crassa*, *Nature*, 182 (1958) 1249–1250.
8 GOODGAL, S. H., The effect of photoreactivating light on the survival and frequency of mutations in UV-irradiated microconidia of *N. crassa*, Ph. D. Thesis, Johns Hopkins University, Baltimore, Md., 1950.
9 JAGGER, J., Photoreactivation, *Bacteriol. Rev.*, 22 (1958) 99.
10 JAGGER, J., A small and inexpensive UV dose-rate meter useful in biological experiments, *Radiation Res.*, 14 (1961) 394–403.
11 SETLOW, J. K., The wavelength-dependent fraction of biological damage due to thymine dimers and other types of lesion in UV-irradiated DNA, *Photochem. Photobiol.*, 2 (1963) 393–399.
12 For summary and references see: J. K. SETLOW, The molecular basis of biological effects of UV radiation and photoreactivation, in M. EBERT AND A. HOWARD (Eds.), *Current Topics in Radiation Research*, Vol. II, North-Holland, Amsterdam, in the press.
13 TERRY, C. E., B. J. KILBEY AND H. BRANCH HOWE JR., The mechanism of photoreactivation in *Neurospora crassa*, *Radiation Res.*, submitted for publication.
14 WEBBER, B. B., AND F. J. DE SERRES, Induction kinetics and genetic analysis of X-ray induced mutations in the *ad-3* region of *N. crassa*, *Proc. Natl. Acad. Sci. (U.S.)*, 53 (1965) 430–437.

DIFFERENTIAL PHOTOREVERSIBILITY OF ULTRAVIOLET-INDUCED PREMUTATIONAL LESIONS IN NEUROSPORA*

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SUMMARY

The genetic effects of UV light (UV) and UV followed by photoreactivation (UV + PR) have been studied in *Neurospora crassa*. A two-component heterokaryon was used to examine the effect of these 2 treatments on (1) inactivation of the heterokaryotic conidia, and (2) the induction of recessive lethal mutations at 2 specific loci. The heterokaryon is heterozygous for 2 closely linked loci, *ad-3A* and *ad-3B*, in the *ad-3* region. The specific-locus mutations can result from either point mutation or chromosome deletion. After UV or UV + PR both point mutations and chromosome deletions were recovered in a ratio of about 24:1. Point mutations and chromosome deletions both increase as the square of the dose, so the spectrum of UV-induced mutation is dose-independent. These experiments also show that PR results in dose-reduction factors (DRFs) of about 0.6 at 60% survival for inactivation of conidia and 0.66 for the overall induction of *ad-3* mutations. Genetic characterization of the *ad-3* mutations showed no difference in the effect of PR on point mutations at the *ad-3A* or *ad-3B* locus; all subclasses of point mutations show a mean DRF of about 0.66. *Ad-3* mutations resulting from chromosome deletion, however, show a significantly lower mean DRF of about 0.50. These results show that PR has a greater effect on damage leading to chromosome deletions than on damage leading to point mutations. The difference between the DRFs obtained for point mutations and chromosome deletions is interpreted as an indication that they either result from qualitatively different lesions or from the same lesions repaired differentially.

We[17] have already shown in an experiment with a haploid wild-type strain that photo-repair of that UV-induced premutational damage resulting in point mutations in the *ad-3* region (*ad-3*[R]) affects all types of mutants similarly. No difference was found between the spectra of *ad-3* mutants induced by UV alone or UV followed by PR with regard to the ratio of *ad-3A* to *ad-3B* mutants or the percentages and types of complementation patterns among *ad-3B* mutants. Forward mutations in the

ad-3 region in a two-component heterokaryon, however, result both from alteration of these genes (point mutation) as well as their physical removal from the chromosome[12] (chromosome deletion, *ad-3*[IR]). These 2 types of mutation respond very differently to high (1000 R/min) and low (10 R/min) dose rates of X-irradiation[13]; with a 100-fold reduction in dose rate there was a 10-fold reduction in the frequency of *ad-3*[IR] mutations but no change in the frequency of *ad-3*[R] mutations. This is the first line of experimental evidence indicating that these 2 classes of mutation may be subject to different mechanisms of repair.

In the present experiment with a two-component heterokaryon we are attempting to study the effect of PR on UV-induced damage leading to *ad-3*[IR] mutations, to answer the following questions: What are the kinetics of induction of this type of damage? Is this type of damage repaired with PR? Is it repaired with the same efficiency as the damage that produces *ad-3*[R] mutations?

MATERIALS AND METHODS

Strains

The strain used for this study was a two-component heterokaryon (wild-type strain 12) with the following genetic markers in each component: I (strain 74-OR60-29A), *A, hist-2, ad-3A, ad-3B, nic-2, ad-2, inos*; and II (strain 74-OR31-16A), *A, al-2, cot, pan-2*. The use of this heterokaryon for assay of inactivation and mutation induction has been described previously[24].

Preparation of experimental material

Cultures were grown from single-colony isolates of heterokaryotic colonies after the conidia had been plated from a silica-gel stock culture of strain 12 on minimal medium. Conidia were harvested from 7-day-old cultures grown for 3 days at 35° and 4 days at 23°. Sterile distilled water suspensions were filtered 5 times through layers of cotton and then washed by centrifuging twice. The washed suspensions were adjusted to a count of $4.5 \cdot 10^6$ conidia/ml and kept at ice-water temperature prior to use.

Exposure to UV and postirradiation treatment

The conidial suspension was irradiated at room temperature (24–25°) in open petri dishes, 85 mm in diameter, with stirring provided by a magnetic stirrer. Irradiation was performed with a G. E. Germicidal Lamp (15 W, No. G15-T8) at a dose rate of 21.27 erg·mm^{-2}·sec^{-1} as measured by a Jagger-type dose rate meter[15]. All suspensions were kept in the dark following irradiation and cooled to ice-water temperature.

Each suspension was then divided into 2 parts, one to a tube completely enclosed in aluminum foil and the other to a tube without the foil. Both tubes were placed in a water bath at 30° at a distance of 8.5 cm from a 300-W incandescent lamp for a period of 35 min.

Isolation of ad-3 mutants and measurement of survival

The technique used in forward-mutation experiments to determine the frequency of recessive lethal mutations in the *ad-3* region in a two-component heterokaryon

have been summarized[11] and will be mentioned only briefly here. Treated conidia were inoculated into 12-l Florence flasks containing 10 l of medium in order to isolate *ad-3* mutants by the direct method[10]. After 7–8 days' incubation, purple colonies were isolated from each flask and aliquots of the background colonies were taken so that estimates of the total number of colonies per flask could be made. Calculations can then be done to estimate the frequency of *ad-3* mutations and survival of the heterokaryotic fraction of conidia. Each purple colony was subcultured and made homokaryotic for the adenine requirement by repeated plating on Fries' minimal medium supplemented with 1.0% sorbose, 0.1% sucrose, and 2 μg/ml adenine sulfate. On this medium, adenine-requiring homokaryotic mutants are morphologically distinguishable.

Genetic analysis of ad-3 mutants

Recessive lethal mutations in the *ad-3* region can result from point mutation or chromosome deletion. A series of genetic tests are performed to distinguish point mutations at the *ad-3A* and *ad-3B* loci from chromosome deletions covering each locus separately or both simultaneously, and to study allelic complementation between the point mutations at the *ad-3B* locus.

(*1*) *Dikaryon test*. This test, described in detail by DE SERRES[9], is designed to determine whether the *ad-3* mutations have resulted from point mutation at the *ad-3A* or *ad-3B* locus or from some more complex events. The *ad-3* mutants are induced in component II of heterokaryon 12, which carries the *cot* mutation (colonial morphology, temperature-sensitive). Heterokaryons that produce *cot* colonies when plated on Fries' minimal medium supplemented with adenine and pantothenate, are homokaryotic viable and have resulted from point mutation at the *ad-3A* or *ad-3B* locus. Heterokaryons that produce no *cot* colonies are homokaryotic lethal.

(*2*) *Trikaryon test*. Homokaryotic lethal mutants are further classified to determine whether they are chromosome deletions in the *ad-3* region (*ad-3*[IR]) or point mutations with a separate site of recessive lethal damage elsewhere in the genome (*ad-3*[R]+*RL*). In the trikaryon test (see ref. 9 for further details) the homokaryotic lethal mutations are tested for homology with a series of testers carrying chromosome deletions that cover different parts of the *ad-3* region. The test for homology (looking for the presence of *cot* colonies) is done by plating conidia from a trikaryon formed between the mutant with the unknown lethal mutation and one of the testers. Strains used as testers are: 12-1-18, which carries a deletion covering both *ad-3A* and *ad-3B*; 12-7-215, which carries a deletion covering *ad-3A*; and 12-7-182, which carries a deletion covering *ad-3B*. The lethal mutations were classified into 2 categories as follows: (I) *ad-3A*[IR], no *cot* colonies formed with 12-7-215 or 12-1-18 but *cot* colonies formed with 12-7-182 are adenine-independent; *ad-3B*[IR], no *cot* colonies formed with 12-7-182 or 12-1-18, but *cot* colonies formed with 12-7-215 are adenine-independent; *ad-3A ad-3B*[IR], no *cot* colonies formed with 12-7-182, 12-7-215 or 12-1-18; (II) *ad-3A*[R] +*RL*, adenine-requiring *cot* colonies formed with 12-7-215 and adenine-independent *cot* colonies with 12-7-182; *ad-3B*[R]+*RL*, adenine-requiring *cot* colonies formed with 12-7-182 and adenine-independent *cot* colonies formed with 12-7-215.

(*3*) *Genotype and complementation test*. The genotype of the *ad-3* mutants and type of complementation pattern among point mutations at the *ad-3B* locus were determined simultaneously by heterokaryon tests (for procedures see refs. 7 and 8).

In the genotype test the strains used as testers are 1-68-13 (*ad-3A*); 1-112-2 (*ad-3B*, noncomplementing); 74-OR33-3A (*hist-2, nic-2, al-2*); and 74-OR60-44A (*ad-2, inos*). The last strain is included as a control to check the ability of tested mutants to form heterokaryons; all mutants should give positive tests with this strain. In typical experiments the following genotypes are found: *ad-3A*, *ad-3B*, *ad-3B nic-2*, *ad-3A ad-3B*, *ad-3A ad-3B nic-2*.

For classification of the type of complementation patterns among point mutations at the *ad-3B* locus[14] the following strains are used as testers: 2-17-258 (complon 1); 2-17-128 (complon 2); 2-31-8 (complon 10-11); 2-32-3 (complon 15); and 2-32-5 (complon 16–17). Depending on the response with these testers *ad-3B* mutants are classified as complementing with nonpolarized (NP) or polarized (P) patterns or noncomplementing (NC). In brief, complementing mutants that gave a positive response with all other testers and a negative test with 2-32-5 alone, with both 2-32-5 and 2-32-3, or with 2-32-5, 2-32-3 and 2-31-8 were classified as polarized. All other combinations of positive responses were classified as nonpolarized. Mutants which gave a negative response with all 5 testers were classified as noncomplementing.

Estimation of dose–effect curves for mutation induction

The genetic characterization of the *ad-3* mutants obtained in forward-mutation experiments enables us to resolve the overall forward-mutation frequencies for *ad-3* mutations into their different components. This analysis allows us to determine that fraction of the total frequency attributable to a particular mutant class and to obtain dose–effect curves for each class of event.

Curves were fitted to the data from the different exposures within each of the 2 treatments by regression analysis. Confidence intervals were determined by a modification of the methods described by BIRNBAUM[4]. The dose–effect curves for a particular class of mutation were compared for a difference in slope by means of a *t* test on the regressions and for a difference in distance by an *F* test from a covariance analysis. The dose-reduction factors (DRFs) were determined by a comparison at a given forward-mutation frequency at the midpoints of the dose–effect curves. The *t* test was also used to determine the significance of any difference between DRFs.

The DRF as described by JAGGER[16] is defined as

$$\frac{\text{Dose required in the dark for a given biological endpoint}}{\text{Dose required in the light to achieve the same endpoint}}$$

RESULTS

Effects on the curves for survival and the overall induction of ad-3 mutations

Mutagenic treatments with UV alone and with UV followed by PR were planned so that a series of samples of *ad-3* mutants from each treatment could be obtained for analysis from nearly comparable forward-mutation frequencies. The percentage survival, total number of purple colonies isolated, and the forward-mutation frequency obtained with each treatment are given in Table I.

The dose–effect curves for survival of the heterokaryotic fraction of conidia are given in Fig. 1. These curves show that UV + PR gives higher levels of survival than UV alone. The dose-reduction factor for inactivation at 60% survival is about 0.6.

TABLE I

SURVIVAL OF HETEROKARYOTIC CONIDIA, FORWARD-MUTATION FREQUENCIES OBSERVED WITH UV ALONE AND WITH UV FOLLOWED BY PR, AND THE NUMBERS OF PURPLE COLONIES OBSERVED AND ANALYZED

Treatment	Dose $(erg \cdot mm^{-2})$	Survival $(\%)$	Number of purple colonies		Forward-mutation frequency $\times 10^6$ survivors
			Observed	Analyzed	
Control	0	100.0	2	2	0.41
UV	500	95.1	143	136	11.70
	1000	87.4	376	146	62.00
	2000	61.4	970	150	198.84
	3000	38.1	838	149	487.61
	4000	14.9	2009	—	844.61
UV + PR	1000	95.5	252	148	20.90
	2000	81.4	351	214	88.82
	3000	87.9	451	145	176.21
	4000	56.2	820	151	375.72
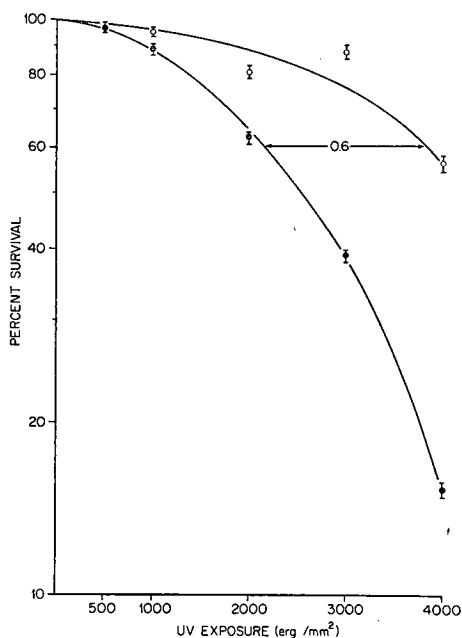
Fig. 1. Comparison of the dose–effect curves for survival of heterokaryotic conidia after UV alone and after UV + PR (●, UV; ○, UV + PR).

The dose–effect curves for the overall induction of purple colonies (presumptive *ad-3* mutations) with each of the 2 treatments is shown in Fig. 2. These curves show that UV-induced *ad-3* mutations increase in proportion to $(dose)^{2.02}$ and with PR in proportion to $(dose)^{2.04}$. Thus the dose–effect curves show no difference in slope $(P > 0.90)$, but there is a significant reduction in the overall mutation frequencies after PR $(P < 0.001)$. The DRF is 0.66 (Table II).

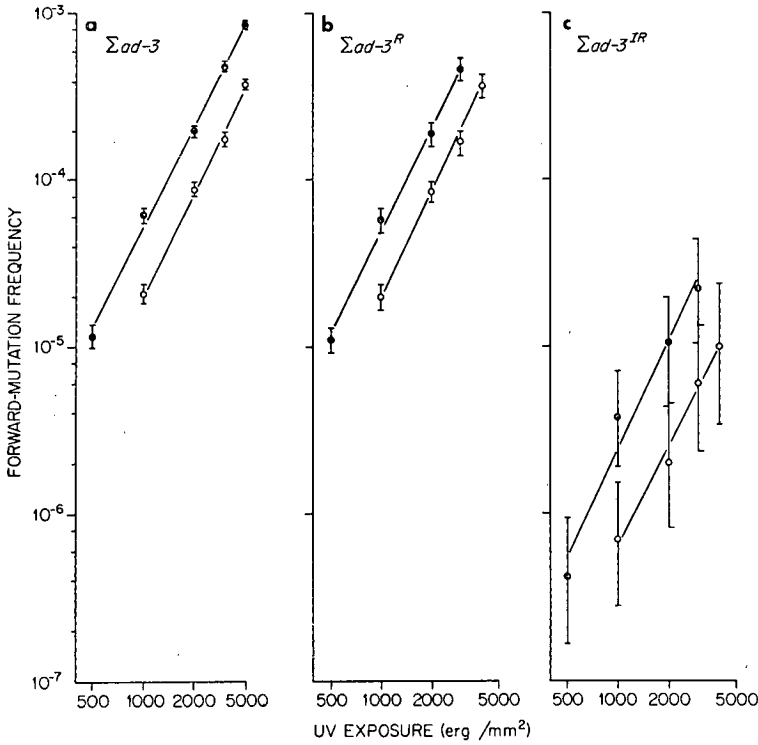Thus PR provides a means of repairing the genetic damage resulting in inacti-

Fig. 2. Comparison of the dose–effect curves for the overall induction of (a) all *ad-3* mutations (Σ *ad-3*), (b) all point mutations (Σ *ad-3*^{R}), and (c) all chromosome deletions (Σ *ad-3*^{IR}) after UV alone and after UV + PR (●, UV; ○, UV + PR).

TABLE II

COMPARISON OF DOSE–RESPONSE CURVES FOR DIFFERENT CLASSES OF MUTATIONS IN THE *ad-3* REGION

Mutation class	Slope of dose–effect curve[a]		Dose reduction factor (DRF)	Probability values of similarity between	
	UV	*UV + PR*		*Slope*	*Distance*
ad-3	2.02 ± 0.23	2.04 ± 0.38	0.66	>0.900	<0.001
ad-3^R	2.03 ± 0.43	2.05 ± 0.42	0.66	>0.900	<0.001
ad-3^{IR}	2.16 ± 1.18	1.95 ± 0.67	0.50	0.590	<0.001
ad-3A^R	2.08 ± 0.68	2.15 ± 1.53	0.65	0.854	0.007
ad-3B^R	2.02 ± 0.35	1.99 ± 0.24	0.66	0.820	<0.001
ad-3A^{IR}	1.89 ± 1.70	—	—	—	—
ad-3B^{IR}	2.11 ± 0.63	2.41 ± 1.38	0.50	0.427	0.003
(ad-3A ad-3B)^{IR}	2.48 ± 2.52	2.16 ± 1.92	0.50	0.708	0.015
ad-3B^{R-NP}	1.82 ± 0.36	2.21 ± 1.11	0.64	0.196	0.007
ad-3B^{R-P}	2.12 ± 1.07	2.17 ± 0.37	0.70	0.867	0.012
ad-3B^{R-NC}	2.16 ± 0.78	1.82 ± 0.41	0.65	0.218	0.006

[a] With 95% confidence limits.

vation of the cell as well as the genetic damage resulting in the production of recessive lethal mutations at 2 specific loci in the *ad-3* region.

TABLE III

PERCENTAGES OF UV-INDUCED RECESSIVE LETHAL MUTATIONS IN THE *ad-3* REGION OF *Neurospora crassa* RESULTING FROM POINT MUTATION AND CHROMOSOME DELETION WITH AND WITHOUT PR

Data for point mutations and chromosome deletions given in percent and number (in parentheses).

Treatment	Dose $(erg \cdot mm^{-2})$	Total number of ad-3 mutants analyzed	Total point mutations $(ad\text{-}3^R)$	Total chromosome deletions $(ad\text{-}3^{IR})$	Point mutations		Chromosome deletions		
					$ad\text{-}3A^R$	$ad\text{-}3B^R$	$ad\text{-}3A^{IR}$	$ad\text{-}3B^{IR}$	$(ad\text{-}3A\ ad\text{-}3B)^{IR}$
UV	500	136	96.3 (131)	3.7 (5)	25.7 (35)	70.6 (96)	0.7 (1)	2.2 (3)	0.7 (1)
	1000	146	93.8 (137)	6.2 (9)	28.1 (41)	65.8 (96)	1.4 (2)	1.4 (2)	3.4 (5)
	2000	150	94.7 (142)	5.3 (8)	24.7 (37)	70.0 (105)	0.7 (1)	2.7 (4)	2.0 (3)
	3000	149	95.3 (142)	4.7 (7)	30.0 (44)	65.8 (98)	0.7 (1)	2.0 (3)	2.0 (3)
Total		581	95.0 (552)	5.0 (29)	27.0 (157)	68.0 (395)	0.9 (5)	2.1 (12)	2.1 (12)
UV+PR	1000	148	96.6 (143)	3.4 (5)	25.0 (37)	71.6 (106)	1.4 (2)	1.4 (2)	0.7 (1)
	2000	214	97.7 (209)	2.3 (5)	23.8 (51)	73.8 (158)	0.0 (0)	0.9 (2)	1.4 (3)
	3000	145	96.6 (140)	3.4 (5)	19.3 (28)	77.2 (112)	0.0 (0)	2.1 (3)	1.4 (2)
	4000	151	97.4 (147)	2.6 (4)	34.4 (52)	62.9 (95)	0.0 (0)	1.9 (3)	0.7 (1)
Total		658	97.1 (639)	2.9 (19)	25.5 (168)	71.6 (471)	0.3 (2)	1.5 (10)	1.1 (7)
Combined total		1239	96.1 (1191)	3.9 (48)	26.2 (325)	69.9 (866)	0.6 (7)	1.8 (22)	1.5 (19)

Effects of the overall induction of recessive lethal mutations resulting from point mutation (ad-3^R) and chromosome deletion (ad-3^{IR})

The genetic tests described in MATERIALS AND METHODS enables us to distinguish point mutations from chromosome deletions in the *ad-3* region.

Fig. 2 shows the dose–effect curves for these 2 classes of mutations with increasing UV dose with and without PR. UV-induced *ad-3*^{R} mutations increase in proportion to (dose)$^{2.03}$ with no PR, and in proportion to (dose)$^{2.05}$ with PR. There is no significant change in slope as a result of PR ($P > 0.90$), but it causes a highly significant reduction in mutation frequency ($P < 0.001$). *Ad-3*^{IR} mutations increase in proportion to (dose)$^{2.16}$ with UV alone and to (dose)$^{1.95}$ when UV is followed by PR. These slopes are not significantly different from each other ($P = 0.59$); but, again, PR causes a significant reduction in the mutation frequency ($P < 0.001$).

These data also show that, whether PR is used or not, there is no apparent change in the proportion *ad-3*^{R} : *ad-3*^{IR} with increasing doses of UV within each treatment series (Table III; for UV alone, $\chi^2_{3 \text{ d.f.}} = 0.98$, $P = 0.80$ at the 95% level; for UV + PR, $\chi^2_{3 \text{ d.f.}} = 0.55$, $P = 0.91$). These data show that within each treatment series, the relative rate of repair of damage leading to *ad-3*^{R} and *ad-3*^{IR} mutations is constant and does not change as a function of dose. However, the χ^2 test demonstrated a lower proportion of chromosome deletions in photoreactivated samples than in unphotoreactivated ones ($\chi^2_{1 \text{ d.f.}} = 3.12$, $P = 0.08$). This shows that damage resulting in chromosome deletions is more susceptible to PR than that resulting in point mutations. This fact is also apparent from the DRFs calculated for the 2 mutational classes (Table II). For *ad-3*^{R} mutations the DRF is 0.66 while for *ad-3*^{IR} mutations it is 0.50. A *t* test on the adjusted means of the dose–effect curves showed that these DRFs were significantly different ($P = 0.02$).
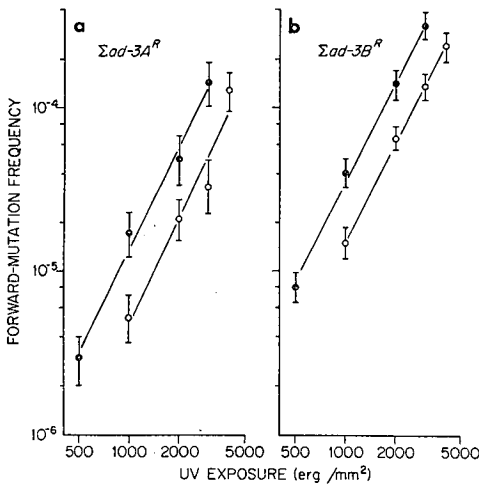

Fig. 3. Comparison of the dose–effect curves for (*a*) all point mutations at the *ad-3A* locus (Σ *ad-3A*^{R}) and (*b*) all point mutations at the *ad-3B* locus (Σ *ad-3B*^{R}) after UV alone and after UV + PR (●, UV; ○, UV + PR).
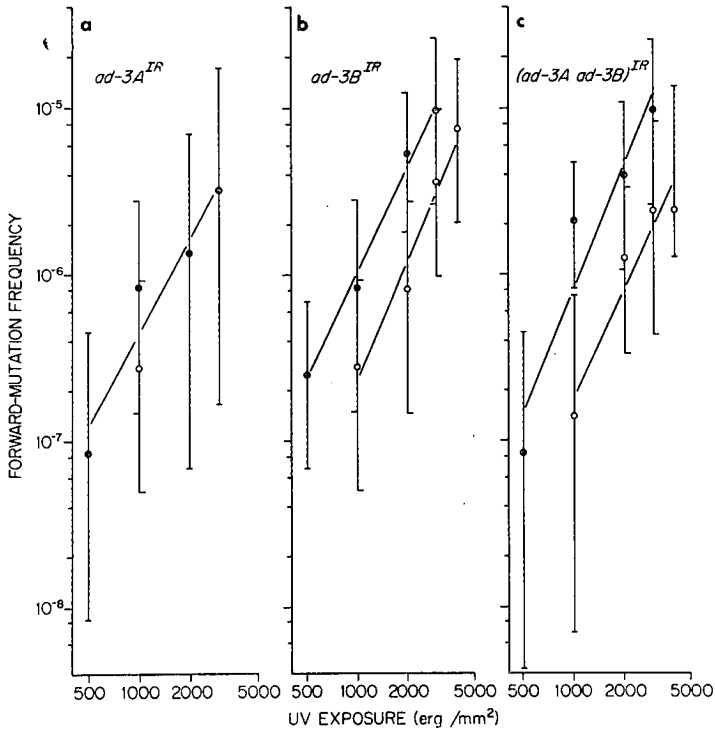
Fig. 4. Comparison of the dose–effect curves for (a) all chromosome deletions covering only the ad-3A locus (ad-3A^{IR}), (b) all chromosome deletions covering only the ad-3B locus (ad-3B^{IR}), and (c) all chromosome deletions covering both loci simultaneously (ad-3A ad-3B^{IR}) after UV alone and after UV + PR (●, UV; ○, UV + PR).

Effects on the induction of point mutations and chromosome deletions at the ad-3A locus and the ad-3B locus

Both ad-3^{R} and ad-3^{IR} mutations can be assigned to the genetic loci ad-3A and ad-3B. The results of these allocations are given in Table III and in Figs. 3 and 4. The slopes of the induction curves for each class with and without PR are summarized in Table II. In the 4 classes, where a comparison is possible, PR causes a highly significant reduction in the frequency of mutation. The DRFs for ad-3A^{R} and ad-3B^{R} mutations do not differ from each other, nor do the DRFs of the ad-3B^{IR} and (ad-3A ad-3B)^{IR} mutations; but the DRFs for the former class of mutations, of course, differ from the latter class.

The allocations (Table IV) also show that there is no change in the proportions of ad-3A^{R} and ad-3B^{R} mutations as a function of UV dose in the absence of PR ($\chi^2{}_3$ d.f. = 1.19, P = 0.76). However, there is some evidence of heterogeneity between this proportion at different doses when PR follows UV treatment ($\chi^2{}_3$ d.f. = 9.52, P = 0.02). There is no obvious trend and this probably represents an undertermined sampling problem. There is also no evidence (Table III) of any change in the proportions of ad-3A^{IR}, ad-3B^{IR}, and ad-3A ad-3B^{IR} mutations with a dose of UV either without PR ($\chi^2{}_6$ d.f. = 2.68, P = 0.84) or with PR ($\chi^2{}_6$ d.f. = 7.61, P = 0.27).

TABLE IV

PERCENTAGES OF POINT MUTATIONS AT THE *ad-3B* LOCUS OF COMPLEMENTING MUTANTS WITH NON POLARIZED OR POLARIZED COMPLEMENTATION PATTERNS AND NONCOMPLEMENTING MUTANTS

Treatment	Dose ($erg \cdot mm^{-2}$)	Total $ad\text{-}3B^R$ mutations	Type of complementation pattern					
			Nonpolarized		Polarized		Noncomplementing	
			Number	%	Number	%	Number	%
UV	500	96	46	47.9	12	12.5	38	39.6
	1000	96	36	37.5	6	6.3	54	56.2
	2000	105	43	41.0	10	9.5	52	49.5
	3000	98	30	30.6	14	14.3	54	55.1
Total		395	155	39.3	42	10.6	198	50.1
UV+PR	1000	106	29	27.4	12	11.3	65	61.3
	2000	158	73	46.2	19	12.0	66	41.8
	3000	112	42	37.5	17	15.2	53	47.3
	4000	95	36	37.9	13	13.7	46	48.4
Total		471	180	38.2	61	13.0	230	48.8
Combined total		866	335	38.7	103	11.9	428	49.4

Effects on the induction of point mutations at the ad-3B locus with nonpolarized ($ad\text{-}3B^{R-NP}$) or polarized ($ad\text{-}3B^{R-P}$) complementation patterns and noncomplementing ($ad\text{-}3B^{R-NC}$) mutants

Point mutations at the *ad-3B* locus show allelic complementation[14] and are classified as complementing with either (a) nonpolarized patterns, $ad\text{-}3B^{R-NP}$, or (b) polarized patterns, $ad\text{-}3B^{R-P}$, or as noncomplementing, $ad\text{-}3B^{R-NC}$. Since a correlation has been found between complementation pattern and genetic alteration at the molecular level[18–22], a change in the relative frequencies of mutants in each of these
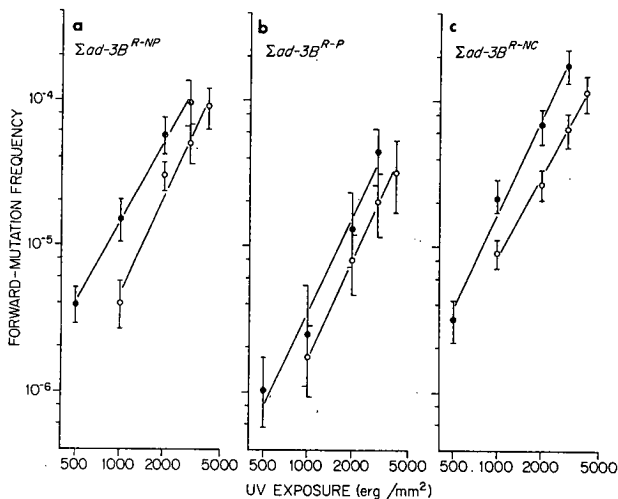

Fig. 5. Comparison of the dose–effect curves for (a) all point mutations at the *ad-3B* locus with nonpolarized complementation patterns ($\Sigma\ ad\text{-}3B^{R-NP}$), (b) all point mutations at the *ad-3B* locus with polarized complementation patterns ($\Sigma\ ad\text{-}3B^{R-P}$), and (c) all point mutations at the *ad-3B* locus that are noncomplementing ($\Sigma\ ad\text{-}3B^{R-NC}$) after UV alone and after UV + PR (●, UV; ○, UV + PR).

3 classes is interpreted as indicating a change in the spectrum of genetic alterations at the molecular level.

The slopes of the induction curves for each of the 3 classes of UV-induced $ad\text{-}3B^R$ mutations with and without PR are summarized in Table II. All 3 classes increase in proportion to $(dose)^{2.0}$ both with and without PR. In each of these classes PR causes a significant reduction in the frequency of mutation obtained with the same dose of UV (Fig. 5). The DRFs for $ad\text{-}3B^{R-NP}$, $ad\text{-}3B^{R-P}$, and $ad\text{-}3B^{R-NC}$ mutations do not differ significantly from each other ($P = 0.70, 0.70, 0.80$). The data on the number of mutants in each of these classes obtained with each treatment (Table IV) provides no clear evidence for any change in the proportion of these mutations with dose of UV either without PR ($\chi^2_{6\ d.f.} = 10.6$, $P = 0.10$) or with PR ($\chi^2_{6\ d.f.} = 11.8$, $P = 0.07$). In neither treatment series is there any general trend indicated by the variation in the percentages of mutants of each genotype, and the low P values could be due to some minor sampling problem. There is, however, no difference between the distributions of the total number of mutants in each of these 3 classes in the 2 treatment series ($\chi^2_{2\ d.f.} = 1.10$, $P = 0.59$).

DISCUSSION

The data from the present experiment show that PR has a differential effect on the UV-induced lesions resulting in point mutation ($ad\text{-}3^R$) and chromosome deletion ($ad\text{-}3^{IR}$) in the $ad\text{-}3$ region, with the latter class of mutation being the more photoreactivable. All classes of point mutations are affected in the same way by PR. These data from an experiment on a two-component heterokaryon of Neurospora both confirm and extend our earlier study[17] with a haploid wild-type strain.

Genetic effects of UV on inactivation of cells and the induction of recessive lethal mutations

The inactivation of heterokaryotic conidia of a two-component heterokaryon has been studied extensively by ATWOOD[1-3]. In the present experiments the multi-hit survival curve obtained (Fig. 1) with UV treatment is that expected on the basis of ATWOOD's earlier work.

Our results show that UV treatment induces recessive lethal mutation both by point mutation and chromosome deletion in Neurospora. From an analysis of the mutations obtained, it has been shown that point mutations predominate, with approx. 24 times as many of them as chromosome deletions. The proportion $ad\text{-}3^R : ad\text{-}3^{IR}$ remains the same as the UV dose increases within each treatment series. The same is true of the proportions $ad\text{-}3A^R : ad\text{-}3B^R$ and $ad\text{-}3B^{R-NC} : ad\text{-}3B^{R-P} : ad\text{-}3B^{R-NP}$. There is also no change in the proportion $ad\text{-}3A^{IR} : ad\text{-}3B^{IR} : ad\text{-}3A\ ad\text{-}3B^{IR}$. All these mutant types increase as the square of the UV dose.

Although the relative proportions of the different mutant types remains the same with increasing UV dose within each treatment series, PR alters the ratio between $ad\text{-}3^R$ and $ad\text{-}3^{IR}$ mutants. It appears that damage leading to chromosomal deletions is photorepaired more readily than damage giving point mutations. This holds true not only for the class as a whole, but also for the individual subclasses.

These results pose 2 main questions: (*a*) Why do *all* types of mutants increase in proportion to the square of the dose; and (*b*) why is the PR of damage giving point mutations less effective than that of damage giving chromosomal deletions?

TABLE V

PERCENTAGES OF CHROMOSOME DELETIONS COVERING THE *ad-3A* LOCUS, THE *ad-3B* LOCUS, OR BOTH LOCI SI TANEOUSLY

Mutagen	Dose rate	Total *ad-3*^{IR} mutations	Chromosome deletions					
			ad-3A^{IR}		*ad-3B*^{IR}		(*ad-3A ad-3B*)^{I}	
			Number	%	Number	%	Number	%
UV	21 erg·mm^{-2}·sec^{-1}	48	7	14.6	22	45.8	19	39.6
X-Rays^{a}	1000 R/min	845	141	16.7	277	32.8	427	50.5

^{a} F. J. DE SERRES, unpublished.

TABLE VI

COMPARISON OF TESTS FOR ALLELIC COMPLEMENTATION ON UV-INDUCED *ad-3B* MUTANTS INDUCED IN W TYPE STRAIN 74-OR23-1A OR HETEROKARYON 12

Strain	Total number of *ad-3B* mutants	Type of complementation pattern						
		Nonpolarized		Polarized		Noncomplementing		t
		Number	%	Number	%	Number	%	
74-OR23-1A (ref. 17)	899	301	33.5	120	13.3	478	53.2	P =
Heterokaryon 12	866	335	38.9	103	11.9	428	49.4	

With X-irradiation, WEBBER AND DE SERRES[24] have shown that point mutations increase in proportion to the dose while chromosome deletions increase in proportion to the dose squared. The data presented here do not reveal any striking differences between the types of chromosomal deletions obtained with UV and those obtained with X-rays (Table V). It is therefore not unreasonable to suppose that UV could have also produced deletions, by some two-event mechanism involving chromosome breakage. However, this raises the problem of the two-hit kinetics found for point mutations. Such induction curves are not uncommon for reverse mutations[5,6] and are generally interpreted in terms of the saturation or inactivation of cellular repair systems at higher UV doses. It is also possible that the two-event kinetics are related in some way to recombination events at the DNA level, which have been postulated as necessary for UV-induced mutagenesis[25], although it is not obvious how. At present we have no evidence to favor either possibility, but it is clear that just because these 2 classes of mutation show similar induction kinetics they do not necessarily result from the same mutational mechanism.

The PR of damage leading to chromosomal deletion is more extensive than PR of damage resulting in point mutations. This is reflected in a change in the proportions of the 2 classes of mutations, so that without PR the ratio is 1:24 while with PR it drops to 1:30. That these 2 classes are essentially different in their responses to PR is emphasized by the observation that all *ad-3B*^{R} mutant classes respond similarly. This agrees well with our earlier data[17] obtained with the homokaryotic wild-type strain 74-OR23-1A.

Explanations of this result fall into 2 basic groups; either the damage producing chromosomal deletions differs qualitatively from that producing point mutations (being intrinsically more photoreactivable), or it is identical and the difference in photoreactivability is due to some secondary factor (*e.g.*, accessibility of the lesions to the photoreactivating enzyme). Our present data do not permit a decision on this point, but by studying the action spectra for the induction of point mutations *versus*

deletions or by using methods which result in the preferential induction of a specific photoproduct[23], it may be possible to resolve this problem.

The data from the present experiment make it possible to compare the genetic effects of UV in different strains of Neurospora. In Table VI the data from tests for allelic complementation on UV-induced *ad-3B* mutants induced in the haploid wild-type strain 74-OR23-1A and the two-component heterokaryon strain 12 are compared. These data show that there are only small differences in the percentages of each type of complementation pattern, and a χ^2 test for a difference in the distribution of mutants in these 3 classes shows that the small difference in the observed percentages of each class are only significant at the 7% level. Since we have found a correlation between genetic alteration at the molecular level and complementation pattern[19-22], these data seem best interpreted as indicating that UV produces essentially the same spectrum of genetic alterations in both strains. There is certainly no striking difference between strains, in this comparison, and experiments are in progress in this laboratory to explore this possibility with strains carrying mutations that make them more sensitive to UV-induced inactivation.

REFERENCES

1 ATWOOD, K. C., The role of lethal mutation in the killing of Neurospora conidia by ultra-violet light, *Genetics*, 35 (1950) 95–96.
2 ATWOOD, K. C., Different action of ultraviolet and X-rays revealed by heterokaryon methods, *Genetics*, 37 (1952) 564.
3 ATWOOD, K. C., Ultraviolet effects on Neurospora heterokaryons, in *Proc. 1st Intern. Photobiol. Congr., Amsterdam*, 1954, pp. 143–145.
4 BIRNBAUM, A., Statistical methods for poisson processes and exponential populations, *J. Am. Statist. Assoc.*, 49 (1954) 254.
5 BRIDGES, B. A., A note on the mechanism of UV mutagenesis in *Escherichia coli, Mutation Res.*, 3 (1966) 273–279.
6 BRIDGES, B. A., Mechanisms of radiation mutagenesis in cellular and subcellular systems, *Ann. Rev. Nucl. Sci.*, 19 (1969) 139–178.
7 DE SERRES, F. J., A procedure for making heterokaryon tests in liquid minimal medium, *Neurospora Newsletter*, 1 (1962) 9–10.
8 DE SERRES, F. J., Studies with purple adenine mutants in *Neurospora crassa*, V. Evidence for allelic complementation among *ad-3B* mutants, *Genetics*, 48 (1963) 351–360.
9 DE SERRES, F. J., Genetic analysis of the extent and type of functional inactivation in irreparable recessive lethal mutations in the *ad-3* region of *Neurospora crassa, Genetics*, 58 (1968) 69–77.
10 DE SERRES, F. J., AND H. G. KØLMARK, A direct method for determination of forward-mutation rates in *Neurospora crassa, Nature*, 182 (1958) 1249–1250.
11 DE SERRES, F. J., AND H. V. MALLING, Measurement of recessive lethal damage over the entire genome and at two specific loci in the *ad-3* region of *Neurospora crassa* with a two-component heterokaryon, in A. HOLLAENDER (Ed.), *Environmental Chemical Mutagens*, Plenum, New York, 1971, in the press.
12 DE SERRES, F. J., AND R. S. OSTERBIND, Estimation of the relative frequencies of X-ray-induced viable and recessive lethal mutations in the *ad-3* region of *Neurospora crassa, Genetics*, 47 (1962) 793–796.
13 DE SERRES, F. J., H. V. MALLING AND B. B. WEBBER, Dose-rate effects on inactivation and mutation-induction in *Neurospora crassa, Brookhaven Symp. Biol.*, 20 (1968) 56–76.

14 DE SERRES, F. J., H. E. BROCKMAN, W. E. BARNETT AND H. G. KØLMARK, Allelic complementation among nitrous acid-induced *ad-3B* mutants of *Neurospora crassa, Mutation Res.*, 4 (1967) 415–424.

15 JAGGER, J., A small and inexpensive UV dose-rate meter useful in biological experiments, *Radiation Res.*, 14 (1961) 394–403.

16 JAGGER, J., *Introduction to Research in Ultraviolet Photobiology*, Prentice-Hall, Englewood Cliffs, N.J., 1967.

17 KILBEY, B. J., AND F. J. DE SERRES, Quantitative and qualitative aspects of photoreactivation of premutational damage at the *ad-3* loci of *Neurospora crassa, Mutation Res.*, 4 (1967) 21–29.

18 KILBEY, B. J., F. J. DE SERRES AND H. V. MALLING, Identification of the genetic alteration at the molecular level of ultraviolet-light induced *ad-3B* mutants in *Neurospora crassa, Mutation Res.*, 12 (1971) 47–56.

19 MALLING, H. V., AND F. J. DE SERRES, Relation between complementation patterns and genetic alterations in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa, Mutation Res.*, 4 (1967) 425–440.

20 MALLING, H. V., AND F. J. DE SERRES, Correlation between base-pair transition and complementation pattern in nitrous acid-induced *ad-3B* mutants of *Neurospora crassa, Mutation Res.*, 5 (1968) 359–371.

21 MALLING, H. V., AND F. J. DE SERRES, Identification of genetic alterations induced by ethyl methanesulfonate in *Neurospora crassa, Mutation Res.*, 6 (1968) 181–193.

22 MALLING, H. V., AND F. J. DE SERRES, Genetic effect of N-methyl-N'-nitro-N-nitrosoguanidine in *Neurospora crassa, J. Gen. Mol. Genet.*, 106 (1970) 195–207.

23 MENNIGMANN, H. D., AND W. WACKER, Photoreactivation of *Escherichia coli* Bs-3 after inactivation by 313 nm radiation in the presence of acetone, *Photochem. Photobiol.*, 11 (1970) 291–296.

24 WEBBER, B. B., AND F. J. DE SERRES, Induction kinetics and genetic analysis of X-ray-induced mutations in the *ad-3* region of *Neurospora crassa, Proc. Natl. Acad. Sci. (U.S.)*, 53 (1965) 430–437.

25 WITKIN, E. M., Ultraviolet-induced mutation and DNA repair, *Ann. Rev. Genet.*, 3 (1969) 525–552.

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AN INVESTIGATION INTO THE MUTAGENIC AFTER-EFFECT OF BUTADIENE DIEPOXIDE USING *NEUROSPORA CRASSA*

By

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With 3 Figures in the Text

Introduction

In the course of experiments carried out in 1958 at the Oak Ridge National Laboratory, KØLMARK and AUERBACH observed an after-effect of diepoxybutane (DEB) in *Neurospora crassa* conidia. When treated, washed spores were kept in water before plating, the frequency of adenine reversions continued to increase for many hours. Plating immediately after treatment prevented this increase in mutations. A preliminary account of these observations was given in 1960 but at that time further work along these lines was discontinued. When work on the same system was resumed here, it was considered necessary to define the conditions under which the after-effect of DEB occurred so that it could be avoided or at least corrected for in future experiments. To this practical interest is added a theoretical one since various authors have detected a similar after-effect for other alkylating agents (IYER and SZYBALSKI 1958, SZYBALSKI 1960 and STRAUSS 1962) and have interpreted it in terms of the reaction of these mutagens with DNA or DNA precursors. It therefore seemed of interest to analyse as far as possible the causes of the after-effect in our system.

Materials and Methods

1. Strain

The strain K 3/17 ad—3 A (38701) inos (37401), auxotrophic for adenine and inositol, was used throughout. It is macroconidiating and grows in small, distinct colonies on solid agar medium without growth inhibiting substances. The adenineless and inositolless mutations revert spontaneously with frequencies of about 5 and 1 per 10^8 conidia respectively. Previously it had been found that the double mutant conidiated poorly on the "complete" medium of Fries (BEADLE and TATUM 1945) but this was overcome by adding a supplement of chopped Neurospora mycelium (KØLMARK and WESTERGAARD 1953). At the commencement of the present series of experiments, it was found that the strain would grow with a rich yield of conidia on the medium described below, that is, without the addition of either mycelium or the vitamins used in the Fries "complete" medium. Whether the improved conidiation is due to some unidentified change in the composition of the medium or a change in the strain itself is not certain. Reisolates have been made deliberately from colonies which were small, had a sharp outline and a rich conidiation. These preferred characters have presumably accumulated as a result of ten years' selection and may well have led to the improved conidiation now found on less fortified medium. Selection for colony type has not, however, led to any conspicuous change in reversion frequencies of the adenine and inositol mutants. The very low response of the inositol mutant to DEB (Table 1) makes it difficult to obtain quantitatively significant results with mutations of this kind. Most of the data are, therefore, concerned with adenine reverse mutations.

2. *Media*

The basic medium used throughout was Fries' minimal salts medium. For conidial production each litre of minimal medium was supplemented with 2.5 g. Difco powdered yeast extract, 1.0 g casein hydrolysate (British Drug Houses Ltd.), 5.0 g malt extract, 15.0 g glycerol, 4 μg biotin, 100 mg adenine sulphate, 8 mg inositol and 15 g Difco Bacto agar.

Survival was scored on minimal medium containing 10 g glucose and 4 μg biotin per litre. This was supplemented with 40 mg adenine sulphate and 20 mg meso-inositol. Adenine reversions were scored on the same medium without adenine sulphate and inositol reversions on survival medium minus meso-inositol.

3. *Preparation and treatment of the conidia*

Conidia were harvested in water from slants grown for 7—10 days at 25° C. The suspension was filtered through cotton wool to remove fragments of mycelium and washed twice with water before mutagenic treatment. Conidia were treated with 0.1 M DEB for 60 min at 21° C at a density of 1×10^7 per ml. They were then centrifuged out and the solution of DEB was decanted off. The chemical treatment was considered terminated at the moment when the conidia were resuspended by the addition of water. The treated conidia were washed twice before post-treatments or plating. Any deviations from this procedure will be described where necessary.

4. *Post-treatment*

When the conidia are plated, any further production of mutations is arrested. The procedure for studying the after-effect has, therefore, been to keep treated washed conidia in centrifuge tubes in water at 25° C and remove samples for plating after the desired times.

5. *Scoring*

Survival and mutations were counted after 5—7 days' incubation at 25° C. Mutations are presented in terms of adenine reversions per 10^6 cells initially viable (giving a value directly proportional to the number of colonies on the plates) or per 10^6 surviving conidia (that is, the proportion of mutants in the viable population after a given treatment).

6. *DEB*

Diepoxybutane was obtained from Columbia Organic Chemicals Co. Inc. It consists of D-L butadiene diepoxide with not more than 3 % of the meso-form.

Results

1. *The After-effect*

The after-effect was first observed in an experiment in which treated, twice washed conidia were resuspended in water and kept for various lengths of time before plating. Three different post-treatment incubation temperatures were used, 4, 20 and 30° C. For comparison, corresponding samples were taken from an aliquot of the conidial suspension which had been treated with ultra violet light instead of DEB. These results are given in Fig. 1 and Table 1.

In this experiment the duration of the DEB treatment was only 35 min. As a result, very little reduction in survival occurred even under the conditions giving the greatest mutational response. The increase in mutations among survivors, which constitutes the after-effect, cannot be wholly due to differential survival of the mutants since the number of ad$^+$ mutations actually counted increased five times during the post-incubation period of 9 hours at 30° C.

The magnitude of the after-effect was found to be dependent on the temperature during post-treatment. No after-effect was apparent if the conidia were kept for 9 hours at 4° C. In subsequent experiments, storage of the conidia at this temperature was found to be a convenient way of preventing further action of the chemical prior to plating. Neither of the mutations studied exhibited an after-effect when U.V. doses were used which resulted in approximately the same survival as the DEB treatment.
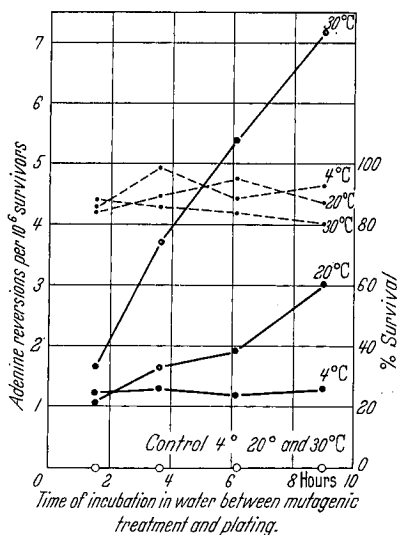
2. The efficiency of the washing procedure

Before invoking any special chemical hypotheses as explanations of the after-effect, a critical examination of the washing procedure was made. Two tests were employed, (i) the number of washings was increased and (ii) a direct test was made for extracellular mutagenic activity in the suspensions of treated conidia used for plating.

A test of the first type has already been reported by KØLMARK and AUERBACH (1960). Conidia treated with diepoxybutane (0.1 M, 20° C, 70 min) were washed twice or five times before transferring them to water for periods of up to 25 hours. During this period adenine reversions per 10^6 survivors increased to a greater extent in the conidia washed five times compared with those washed only twice. The number of adenine reversions *counted* however did not increase. Survival varied between 30 and 3%. The absence of an absolute increase in adenine reversions

Fig. 1. DEB after-effect at different temperatures. Conidia treated with 0.1 M DEB at 20° C for 35 min. This treatment was stopped by washing with distilled water at time 0 in the graph. After one additional wash aliquots were resuspended in water at 4, 20 and 30° C 36 min after the first wash. Samples were removed for plating at the times plotted. ●——● Adenine reversions per 10^6 survivors; ● — — ● per cent survival

and the low survival levels encountered makes an interpretation of this experiment difficult. It is also possible that during the washing procedure the extended centrifugation as such may have influenced the result, but this effect was not studied further.

In order to test for extracellular mutagenic activity in the plating suspension, the following method was used.

a) Treated and untreated conidia were washed twice and resuspended in water. The liquid from each suspension was then separated from the conidia by millipore filtration.

b) The treated conidia were divided into two parts, one of which was plated at once to determine the mutation frequency immediately after treatment.

The second part was incubated in water for ten hours before plating, to provide an estimate of the after-effect. This was also done for the untreated conidia.

c) The filtrates were tested for mutagenic activity by adding them to freshly prepared conidia and incubating the mixture for ten hours. After incubation the

Table 1. *Effect of postincubation in water at different temperatures on inositol and adenine reverse mutations and survival after treatment with DEB and ultraviolet light*

Mutagenic treatment	Post treatment in H_2O		Survival	Reversions			
				inos⁺		ad⁺	
20° C	° C	Time	%	Counted	per 10⁶ survivors	Counted	per 10⁶ survivors
H_2O control	4	1 hr 30 min	100	0	—	0	—
		4 hrs 00 min	98	0	—	2	0.04
		6 hrs 45 min	114	0	—	0	—
		9 hrs 35 min	100	0	—	0	—
H_2O control	20	1 hr 30 min	101	0	—	1	0.02
		4 hrs 00 min	108	0	—	1	0.02
		6 hrs 45 min	106	0	—	0	—
		9 hrs 35 min	102	0	—	0	—
H_2O control	30	1 hr 30 min	100	0	—	3	0.07
		4 hrs 00 min	109	0	—	1	0.02
		6 hrs 45 min	104	0	—	1	0.02
		9 hrs 35 min	104	1	0.02	0	—
DEB 0.1 M., 35 min	4	1 hr 30 min	86	2	0.05	49	1.23
		3 hrs 40 min	100	1	0.02	61	1.32
		6 hrs 10 min	90	0	—	50	1.20
		9 hrs 00 min	94	0	—	57	1.31
DEB 0.1 M., 35 min	20	1 hr 30 min	84	0	—	43	1.10
		3 hrs 40 min	90	2	0.05	69	1.67
		6 hrs 10 min	96	2	0.05	85	1.91
		9 hrs 00 min	87	1	0.03	123	3.05
DEB 0.1 M., 35 min	30	1 hr 30 min	88	1	0.03	72	1.69
		3 hrs 40 min	87	1	0.03	149	3.71
		6 hrs 10 min	85	5	0.13	213	5.41
		9 hrs 00 min	81	0	—	268	7.18
UV 5 min, 34 cm	4	1 hr 30 min	91	209	5.0	129	3.07
		3 hrs 40 min	93	196	4.6	130	3.03
		6 hrs 10 min	96	206	4.7	150	3.38
		9 hrs 15 min	83	239	6.4	130	3.48
UV 5 min, 34 cm	20	1 hr 30 min	90	206	5.0	129	3.08
		3 hrs 40 min	89	245	6.0	134	3.26
		6 hrs 10 min	93	247	5.7	136	3.14
		9 hrs 15 min	85	206	5.2	133	3.37
UV 5 min, 34 cm	30	1 hr 30 min	91	232	5.5	132	3.12
		3 hrs 40 min	89	238	5.8	132	3.20
		6 hrs 10 min	82	195	5.2	92	2.43
		9 hrs 15 min	92	209	4.9	103	2.43

conidia were plated. The results are presented in Table 2. The after-effect is seen by comparing series 4 with series 3. Nevertheless, no mutagenic effect could be demonstrated in freshly prepared conidia treated for a similar period with the filtrate from DEB-treated, twice washed and resuspended conidia (series 6). It is, therefore, concluded that extracellular mutagen left by inefficient washing is not sufficient to produce the after-effect.

3. The possible effects of anoxia on the after-effect

In the experiments described thus far, the after-effect was observed when treated and washed conidia were left undisturbed in a centrifuge tube. Unless

Table 2. *Test for extracellular mutagenic activity in the suspensions of DEB treated and untreated twice washed and resuspended conidia*

Series number	Treatment		Hours of incubation between treatment and plating	per cent survival	ad+ counted	ad+/10⁶	
	Agent	Hours				initial	survivors
1	H$_2$O	1	none	100	3	0.085	0.085
2	H$_2$O	1	10	99	0	—	—
3	0.1 M DEB	1	none	70.3	857	24.3	34.5
4	0.1 M DEB	1	10	62.4	1568	44.4	71.3
5	Filtrate from Series 1	10	none	100	6	0.14	0.14
6	Filtrate from Series 3	10	none	102.2	3	0.06	0.06

they are in some way stirred, the conidia quickly settle and form a layer at the bottom of the tube. It is known that, under these conditions, anoxia rapidly develops in the suspension (Kølmark, unpublished data) and the possibility was considered that anoxic conditions after DEB treatment might give rise to the after-effect. This was apparently supported by the finding that no after-effect occurred when the treated conidia were kept agitated for four hours in a shallow dish at 25° C. However, this experiment did not decide whether the prevention of anoxia or the prevention of the spores from settling is the important factor in abolishing the after-effect. The role played by anoxia was studied in the following experiment. Two samples of treated, twice washed conidia were transferred to large test tubes. Air was bubbled through one and nitrogen through the other at rates which kept the conidia in suspension. Other samples were transferred to test tubes which were left undisturbed so that the conidia settled to the bottom of each tube. All the tubes were incubated at 25° C. After various intervals samples were withdrawn for plating to determine the survival and mutation frequency. The results are given in Fig. 2.

Whether expressed as an increase in mutations or a drop in survival, the after-effect was found only in those series in which the conidia were allowed to settle during post-treatment. In these series survival dropped steadily from 80 to 20% in 24 hours, and, in doing so, gave play to selective forces which complicated the after-effect on mutation frequency. During the first eight hours there was a clear increase not only in the frequency of adenine reversions among survivors, but also in the absolute numbers of revertants scored. In the period from twelve to 24 hours the mutation frequency among survivors fell rapidly indicating selection against the newly arisen mutants.

Both expressions of the after-effect, the drop in survival and the increase in mutation frequency, were abolished when nitrogen or air was passed through the treated suspensions. Both gases were equally effective in bringing about this abolition. It was concluded that anoxia alone cannot promote the after-effect. The simplest interpretation of the bubbling effect was that ordinary washing does not completely free the conidia from DEB or a mutagenic derivative of it. In well stirred suspensions the remaining mutagen is removed by diffusion. This

is slowed down in undisturbed, settled spores. Two further experiments were designed to test this assumption.

4. The effect of conidial number on the after-effect

If the after-effect is the result of mutagen retained in the cells because of restricted diffusion, its magnitude should increase as the suspension density is increased. Accordingly a suspension of 6.9×10^6 conidia per ml was treated with DEB and washed. A sample was first plated to determine the immediate effect
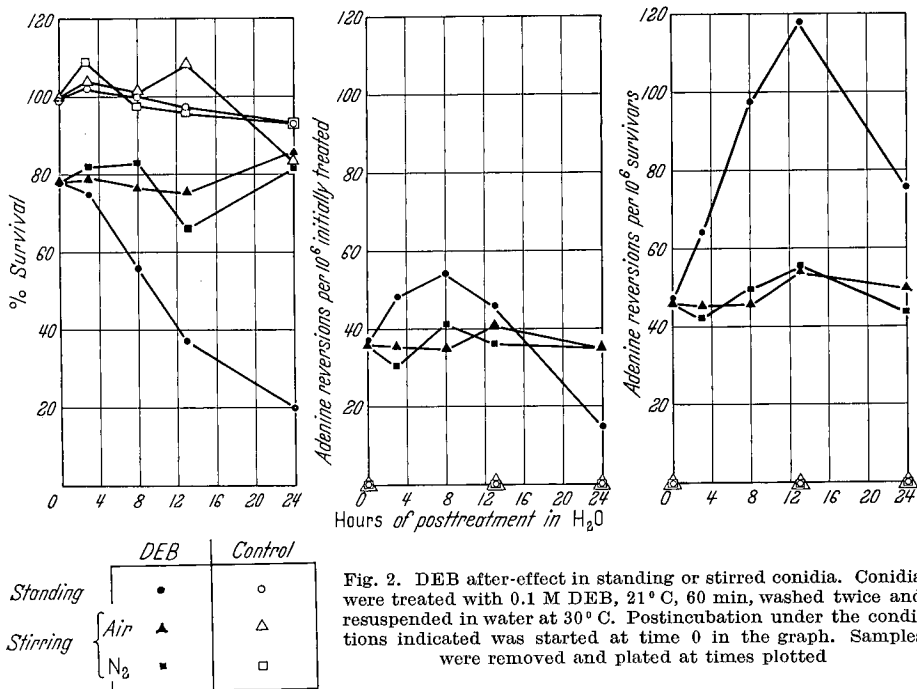

Fig. 2. DEB after-effect in standing or stirred conidia. Conidia were treated with 0.1 M DEB, 21° C, 60 min, washed twice and resuspended in water at 30° C. Postincubation under the conditions indicated was started at time 0 in the graph. Samples were removed and plated at times plotted

of DEB on mutation frequency and survival. The remainder was divided into three suspensions of different conidial densities; 1.38, 6.9 and 34.5×10^6 per ml. 10 ml samples of each suspension were either allowed to stand, or were stirred for seven hours before plating. The results are given in Table 3. In agreement with the expectation the after-effect increased with increasing suspension density.

5. Cancellation of the after-effect by shaking

If cells in suspension lose the remaining traces of DEB by diffusion, their ability to show an after-effect should decrease in proportion to the time they are kept in suspension before being allowed to settle. Treated, washed conidia were kept suspended by shaking for periods of up to four hours. At different times after the commencement of shaking three samples were removed simultaneously. One of these was plated immediately, the other two were allowed to stand six and twelve hours respectively before plating. The results are shown in Fig. 3.
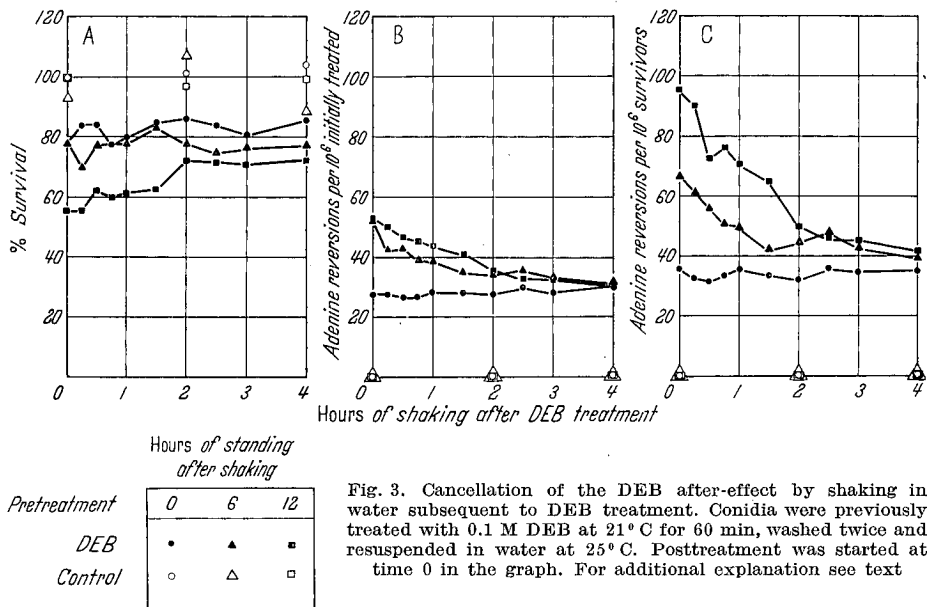
Fig. 3. Cancellation of the DEB after-effect by shaking in water subsequent to DEB treatment. Conidia were previously treated with 0.1 M DEB at 21° C for 60 min, washed twice and resuspended in water at 25° C. Posttreatment was started at time 0 in the graph. For additional explanation see text

Table 3. *The effect of conidial number on the DEB after-effect*

Treatment		Post treatment		Hours of post-treatment	Survival %	ad+ counted[1]	ad+/10⁶	
DEB molar conc.	Conidia/ml	Conidia × 10⁻⁶/ml	Standing or aerated				initial	survivors
0	6.9 × 10⁶	—	—	0	100	2	0.06	0.06
		1.38	St.	7	92.1	0	—	—
		6.9			99.2	5	0.15	0.15
		34.5			89.6	15	0.06	0.07
		1.38	Air		101.4	0	—	—
		6.9			97.9	3	0.09	0.09
		34.5			99.8	5	0.03	0.03
0.1		—	—	0	78.1	1315	38.1	48.8
		1.38	St.	7	55.0	332	48.1	87.5
		6.9			65.8	1995	56.3	85.5
		34.5			50.1	12350	71.6	143.0
		1.38	Air		71.0	262	38.0	53.5
		6.9			69.3	1493	43.3	62.5
		34.5			77.7	6970	40.4	52.0

[1] These numbers represent the colonies found from 5 ml of the suspension at the density used during the post treatment.

Each part of this diagram consists of three curves. One, symbolised by circles, shows survival (part A) or mutation frequency (parts B and C) when the treated conidia were plated immediately after being kept in suspension for a given number of hours (abscissa). The second and third curves (triangles and squares respectively) give the corresponding values when a six- or twelve-hour period of standing was interposed between the shaking and plating. The distance between a circle and the corresponding triangle or square is a measure of the

after-effect still obtainable after a given period of shaking. The after-effect without shaking (points on the ordinate) diminishes progressively as the period of shaking is increased. After four hours' shaking insufficient mutagen remains to produce any appreciable after-effect.

6. A test for extracellular mutagenic activity in suspensions of stirred and unstirred conidia

In section 2 the absence of extracellular mutagenic activity in the plating suspension of treated spores immediately after washing was shown. It seemed possible, however, that during a prolonged post-treatment sufficient mutagen for detection, using the same test, would diffuse from the spores, particularly in stirred suspensions. Conidia at a density of 1×10^7 per ml were treated with DEB, washed and resuspended at a density of 5×10^7 per ml. 10 ml samples were allowed to stand or were stirred for seven hours at 25^0 C. Filtrates from these suspensions were then added to freshly prepared conidia and these were kept stirred for 12 hours before plating. No mutagenic or toxic effects could be detected. It is concluded that any mutagen which the treated conidia might have released into the water is insufficient to elicit a mutagenic response from untreated conidia.

7. An attempt at chemical inactivation of DEB

Ross (1950), using a method of titration of epoxides, added the epoxide to a boiling 0.2 M solution of sodium thiosulphate in 50% acetone. The developing alkalinity was titrated with 0.2 N acetic acid. In the present experiments, DEB-treated conidia were washed twice with sodium thiosulphate at concentrations which varied from 0.025 to 0.4 M at 21^0 C. The conidia were kept in thiosulphate for 20 min and were then suspended in water to test the after-effect in the usual manner. The treatment had no toxic effect and caused no appreciable reduction of the after-effect, probably because, under physiological conditions, the reaction of DEB with thiosulphate is too slow.

Discussion

Mutagenic after-effects in cells stored in water or buffer have been observed by workers with several organisms and following the use of several mutagens. The explanations offerred to account for the phenomenon fall into two classes, a) those which assume the mutations constituting the after-effect are already induced but not completed during treatment, and b) those which assume the mutations are actually induced after the withdrawal of the mutagen. In the last case, either residual mutagen or a mutagenic derivative produced during treatment must be responsible.

BAUTZ and FREESE (1960) suggest an explanation of the first type to account for their results with ethyl ethanesulphonate, EES. Both inactivation and mutations at the rII locus of bacteriophage T 4 continued to increase when the phage particles were kept in buffer at neutral p_H. EES is thought to ethylate guanine residues in the DNA. The product is a resonance hybrid which, because of a weakened bond with the deoxyribose of the DNA backbone, tends to eliminate guanine. In subsequent replications any of the four bases can theoretically

replace guanine and errors in the base order may result. Clearly, the initial ethylation is all that is necessary and the rest of the process would not require the intervention of EES. A similar interpretation has also been used by STRAUSS to account for results obtained by treating *Escherichia coli* with Ethyl methane-sulphonate (STRAUSS 1962).

Although DEB has been shown to react with guanine (BROOKES and LAWLEY 1961), an explanation of the after-effect modelled on that of BAUTZ and FREESE is unacceptable. The after-effect of DEB can be completely prevented by any conditions which keep the conidia in suspension. It is difficult to see why this should prevent the removal of alkylated guanine from DNA. The longer the treated conidia are retained in suspension the less is the after-effect finally obtained. This seems rather to indicate that residual mutagen is left after washing. It would appear that, while the normal washing is able to remove extracellular mutagen efficiently, it cannot remove intracellular mutagen. This is probably so because, during each washing, the conidia are closely packed by centrifugation and diffusion from the spores is restricted. However, as long as the washed conidia are prevented from sedimenting, diffusion is rapid enough to remove the mutagen before the after-effect can occur.

Plating also prevents the after-effect. Resumption of growth does not appear to be responsible for this. Conidia were treated and shaken for periods of up to two and a half hours in either water or liquid growth medium. After this treatment samples were tested for the after-effect. As expected, the magnitude of the after-effect diminished as the time of shaking increased. No differences were detectable between spores suspended in water and those suspended in growth medium. A more acceptable explanation of the plating effect is that, at the density at which the spores are in-plated (6×10^5/ml) diffusion can remove the mutagen rapidly enough to prevent the after-effect.

SZYBALSKI, also, postulates the presence of residual mutagen to account for the after-effect he finds in *Escherichia coli* treated with triethylenemelamine, TEM (SZYBALSKI 1960). Mutations from streptomycin dependence to independence continue to occur for periods of up to ten or twelve days after the extracellular mutagen has been removed. SZYBALSKI suggests that enough TEM is bound to the cells to produce these mutations and that it does so by reacting with DNA precursors. After the altered precursors have been incorporated into the DNA they produce errors in replication at later divisions. Evidence that TEM reacts with thymidine to give a mutagenic product rests on the finding that a mixture of TEM and thymidine is twice as mutagenic as TEM alone. No mutagenic product could be isolated from this mixture (LORKIEWICZ and SZYBALSKI 1961).

The question as to the nature of the mutagen responsible for the DEB after-effect cannot yet be answered. Since it takes such a long time to remove it from the cells by diffusion it might be suspected that DEB does not exist in the free state within the cells. Whether, however, it has reacted with a cell component to give a diffusible mutagenic derivative, or whether it is absorbed in an unreacted form is not known. Spectrophotometric studies have shown that U.V.-absorbing material passes into the surrounding water when treated cells are resuspended. There is no evidence that this material is mutagenic towards freshly prepared conidia and it may have no role in the mutagenic process at all.

Our experiments emphasise the need for caution in interpreting the after-effect phenomenon. Clearly a great deal of care must be taken to ensure that additional mutations are not the result of an inefficient washing process. Inability to detect free mutagen by chemical methods is not necessarily a conclusive test since it may simply mean that the mutagen has been transformed into a state not detected by the particular test employed. Before these problems can be solved the formulation of more elaborate hypotheses must be deferred.

Summary

Butadiene diepoxide (DEB) was used to induce reverse mutations in an *ad—3A* mutant of *Neurospora crassa*. It was found that the mutagenic action of DEB continued when the twice washed and resuspended conidia were left standing in water at room temperature. The after-effect can be prevented if the conidia are kept stirred by shaking or gaseous bubbling for 4—5 hours. The after-effect does not develop if the conidia are plated immediately after washing, or if they are kept at 0—4° C until plating. The experimental data indicate that the after-effect is caused by traces of DEB, or by mutagenic reaction products of DEB, which are not readily removed by the ordinary washing procedure, but which may be removed by diffusion from the cells when these are kept suspended for a prolonged period.

Literature cited

BAUTZ, E., and E. FREESE: On the mutagenic effects of alkylating agents. Proc. nat. Acad. Sci. (Wash.) **46**, 1585—1594 (1960).

BEADLE, G. W., and E. L. TATUM: Methods of producing and detecting mutations concerned with nutritional requirements. Amer. J. Bot. **32**, 678—686 (1945).

BROOKES, P., and P. D. LAWLEY: The alkylation of guanosine and guanylic acid. J. chem. Soc. **766**, 3923—3928 (1961).

IYER, V. N., and W. SZYBALSKI: The mechanism of chemical mutagenesis. I. Kinetic studies on the action of triethylene melamine (TEM) and azaserine. Proc. nat. Acad. Sci. (Wash.) **44**, 446—456 (1958).

KØLMARK, H. G., and C. AUERBACH: Mutagenic after-effect in *Neurospora* treated with diepoxybutane. Microbial Genetics Bull. **17**, 24—25 (1960).

—, and M. WESTERGAARD: Further studies on chemically induced reversions at the adenine locus of *Neurospora*. Hereditas (Lund) **29**, 209—224 (1953).

LORKIEWICZ, Z., and W. SZYBALSKI: The mechanism of chemical mutagenesis. IV. Reaction between triethylene melamine and nucleic acid components. J. Bact. **82**, 195 (1961).

ROSS, W. C. J.: The reactions of certain epoxides in agueous solutions. J. chem. Soc. **464**, 2257—2272 (1950).

STRAUSS, B. S.: Response of *Escherichia coli* auxotrophs to heat after treatment with mutagenic alkyl methanesulphonates. J. Bact. **83**, 241—249 (1962).

SZYBALSKI, W.: The mechanism of chemical mutagenesis with special reference to triethylene melamine action. Developments in Industrial Microbiology, pp. 231—241 (Brinton M. Miller, Ed.). New York: Plenum Press 1960.

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<div align="center">Short Communication</div>

A Mutagenic After-Effect Associated with Ethylene Oxide in *Neurospora crassa*

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Introduction

In our previous publications we have compared the mutagenic characteristics of the bifunctional butadiene diepoxide (DEB) and its monofunctional equivalent (EO) ethyleneoxide (Kølmark, Kilbey and Kondo, 1963; Kølmark and Kilbey, 1968). From this work it appears that the process whereby reversions of the *ad-3A 38701* allele are induced is similar for the two compounds and that bifunctionality is not important for the mutagenic and lethal effects of DEB in this system.

DEB is interesting in another way. It was shown some time ago (Kølmark and Auerbach, 1960) that when treated conidia of *Neurospora* are washed free of DEB and allowed to stand they continue to exhibit reversions to adenine independence. We were subsequently able to show that by continuously stirring the washed spores this mutagenic after-effect was abolished (Kølmark and Kilbey, 1962). It appears that standard washing procedures involving centrifugation or filtration are insufficient to remove the mutagen from the cells. Although the bifunctional nature of DEB is not important for its mutagenic effect, the possibility has been raised that bifunctionality might be significant in promoting the after-effect (Loveless, 1966). This might occur if, for example, the mutagen first alkylated a non-DNA component of the cell, thus becoming "anchored" in the cell, and subsequently alkylated the DNA. We have attempted to investigate this suggestion by testing the monofunctional equivalent of DEB, EO, for evidence of an after-effect.

Materials and Methods

The strain and the techniques employed in this investigation are the same as those described earlier (Kølmark and Kilbey, 1962; Kølmark and Kilbey, 1968).

Results and Discussion

Several experiments of one type were performed to test for an after-effect with EO. In the one presented below 2×150 ml of a conidial suspension containing 1×10^7 conidia per ml were exposed to a 0.14 M solution of EO in water for 10 minutes and 20 minutes respectively. The temperature during exposure was

Table. *The mutagenic after-effect of ethylene oxide in the strain K3/17 ad-3A (38701)*

Mutagenic Treatment	Post treatment conditions	Survival		Ad reversions	
		Counts	%	Counts	ad+/10⁶ surv.
Controls untreated	Plated immediately after treatment	753	100%	0	—
		798	Average	1	0.02
	Plated after	798	survival	1	0.02
	240 minutes	894		0	—
10 minutes, 0.14 M EO, 22°C	Immediate plating	726	87.5	24	0.66
	Stirred samples				
	30 minutes	796	96.0	23	0.58
	60 minutes	714	86.0	27	0.76
	90 minutes	898	108.4	23	0.51
	120 minutes	786	94.9	19	0.48
	180 minutes	777	93.5	11	0.28
	240 minutes	701	84.6	6	0.17
	Standing samples				
	30 minutes	793	95.5	27	0.68
	60 minutes	697	84.2	53	1.52
	90 minutes	716	86.3	73	2.04
	120 minutes	788	95.0	87	2.21
	180 minutes	724	87.2	123	3.40
	240 minutes	738	88.9	161	4.40
20 minutes, 0.14 M EO, 22°C	Immediate Plating	652	80.5	199	6.1
		659	81.2	191	5.8
	Stirred samples				
	30 minutes	lost			
	60 minutes	681	84.2	167	4.9
	90 minutes	686	84.8	159	4.6
	120 minutes	839	103.2	146	3.5
	180 minutes	694	85.7	131	3.8
	240 minutes	707	87.5	143	4.0
	Standing samples				
	30 minutes	649	80.3	240	7.4
	60 minutes	753	93.0	373	9.9
	90 minutes	750	92.7	407	10.7
	120 minutes	695	86.0	558	16.0
	180 minutes	800	98.9	811	20.3
	240 minutes	686	85.2	1010	29.6

22° C. The treatments were terminated when the conidial suspensions were filtered through "Oxoid" membrane filters to remove the mutagenic solution and the spores washed with ice-cold water. The treated conidia were washed off into 150 ml sterile water at room temperature. Immediately after resuspension, samples from each treatment were plated to provide an estimate of the mutation frequency in the absence of an after-effect. The remainder of each suspension was distributed in 10 ml samples to six centrifuge tubes and six test tubes fitted with aerators. The samples in the centrifuge tubes were sedimented and left to stand, the samples in tubes were stirred continuously by aeration to prevent

settling. A standing and a stirred sample was plated at the following times after treatment termination; 30, 60, 90, 120, 180 and 240 minutes.

The results of this experiment are given in the Table and in Fig. 1. From these data it is apparent that under the same conditions used to promote a mutagenic after-effect with DEB, EO also exhibits an after-effect. Furthermore, the conditions which abolish the after-effect with DEB also abolish the after-effect of EO. Bifunctionality is therefore not a prerequisite for the DEB after-effect.

It will be observed that when EO-treated spores are plated after various periods of shaking, the mutation frequency decreases slightly. This tendency was not observed among samples similarly treated with DEB (KØLMARK and KILBEY, 1962, Fig. 2). One interpretation of the present findings is that although a major part of the EO after-effect *is* abolished by plating, traces of the mutagen persist long enough to produce a limited after-effect even on the plates. After prolonged washing this after-effect is also eliminated. For DEB plating appeared to completely arrest the after-effect.

It is important to emphasize the difference between the after-effect described here and the after-effect described by other workers with these substances. WATSON (1964) has described experiments which show that, when DEB-treated sperm of *Droso-phila* are stored in the female, the frequency of translocations (chromo-some breaks) increase with time of storage. In comparison, sex-linked-lethals (mainly point mutations) show only a slight increase over the same period. Similar findings were found with triethylene melamine (WATSON, 1966), but neither EO nor ethyleneimine, the monofunctional counterparts, showed an after-effect.

Fig. 1. The mutagenic after-effect of ethylene oxide in the strain *K3/17 ad-3A (38701)*

Mr. RATNAYAKE working in this laboratory has confirmed the earlier findings of Watson and shown that they also apply to dominant lethals (single chromo-some breaks), (RATNAYAKE, personal communication).

Thus, in *Drosophila* the after-effect is confined principally to events believed to result from chromosome breakage and not point mutations. Only the bi- or polyfunctional agents appear able to give rise to the after-effect. In *Neurospora* the damage giving rise to reversions is probably mainly of the point-mutation type although the sophisticated tests used for *Drosophila* are not available to substantiate this. Both the bi- and monofunctional agents are effective in produc-
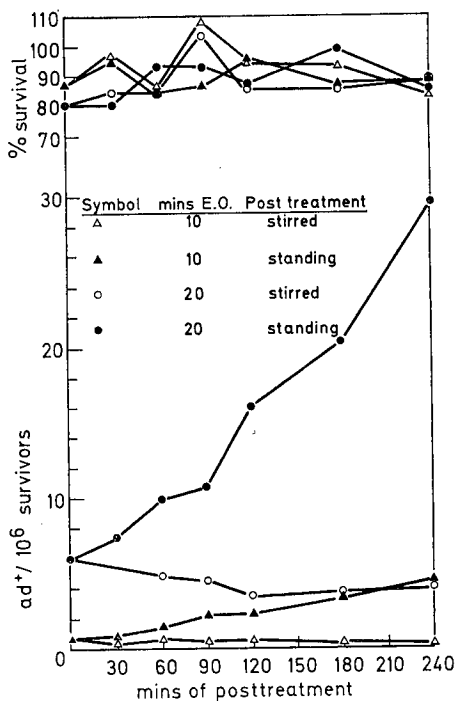
ing an after-effect. We conclude that the present data confirm our earlier supposition that the after-effect found with DEB (and now EO) in *Neurospora* is due to retention of traces of mutagen in spite of the standard washing procedure followed.

References

KØLMARK, H. G., and C. AUERBACH: Mutagenic after-effectital in *Neurospora* treated with diepoxybutane. Microbiol. Gen. Bull. **17**, 24—25 (1960).
—, and B. J. KILBEY: An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa*. Z. Vererbungsl. **93**, 356—365 (1962).
— —, and S. KONDO: Kinetic studies of chemically induced reverse mutations in *Neurospora crassa*. Genetics Today Vol. I, 61—62. Pergamon Press 1963.
— — Kinetic studies of mutation induction by epoxides in *Neurospora crassa*. Molec. Gen. Genetics. 1968.
LOVELESS, A.: Genetic and allied effects of alkylating agents, p. 88—89. London: Butterworths 1966.
WATSON, W. A. F.: Evidence of an essential difference between the genetical effect of mono- and bi-functional alkylating agents. Z. Vererbungsl. **95**, 374—378 (1964).
— Further evidence of an essential difference between the genetical effects of mono- and bi-functional alkylating agents. Mutation Research **3**, 455—457 (1966).

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Kinetic Studies of Mutation Induction
by Epoxides in *Neurospora crassa*

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Summary. 1. The macroconidial strain *K 3/17 ad-3 A (38701)* was used in reverse mutation experiments comparing the mutagenic properties of the epoxides diepoxybutane (DEB) and ethyleneoxide (EO). A microconidial strain with the same *ad* allele was also used.

2. The kinetics of mutation induction for both the monofuctional compound EO and the difunctional compound DEB follows the same generalized equation:

$$ad^+ \times 10^{-6} \text{ survivors} = a(ct)^b.$$

where a and b are constants for a given epoxide, when the strain and the experimental conditions are defined. The product (ct) is defined as the "dose", where c and t are the molar concentration of the mutagen and the duration of the treatment, respectively.

3. The mutagenic response for a given mutagen is independent of the dose rate, except where the treatment is for an extended duration (t very large), in which case the effect is diminished.

4. EO is about 20 times more "effective" than DEB when comparison of mutation frequencies is based on equal doses. The two compounds are equally "efficient", when mutation frequencies are compared at equal survivals.

5. The significance of the kinetic data is considered in relation to some possible models of mutation mechanism. The results are discussed in relation to previous data with this system, and to work by other authors.

Introduction

The epoxide, diepoxybutane, DEB, has been shown to be a potent inducer of reversions of the mutant *ad-3 A (38701)* of *Neurospora crassa* (Kølmark and Westergaard, 1953; Kølmark, 1953; Kølmark and Kilbey, 1962). Mutation induction proceeds at an ever increasing rate as treatment time increases (see Kølmark and Giles, 1955, Fig. 1).

This type of mutation curve suggests the requirement for two or more independent events to take place in order to induce a mutation. The present experiments were designed to investigate this in more detail by varying the dose rates of treatment over wide ranges.

At the same time it seemed important to compare the data obtained by the difuctional compound DEB with those obtained using the monofuctional equivalent, ethylene oxide, EO. Some preliminary data were reported elsewhere (Kølmark, Kilbey and Kondo, 1963).

Materials and Methods

1. Strains

The strain *K 3/17, ad-3 A (38701) inos (37401)*, auxotrophic for both adenine and inositol, was used in most of the experiments. This strain is macroconidial, and it grows with small compact colonies in the absence of growth inhibiting substances. A detailed description

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of this material may be found in Kølmark and Kilbey (1962), but it may be noted here that the *ad* and *inos* mutants revert spontaneously with frequencies of 5 and 1 per 10^8 conidia, respectively.

The microconidial strain *398-28 A, ad-3 A (38701)* was also used in some experiments. It has a colonial growth habit which is very similar to that of strain *K 3/17*.

2. Media

For conidial production each litre of Fries' minimal medium (Beadle and Tatum, 1945) was supplemented with 2.5 gms Difco powdered yeast extract, 1.0 gms casein hydrolysate (British Drug Houses Ltd.), 5.0 gms malt extract, 15 ml glycerol, 100 mg adenine sulphate, 10 mg meso-inositol, and 15 gms Difco Bacto agar.

Survival was scored on Fries, minimal salts medium containing 10 gms glucose per litre, and supplemented with 40 mg adenine sulphate and 20 mg meso-inositol. Adenine reversions were scored on the same medium without adenine sulphate.

3. Mutagens

Diepoxybutane, $H_2C - CH - CH - CH_2$, (DEB), was obtained from Columbia Organic
$\backslash O /\backslash O /$
Chemicals Co., Inc. It consists of D—L butadiene-diepoxide with not more than 3% of the meso-form.

Ethyleneoxide, $H_2C - CH_2$, (EO), was obtained from Imperial Chemical Industries Ltd.
$\backslash O /$

The compressed gas was dissolved in distilled water and the concentration of EO determined by titration with 0.1 N HCl in the presence of excess KCl. The indicator used was methyl red and the reaction mixture was maintained at 50°C during titration. After the actual concentration had been determined, suitable dilution of the solution was made to obtain the desired concentration of the mutagen.

4. Mutagenic Treatment

Conidia were collected in water from slants grown for one week at 25°C. The suspension was filtered through cotton to remove mycelial fragments, and then washed twice with water before mutagenic treatment. Conidial concentration was estimated using a haemocytometer and the final concentration of conidia was adjusted to 1×10^7/ml.

Aliquots of this suspension were treated in centrifuge tubes in most cases, although in one or two experiments larger volumes of suspension were treated in flasks. In the first case treatment and washing were carried out in the same vessel, in the second case the treatment was terminated by washing the conidia on "Oxoid" membrane filters.

The treatment was timed from the point of addition of the chemical. Before and during treatment the conidia were maintained at 22°C.

The conidia in the tubes were kept stirred throughout exposure to the chemical by shaking the tubes in a waterbath. It was found necessary to incorporate a "floating stirrer" device in addition to the shaking since conidia still tended to settle during treatment. The stirrer was made of 5 mm wide glass tubing, on one end of which was blown a bulb of c. 1 cm diameter. At the opposite end the tube was flattened for about 1 cm. The length of the device was found to be right for an efficient stirring when the tip of the paddle kept floating a few mm above the bottom of the tube. A hook made of glass was used to insert and remove the stirrer.

The treatment was terminated by centrifuging to sediment the conidia and then pouring off the mutagenic solution. Ice cold water was added to resuspend the conidia at the point of termination. The conidia were washed twice with water and were finally suspended in ice cold water and kept at approximately 3°C until plating. There is no lethal or mutagenic "after-effect" under these conditions (Kølmark and Kilbey, 1962).

Diluted and undiluted series were plated for survival and mutation to adenine independence, respectively. The plates were incubated for 6—8 days at 25°C before scoring.

Results

1. Kinetic Formulation of Mutation Induction by DEB .

In experiments concerned with the kinetics of mutation induction three of the most important variables would appear to be the concentration of the mutagen, c, the duration of the treatment, t, and the temperature during treatment. The experiments to be described were performed at a constant temperature but both the concentration and the duration of treatment were varied.

Table 1 shows data from an experiment in which conidia were treated for four different periods at each of four different concentrations of DEB. The mutation frequencies and survival percentages from this experiment are plotted as functions of time and of dose (ct), in Fig. 1a, b and c.

Table 1. *Induction of reverse mutations by DEB in strain K 3/17 ad-3A (38701)*

Series no.	c DEB molar conc.	t min and sec	ct mol × min	Survival %	ad^+ reverse mutations counted	per 10^6 survivors
1	1×10^{-2}	180.00	1.8	95.8	33	0.96
2	1×10^{-2}	360.00	3.6	92.5	117	3.51
3	1×10^{-2}	540.00	5.4	83.1	157	5.25
4	1×10^{-2}	720.00	7.2	61.7	176	7.93
5	3.16×10^{-2}	60.00	1.9	112.0	31	0.73
6	3.16×10^{-2}	120.00	3.8	91.3	122	3.71
7	3.16×10^{-2}	180.00	5.7	88.6	349	10.9
8	3.16×10^{-2}	240.00	7.6	72.0	550	21.2
9	1×10^{-1}	20.00	2.0	88.7	35	1.09
10	1×10^{-1}	40.00	4.0	95.8	190	5.50
11	1×10^{-1}	60.00	6.0	83.1	421	14.1
12	1×10^{-1}	80.00	8.0	58.4	658	31.4
13	3.16×10^{-1}	6.40	2.1	89.5	22	0.69
14	3.16×10^{-1}	13.20	4.2	86.1	158	5.10
15	3.16×10^{-1}	20.00	6.3	83.3	447	14.9
16	3.16×10^{-1}	26.40	8.4	65.8	567	23.9
	Controls in H_2O					
17	0	0.00	—	105.2	1	0.03
18	0	180.00	—	100.5	0	—
19	0	360.00	—	90.0	2	0.06
20	0	540.00	—	100.5	2	0.06
21	0	720.00	—	100.5	2	0.06

Fig. 1a is a plot of mutation frequency as a function of time. It is seen that the rate of induction of mutations slows down as the concentration of DEB is decreased. The same data are redrawn in Fig. 1b where, instead of time, total dose (ct) of the mutagen is used as the abscissa.

The result of plotting the data in this way is to superimpose the induction curves arising from the three highest concentrations of DEB. The curve obtained

from using 0.01 M DEB does not follow this curve and appears to have an almost linear form.

From these data it may be concluded that the mutation frequency over a wide range of c and t values depends entirely on the total dose of chemical administered. Thus at the lower doses of DEB the mutation frequency is unchanged when the concentration is varied by a factor of 30, provided the time of treatment is raised or lowered in an inverse manner. At the higher DEB concentrations c and t may be inversely varied by a factor of at least 10 without altering the mutational effect. At the lowest DEB concentration, and for extended times of treatment, the mutation yield is lower than at equivalent doses administered at the higher dose-rates. At the highest concentration and shortest times there is no lag in mutation induction to indicate that time as such has reached a lower limiting value. The only limitation found here is the purely practical one set by the time taken to apply and remove the chemical. Survival is also determined by the dose, since these data can also be superimposed when effect is plotted as a function of dose (Fig. 1a and b). At ct values of 6 and 8 there is no difference between the curves produced by the three highest DEB concentrations and the lowest concentration as was the case for mutation.

The upward-bending development of the mutation induction curve in Fig. 1a suggests that mutation induction follows a higher than first order mechanism. In Fig. 1c the logarithm of mutation frequency is plotted as a function of the logarithm of the dose (ct). Within the dose range and concentrations used (with the exclusion of 0.01 M DEB and extended duration of treatment), the mutations fall on what appears to be a straight line. A "least-squares" regression analysis was used to calculate the line which best fits the data. The line is described by the equations

$$\log (ad^+ \times 10^{-6} \text{ survivors}) = 0.76 + (2.39 \log ct) \qquad (1)$$

or
$$ad^+ \times 10^{-6} \text{ survivors} = 0.17 \ (ct)^{2.39} \qquad (2)$$

(DEB, macroconidia).

The factor 0.17 in (2) is the interception of the line with the ordinate at $ct=1$; the exponent 2.39 is the slope of the line.

This simple formula describes in exact terms the yield of adenine reversions when a chemically defined mutagen DEB reacts with a genetically defined mutant ad-3A (38701) of Neurospora crassa when the conditions of the experiment simulate those used here. An alteration of the conditions of treatment or of the physiological state of the material may modify the values given above.

2. Mutation Induction with EO

The results obtained when K3/17 (macroconidia) is treated with EO using different concentrations and duration of treatments are given in Fig. 2. Five different concentrations of EO were used in two different experiments, the molarity ranging from 0.0015 to 0.15 M. For the two highest EO concentrations the points follow a linear progression when $\log ad^+ \times 10^{-6}$ is plotted against log dose (molarity × minutes). Here, too, the curves are superimposible as for DEB.
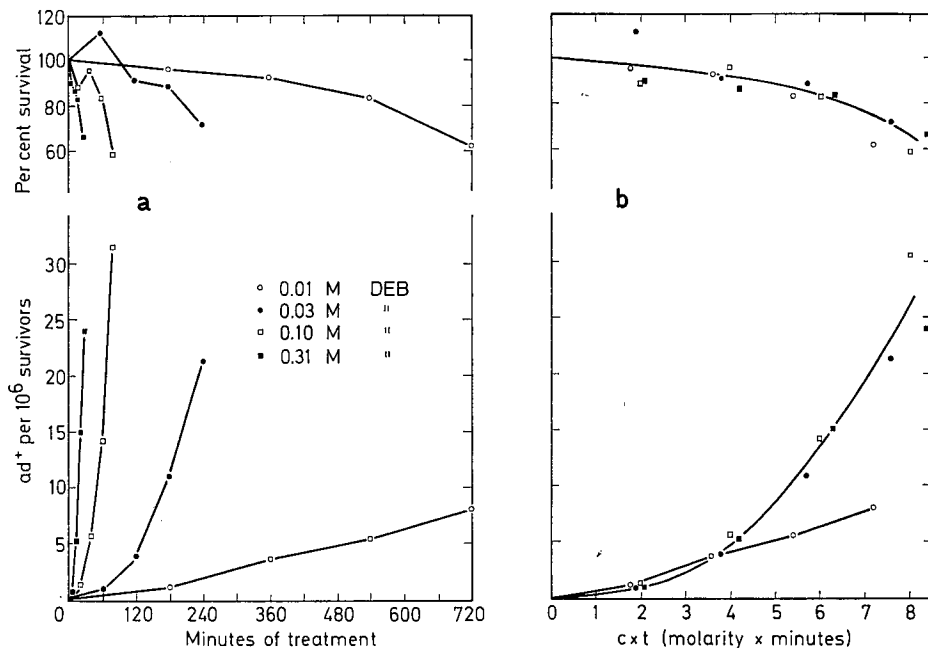
The points obtained in the series using lower molarities of EO are somewhat scattered; however, as with DEB, there is a general tendency to produce lower frequencies of mutation than expected when the time of treatment is extended. The regression line calculated using the two highest EO concentrations has the equation

$$ad^+ \times 10^{-6} \text{ survivors} = 2.61 \ (ct)^{2.69} \quad (3)$$

(EO, macroconidia).

3. Mutation Induction with DEB Using Microconidia

The data presented in the previous sections have made it possible to derive equations which describe the kinetics of
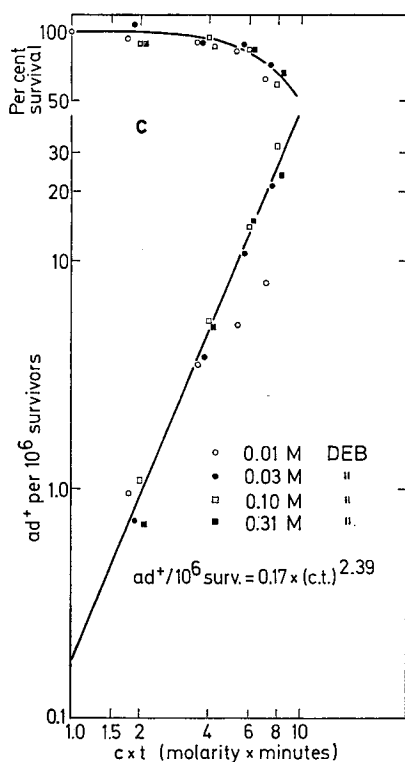
Fig. 1. Reverse mutations and survivals of strain *K3/17 ad-3A (38701)* treated with DEB at various molar concentrations and for varied durations. The same data were plotted in different functional relationships and on various co-ordinates in the partial Figures 1a, b, and c, as seen on the graphs and explained in the text
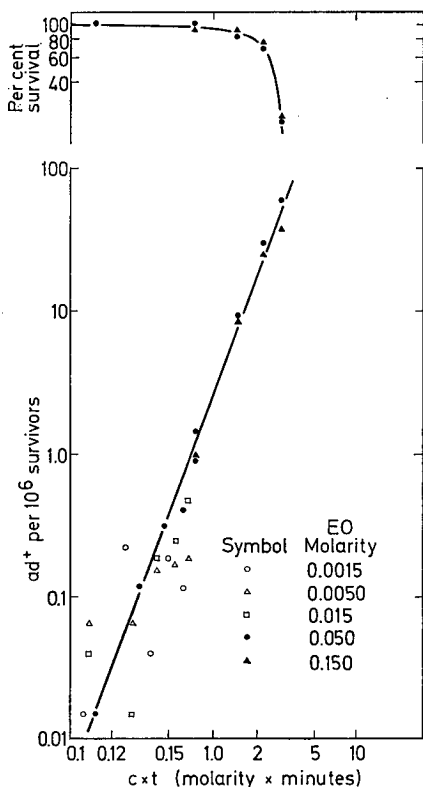
Fig. 2
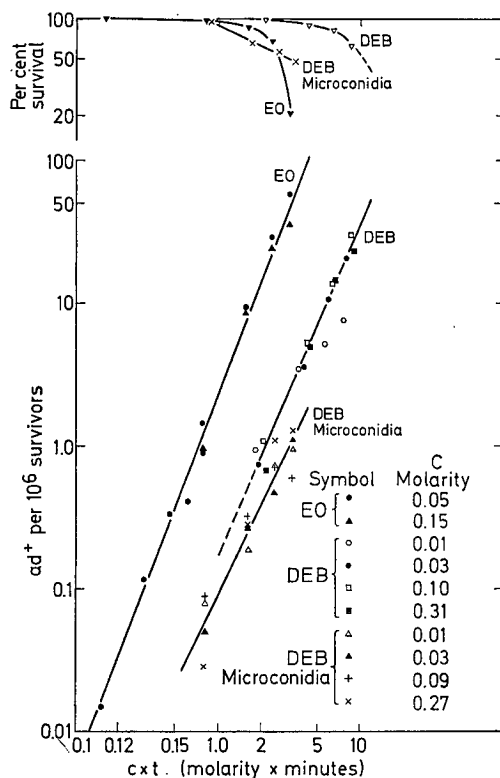
Fig. 3

Fig. 2. Reverse mutations and survivals of strain $K3/17$ ad-$3A$ (38701) after treatment with various doses of EO

Fig. 3. Comparison of mutation and survival effect in strain K $3/17$ ad-$3A$ (38701) after treatment with various doses of DEB and EO. Also, comparison of these effects after DEB treatment, when the same ad-$3A$ (38701) allele is incorporated into macro- and microconidial strains

mutation under defined conditions. The last part of this work was designed to determine the effects of changed physiological conditions on the kinetics of mutation induction. Microconidia were used which have 1 nucleus per spore as compared with 2—3 in macroconidia (HUEBSCHMAN, 1952). This difference in nuclear number might influence the production or recovery of mutants.

The microconidiating strain 398-$28A$, ad-$3A$ (38701) was treated with DEB at concentrations which ranged from 1×10^{-2} M to 2.7×10^{-1} M and the treatment times were varied such that the same four doses (ct) were given at each of the four concentrations used. Microconidia are rather more sensitive to the lethal effects of a variety of physical and chemical treatments so the doses used were somewhat lower than in the experiments with macroconidia although the ranges of concentrations were comparable.

The results of this determination are plotted in Fig. 3 together with the data from the previous experiments using macroconidia. It is clear that the micro-

conidia also show a linear increase in mutation frequency when $\log ad^+ \times 10^{-6}$ is plotted as a function of log dose. The relationship between mutation frequency and DEB dose is given as

$$ad^+ \times 10^{-6} \text{ survivors} = 0.09 \ (ct)^{2.1} \qquad (4)$$
$$\text{(DEB, microconidia)}.$$

Discussion

1. Comparison between the Actions of DEB and EO

EHRENBERG, LUNDQUIST and STRÖM (1958) proposed the use of two terms to compare the mutagenicity of different mutagenic agents. The first, the "effectiveness" of the treatment is based on a comparison of the mutagenic effects at equimolar doses of the agents. Such a measure does not take into account the differences in stability and penetration which may exist between the compared substances. They, therefore, also propose that mutagenic effect be related to other effects of the mutagen e.g. its lethality. This ratio may be termed the mutagenic "efficiency" of the agent. As will be seen different degrees of effectiveness are by no means always paralleled by corresponding values of efficiency.

From the purely practical standpoint, efficiency is clearly the more important parameter to consider, since, however effective a mutagen may be, it is of little value if it is also highly toxic. Lethality is often used as a basis for calculating mutagenic efficiency, e.g. workers with bacteriophage express the mutant frequencies as mutations per lethal hit. In some instances, however, lethality of a treatment cannot be estimated. Under these circumstances other means of obtaining a measure of mutagenic effectiveness have been employed. NAKAO and AUERBACH (1961), for example, have compared the chromosome breaking capacities of EO and DEB in *Drosophila*. Here it is impossible to estimate the survival of treated spermatozoa and these workers used recessive lethal mutations to standardise the doses of each chemical reaching the chromosomes. On a purely equimolar dose basis DEB is more effective than EO in producing chromosome breaks. However, when doses are equalised on a basis of recessive lethals, the differences disappear. In fact, this seems an even better basis than lethality for

Table 2. *Relative effectiveness of mutation induction at given doses of EO and DEB*

Mutagen	Dose ct	Frequency $ad^+/10^6$ S	Relative effectiveness EO/DEB	Increase
EO	1	2.613		
			15.1	
DEB	1	0.173[a]		
				2.9
EO	2	16.50		
			18.0	
DEB	2	0.92		
				2.8
EO	3	49.0		
			20.8	
DEB	3	2.35		

[a] This figure was found by extrapolation.

standardising the treatment dose which reaches the chromosomes since recessive lethal events are known to be chromosomal whereas lethality, as such, can result from genic and non-genic events.

EHRENBERG and GUSTAFSSON (1957) also compared EO and DEB using barley as a test organism and, although, mutagenically, DEB was more effective than EO, it was also more toxic, so, on balance, EO was somewhat more efficient. In work more closely related to the present study KØLMARK and WESTERGAARD (1953) reported data which show that ad-3A (38701) reversions in Neurospora were induced more effectively by EO, but that DEB was rather more efficient. In these experiments, however, a degree of uncertainty attaches to the data since, at the time, the after-effect associated with DEB (KØLMARK and KILBEY, 1962) was unsuspected and, therefore, was not controlled.
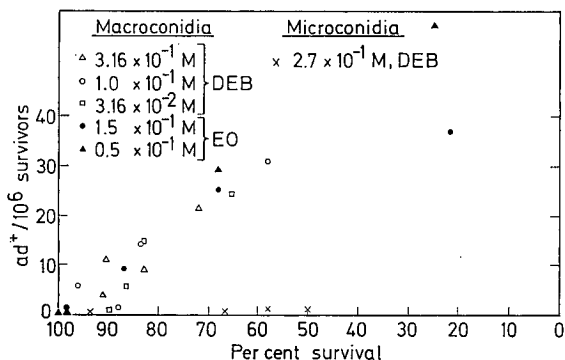
Fig. 4. Reverse mutations as functions of per cent survival. Strain K 3/17 ad-3A (38701), macroconidial, and strain 398-28 ad-3A (38701), microconidial, were treated with various doses of DEB and EO

The data obtained in the present series of experiments show that EO is about 20 times as effective as DEB in inducing adenine reversions. Table 2 shows the relative effectiveness,

$$ad^+ \times 10^{-6} \text{ survivors}/ad^+ \times 10^{-6} \text{ survivors},$$
$$\text{(EO)} \qquad\qquad\qquad \text{(DEB)}$$

for the ct values of 1, 2 and 3 using the data obtained from the high dose rate studies. Since the mutation induction curves diverge at a constant small rate the relative effectiveness also increases slowly as the dose increases. It ranges from 15 to 21.

In Fig. 4 the data from the highest dose rates of DEB and EO have been plotted with survival percentages on the abscissa and the corresponding mutation frequencies on the ordinate. It is immediately apparent that the EO data can be superimposed upon the more extensive DEB data, indicating that the two chemicals are of equal mutagenic efficiency.

This figure also demonstrates that microconidia are considerably more sensitive to DEB than macroconidia although, as has been shown, the kinetics of mutation is approximately the same in each case.

From the preceeding data is seen that the actions of DEB and EO exhibit certain similarities:

Thus, the kinetics of mutation induction for both of them (barring low dose rates) fits the generalized equation:

$$ad^+ \times 10^{-6} \text{ survivors} = a(ct)^b \tag{5}$$

where a and b are constants for a given epoxide, when the strain and the experimental conditions are defined, and where the product (ct) is the dose as defined previously.

Also, the two epoxides are equally efficient in inducing adenine reversions, when the frequencies are based on the survival (Fig. 4).

These similarities are most readily accounted for if it is assumed that EO and DEB induce mutations and cause lethality by similar mechanisms. Since EO is a monofunctional agent this suggests that DEB also acts monofunctionally and eliminates crosslinking of the DNA strands as a significant contributor to either mutations or lethality. It is interesting to note in this connection that NAKAO and AUERBACH (1961) reached a similar conclusion concerning chromosome breakage (translocations) in *Drosophila*. The higher effectiveness of EO in the *K3/17* system may be related to the size of the molecules and its ease of penetration.

2. The Mechanism of Mutagenesis

The kinetics of mutation induction by both EO and DEB have exponents which are close to 2. With microconidia the exponent approaches 2 even more closely. Formally this could be interpreted as indicating that two independent events are required in the same cell before an adenine reversion is realised. One may only speculate concerning the nature of these events. They may both occur in the DNA of the gene or one may occur elsewhere in the cell.

One possibility which may help to explain these data arises from the finding of BROOKES and LAWLEY (1961). These workers showed that, after the N-7 position of guanine had been alkylated, further alkylation could take place at the N-9 position. If we postulate that mutations are far more probable after a double alkylation it is possible to predict that quadratic curves similar to the types encountered here would be obtained. This explanation could also account for the results arising from treatment at low dose rates. Under these circumstances only one alkylation might occur leading to a linear induction curve. Although this may be the basic mechanism underlying mutation induction, additional factors of some kind must still be considered probable because the exponents obtained were all greater than 2.

While molecular interpretations of this type must be considered, other explanations must not be ignored. One of these, for which there seems some precedent (see HAYNES, 1966), involves the progressive inactivation of a repair system by the mutational treatment according to a strict kinetic formulation. This could result in an otherwise linear induction curve bending upwards as the dose of treatment increased. Independence of dose rates at higher molarities can be accounted for if the inactivated repair system is replenished slowly. This would also conveniently account for the linear increase of mutations with dose at the lowest rate: Under these conditions replenishment of the repair system can keep pace with its inactivation and maximum repair takes place at all points. An

explanation of this type would also fit observations of Auerbach (personal communication) that a previous exposure of *K 3/17* to DEB sensitises the cells to a second exposure to the same chemical.

At present we can only describe the kinetics of mutagenesis by EO and DEB, no definite mechanism can be indicated. However we can add to the evidence that cross-linking is not an important factor in DEB mutagenesis, and it is hoped that more experiments may indicate whether or not inactivation of repair is a reasonable hypothesis in this context.

References

Beadle, G. W., and E. L. Tatum: Methods of producing and detecting mutations concerned with nutritional requirements. Amer. J. Bot. **32**, 678—686 (1945).

Brookes, P., and P. D. Lawley: The alkylation of guanosine and guanylic acid. J. chem. Soc. **766**, 3923—3928 (1961).

Ehrenberg, L., and A. Gustafsson: On the mutagenic action of Ethylene Oxide and Diepoxybutane in Barley. Hereditas (Lund) **43**, 595—602 (1957).

— U. Lundquist, and G. Ström: The mutagenic action of ethylene imine in barley. Hereditas (Lund) **44**, 330—336 (1958).

Haynes, R. H.: The interpretation of microbial inactivation and recovery phenomena. Radiat. Res., Suppl. **6**, 1—29 (1966).

Huebschman, C.: A method for varying the average number of nuclei in the conidia of *Neurospora crassa*. Mycologia **44**, 599—604 (1952).

Kølmark, H. G.: Differential response to mutagens as studied by the *Neurospora* reverse mutation test. Hereditas (Lund) **39**, 270—276 (1953).

—, and N. H. Giles: Comparative studies of monoepoxides as inducers of reverse mutations in *Neurospora*. Genetics **40**, 890—902 (1955).

—, and B. J. Kilbey: An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa*. Z. Vererbungsl. **93**, 356—365 (1962).

— —, and S. Kondo: Kinetic studies of chemically induced reverse mutations in *Neurospora crassa*. Genetics today, vol. I, p. 61—62. London: Pergamon Press 1963.

—, and M. Westergaard: Further studies on chemically induced reversions at the adenine locus of *Neurospora*. Hereditas (Lund) **39**, 209—224 (1953).

Nakao, Y., and C. Auerbach: Test of a possible correlation between crosslinking and chromosome breaking abilities of chemical mutagens. Z. Vererbungsl. **92**, 457—461 (1961).

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The Stabilisation of a Transient Mutagen-Sensitive State in *Neurospora* by the Protein Synthesis Inhibitor Actidione*

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Summary. Experimental evidence has been obtained to show that a transient mutagen sensitive state, believed to be induced in *Neurospora* by DEB, can be stabilised by the protein synthesis inhibitor actidione. Sensitisation can thus be separated from the complicating effects of traces of the DEB retained by the cells following washing. The bearing of these results on the interpretation of the DEB after-effect and DEB mutation induction curves is briefly discussed.

Introduction

In an earlier paper (Kølmark and Kilbey, 1968) the kinetics of mutation induction by DEB[1] and EO was reported. Three aspects of the mutagenic activity were particularly obvious: (a) The kinetics was such that successive increments of dose produced an ever increasing mutagenic effect; i.e. the curves relating mutation frequency to dose (concentration × time) were upward-bending. (b) At the lowest concentration of DEB used for treatment (0.01 M) the relationship became linear or near-linear. And, (c) apart from the data obtained at the lowest concentration of DEB, the curves relating mutation frequency to dose proved to be superimposible.

An explanation which seemed to encompass these three observations adequately was proposed. It was suggested that, besides inducing mutagenic damage, DEB, and presumably EO, inactivates a cellular process which limits its mutagenic effect. This might be regarded as a repair process although there is no independent evidence for such an assumption. Progressive inactivation of such a system permits subsequent dose increments to have a steadily increasing mutagenic effect. To explain the results obtained at the lowest dose rate it was assumed that the concentration was either too low to inactivate the 'repair' system or, more likely, that the time required for treatment was long enough to enable the cell to replenish the damaged components. In either case, maximum or near-maximum 'repair' occurred to give a linear relationship.

This interpretation leads to several expectations. In the first place, pre-treatment with DEB should sensitise conidia to further mutagenic treatments with DEB and possibly with other mutagenic agents. Secondly, sensitisation should be transient, fading as the cell replaces those elements of the system which have been damaged. Both expectations have been fulfilled: It has been shown that

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1 *Abbreviations*: DEB = diepoxybutane, EO = ethylene oxide.

conidia treated with DEB are sensitised to further small doses of DEB (Auerbach, 1969) and to UV (Auerbach and Ramsay, 1970a). Sensitisation also fades as the interval between the two mutagenic treatments is increased. 2–4 hours are required for complete loss of sensitivity but even a few minutes fractionating a DEB treatment into two parts produces a detectable reduction in effect (Auerbach and Ramsay, 1970b).

Our understanding of the mutagen-sensitive state is complicated by the demonstration that traces of mutagen are retained after treatment with both DEB and EO (Kølmark and Kilbey, 1962; Kilbey and Kølmark, 1968). Cells which are washed and sedimented to give a pellet and stored at room temperature continue to manifest mutations for some hours. If similar cells are maintained in suspension the mutagenic after-effect is not manifested and the capacity to display it steadily declines. The simplest interpretation of this difference is that, in sedimented samples, normal diffusion of the chemical from the cells is reduced while in suspension it is facilitated. Again, three to four hours are required for the complete loss to take place.

The comparability of the times taken for loss of the mutagen-sensitive state on the one hand and the chemical on the other poses several questions: Is it possible, for example, that expression of mutagen sensitivity depends on traces of mutagen retained by the cells or are the two completely independent? Does the decline in the capacity to display an after-effect reflect the decline in sensitivity, or loss of the chemical, or both? Clarification of these and other points would be achieved if the two effects could be dissociated. Conditions might be found, for example, which lead to the perpetuation of sensitivity in spite of loss of the chemical from the cells.

In addition to improving our understanding of the after-effect, the stabilisation of the mutagen-sensitive state should permit a direct test to be made of the hypothesis proposed to account for the kinetics of DEB-induced mutation.

The present report describes conditions in which the stabilisation of the mutagen-sensitive state is achieved with the protein synthesis inhibitor, Actidione (Pall, 1966). The inhibitor was chosen on the assumption that the cellular component suffering damage is a protein and that its replacement would require protein synthesis.

Materials and Methods

The strain of *Neurospora* and the techniques employed were as described earlier (Kølmark and Kilbey, 1962). Actidione (cycloheximide) was obtained from Sigma Chemicals and DEB from Eastman Kodak. A fresh filter-sterilised solution of Actidione was prepared just before use. This stock solution contained 80 µgm per ml. but after dilution into the suspension the final concentration was reduced to 20 µgm per ml. In all cases samples containing actidione were washed free from the drug before plating since it was found that, even at the concentration finally reached on the plates (approximately 1 µgm per ml.), the conidia failed to germinate.

Results

The experiments to be described were all of the same basic design. Their main object was to determine the extent of the DEB after-effect and the degree of sensitisation towards a second mutagenic treatment immediately after the termination of the main DEB treatment and then after four hours had elapsed.
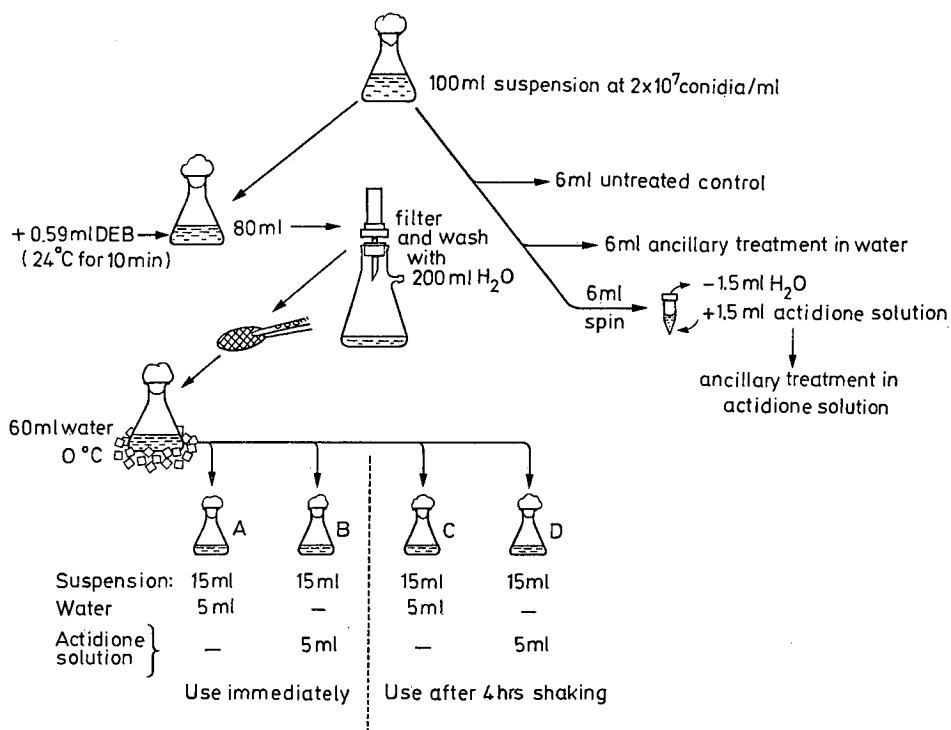
100 ml suspension at 2×10^7 conidia/ml

6 ml untreated control

+ 0.59 ml DEB → 80 ml → filter and wash with 200 ml H_2O
(24°C for 10 min)

6 ml ancillary treatment in water

6 ml spin

− 1.5 ml H_2O
+ 1.5 ml actidione solution

ancillary treatment in actidione solution

60 ml water 0°C

	A	B	C	D
Suspension:	15 ml	15 ml	15 ml	15 ml
Water	5 ml	—	5 ml	—
Actidione solution	—	5 ml	—	5 ml

Use immediately | Use after 4 hrs shaking

Fig. 1. Protocol of the experiments described

During this four hour period the conidia were stored suspended either in water or in a solution of actidione containing 20 μgm per ml of the drug. The experimental protocol is set out diagrammatically in Fig. 1.

Immediately after treatment, the contents of flasks A and B were each treated as follows: Six ml. were plated to determine the basic DEB response, 6 ml. were sedimented and stored at 25°C for four hours to assess the DEB after-effect and 6 ml were exposed to an ancillary mutagenic treatment, either 20 seconds UV at an approximate dose-rate of 20 ergs per mm² second, or 0.01 M DEB for 20 minutes at 25°C. Flasks C and D were treated in exactly the same way after first shaking them for four hours at 25°C.

The following comparisions can be made: A compared with B should show whether actidione affects the response of the strain to DEB at the dose used, the ability to manifest the after-effect and the ability to interact synergistically with the ancillary treatment. A compared with C shows how these responses change during the period of storage in water and B with D shows how actidione effects these changes in response.

Table 1 gives the results of two experiments in which DEB itself was used as the ancillary treatment. It will be seen that actidione has very little effect on the ability of K3/17 to respond to DEB. The slight, but consistent improvement in the mutational response when actidione is present may be connected with the

Table 1. DEB

Flask	A$_{(H_2O)}$	C$_{(H_2O)}$	B$_{(Actidione)}$	D$_{(Actidione)}$
DEB Baseline Response (0.1 M, 10 mins, 24° C)	1.35 *2.33*	1.35 *1.61*	2.16 *3.60*	2.80 *4.00*
DEB After-effect	5.70 *11.90*	2.16 *1.22*	7.55 *14.59*	2.16 *4.3*
Ancillary DEB$_d$ (0.1 M, 20 mins, 25° C)	0.48 *0.92*		0.15 *0.35*	
Σ DEB, ancillary DEB (Expected)	1.83 *3.25*	1.83 *2.53*	2.31 *3.95*	2.95 *4.35*
Interaction (observed)	6.43 (3.5) *11.64 (3.6)*	2.09 (1.2) *1.69 (0.7)*	10.23 (4.5) *12.65 (3.2)*	9.5 (3.2) *16.57 (3.6)*

In both Table 1 and 2 the figures in brackets represent the factors of increase in the observed results of the interaction compared with the expected values baded on additivity. All other figures are the frequencies of ad^+ revertants per 10^6 survivors. 5×10^7—10^8 cells formed the basis of each estimate and survival ranged between 60–100%. Figures in italics are those from experiment 2. Spontaneous reversions have been ignored since in no case were they higher than 0.06 $ad^+/10^6$ survivors.

Table 2. UV

Flask	Expt. No.	A$_{(H_2O)}$	C$_{(H_2O)}$	B$_{(Actidione)}$	D$_{(Actidione)}$
DEB Baseline Response	1	0.71	0.40	0.90	0.40
	2	*1.53*	*1.61*	*2.01*	*1.60*
	3	0.45	0.58	0.73	0.60
DEB After-effect	1	3.28	1.04	3.05	1.32
	2	*11.33*	*1.50*	*9.20*	*2.18*
	3	2.48	1.07	2.27	1.05
UV response	1	1.52		1.57	
	2	*3.04*		—	
	3	2.49		—	
Σ DEB, UV (Expected)	1	2.23	1.92	2.47	1.97
	2	*4.57*	*4.64*	*5.05*	*4.64*
	3	2.94	3.07	3.22	3.09
Interaction (observed)	1	4.48 (2.0)	2.01 (1.0)	3.10 (1.2)	3.60 (1.8)
	2	*9.21 (2.0)*	*3.90 (0.8)*	*9.63 (1.9)*	*7.49 (1.6)*
	3	6.50 (2.2)	2.90 (0.9)	4.66 (1.45)	4.93 (1.6)

effect of the inhibitor on the kinetics of DEB mutagenesis (see Kilbey, 1973). Actidione has no effect on the ability of the strain to manifest an after-effect or to respond synergistically to the ancillary treatment (Columns A and B). In water the ability to display the after-effect and the strong interaction with the ancillary treatment have disappeared by the end of four hours (columns A and C) but in the presence of actidione, the cells are just as sensitive to the second DEB

dose after storage as they were to start with (columns B and D) in spite of the fact that the after-effect has disappeared.

Table 2 presents data of a similar nature but obtained with UV as the ancillary mutagen. Whereas the interaction with ancillary DEB treatments produced very consistent effects, the interaction between DEB and UV in *Neurospora* is somewhat variable and less pronounced. However in a number of experiments a clear positive interaction was observed and, in these cases, it could be unambiguously shown that actidione led to the perpetuation of the sensitivity towards UV. Three experiments are presented in Table 2. Since actidione has no detectable effect on the UV response of the conidia of this strain (see Table 2 and unpublished results) only a single UV control was performed in the second and third of these experiments.

Discussion

The results just described can be summarised as follows: Actidione, a protein synthesis inhibitor active in *Neurospora*, has little effect on the basic response of *Neurospora* to DEB under the conditions of these experiments. Furthermore it neither prevents the expression of the mutagenic after-effect nor the decline in ability to express the after-effect in cells which are maintained in suspension. However, it does prevent decline in sensitivity toward further mutagenic treatments either by UV or DEB.

These results demonstrate unambiguously that the sensitisation-effect can exist independently of the traces of DEB retained by the cells. They also verify the earlier conclusion that loss of the capacity to manifest an after-effect can be related to the loss of chemical from the cells. Maintenance of sensitivity alone is insufficient to produce an after-effect, mutagenic agent must also be present. The reverse situation, retention of chemical without sensitisation, is also probably insufficient for an after-effect, although it is impossible to study it directly: The closest one can, at present, approach to this situation is to study the mutagenic effects of extremely low doses of extraneously administered DEB. These are only effective, as can be seen here, when the cells have been sensitised, and remain so at the time of treatment. Loss of either the chemical or the sensitive state is therefore enough to prevent the expression of the mutagenic after-effect but which factor is more important in determining the rate of decline is at present unclear.

Since the prevention of protein synthesis (which can safely be assumed to occur in the presence of actidione) prevents to loss of mutagen-sensitivity it is implied that the component damaged is either a protein or has an indispensible proteinaceous constituent. The fact that sensitivity extends to UV eliminates the possibility that the observations are simply due to alterations in cell permeability to DEB. The nature of the system suffering inactivation is unknown but it is immediately possible to infer that its sensitivity to DEB is considerably greater than a standard repair system such as photoreactivation. Rannug showed clearly that doses of DEB which produced marked sensitisation to UV left photoreactivation activity unimpaired (Rannug, 1971). A possible feature of its activity which should prove interesting will be the extent to which it is specific for damage causing *ad-3A 38701* reversions. Also of interest will be a comparison of its specificity towards point mutations versus gross chromosomal changes pro-

ducing recessive lethality in *Neurospora*. Photoreactivation has been shown to possess specificity in both situations, in earlier studies (Kilbey, 1967; de Serres and Kilbey, 1971).

Finally, the demonstration that sensitivity can be stabilised, with its implication that the processes which replenish the damaged system are inhibited should facilitate the direct test of the hypothesis by which the DEB dose-effect curves for mutation are explained. This will form the basis of a further report.

References

Auerbach, C.: Analysis of a case of mutagen specificity in Ciba Symposium, on Mutation as a cellular process, p. 66. London 1969.

Auerbach, C., Ramsay, D.: Analysis of a case of mutagen specificity in *Neurospora crassa*. II. Interaction between treatments with diepoxybutane (DEB) and ultraviolet light. Molec. gen. Genet. **109**, 1–14 (1970a).

Auerbach, C., Ramsay, D.: III. Fractionated treatment with Diepoxybutane (DEB). Molec. gen. Genet. **109**, 285–291 (1970b).

De Serres, F. J., Kilbey, B. J.: Differential photoreversibility of ultraviolet-induced premutational lesions in *Neurospora*. Mutation Res. **12**, 221–234 (1971).

Kilbey, B. J.: Specificity in the photoreactivation of premutational damage induced in *Neurospora* by ultraviolet. Molec. gen. Genet. **100**, 159–165 (1967).

Kilbey, B. J.: The manipulation of mutation-induction kinetics in *Neurospora crassa*. Molec. gen. Genet. **123**, 73–76 (1973).

Kilbey, B. J., Kølmark, H. G.: A mutagenic after-effect associated with ethylene oxide in *Neurospora crassa*. Molec. gen. Genet. **101**, 185–188 (1968).

Kølmark, H. G., Kilbey, B. J.: An investigation into the mutagenic after-effect of butadiene epoxide using *Neurospora crassa*. Z. Vererbungsl. **93**, 356–365 (1962).

Kølmark, H. G., Kilbey, B. J.: Kinetic studies of mutation induction by epoxides in *Neurospora crassa*. Molec. gen. Genet. **101**, 89–98 (1968).

Pall, M. L.: The use of cycloheximide as an inhibitor of protein synthesis in Neurospora. Neurospora Newsletter **9**, 16 (1966).

Rannug, J. U.: Photoreactivation of mutational damage produced by the interaction of DEB and UV in *Neurospora*. Molec. gen. Genet. **111**, 194–196 (1971).

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The Manipulation of Mutation-Induction Kinetics in *Neurospora crassa**

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Summary. Conditions have been found which convert the linear dose-effect relationship obtained with DEB[1] at low dose-rates into the upward bending relationship characteristics of the results at higher dose rates. These findings support the earlier suggestion (Kølmark and Kilbey, 1968) that DEB inactivates a repair process, the inactivation and replenishment of which are important factors in determining mutation induction kinetics.

Introduction

Diepoxybutane and Ethylene Oxide both display mutation-induction kinetics of an "upward-bending" type. That-is-to-say, successive dose increments produce ever-increasing mutagenic effects (Kølmark and Kilbey, 1968). At first sight this might be interpreted in terms of a two-hit mechanism for mutagenesis, but such an explanation is difficult to uphold for several reasons. In the first place, the observed relationship is always such that between two and three events would be required. Only in experiments with a micro-conidiating strain was the exponent close to 2. Secondly, for treatments at extremely low dose-rates (in the experiments referred to, a DEB concentration of 0.01 M was used for twelve hours), the dose-effect relationship becomes linear. Consequently an alternative suggestion was formulated for DEB in which the chemical was visualised as progressively inactivating cellular systems which repair or otherwise limit DEB-induced DNA lesions. As a result of this, repair is reduced as treatment is increased and the mutagenic action is steadily enhanced. At the lowest DEB concentration the treatment times are much longer and cells have the opportunity to replace the damaged elements. Maximal, or near-maximal repair is therefore expected, and a simple linear relationship between dose and effect is found.

This hypothesis is amenable to testing since it predicts that any condition which prevents the replacement of the damaged components should convert the linear response at low DEB concentrations into the upward-bending relationship characteristic of the higher concentrations.

Recently data were obtained which showed that the mutagen-sensitivity induced in conidia treated with DEB, can be prevented from declining with time of incubation by the presence of the protein synthesis inhibitor actidione (Kilbey, 1973). The present report is concerned with the effects of actidione on the kinetics of DEB mutagenesis at low dose-rates.

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1 Abbreviations: DEB = diepoxybutane, EO = Ethylene Oxide.

Materials and Methods

The materials (chemicals and fungal strain) and the methods used (culture conditions, preparation of the suspensions and modes of treatment) have all been described in detail elsewhere (Kølmark and Kilbey, 1962).

Results and Conclusions

Suspensions of conidia containing between 1 and 3×10^7 conidia per ml were prepared with and without the addition of 15 µg per ml actidione (cycloheximide). This concentration is slightly lower than that used before (Kilbey, 1973) since it was found that, over the prolonged periods required for DEB Treatment, 20 µg/ml proved slightly toxic. The concentration of DEB used throughout was 0.005 M. This is also less than that used in the first study and the reasons for the lowered tolerance of the strain is unknown. The concentration used was commensurate with linear mutation induction. All treatments were made at 21 °C, samples being withdrawn for washing and plating at the required times. Fig. 1 shows the combined results of three experiments. Good agreement was obtained between them and it is clear that, in the presence of actidione, there is a marked change in the mutation induction kinetics. Instead of the near-linear or linear response typical of treatment at low dose-rates the upward-bending relationship characteristic of higher dose-rates is obtained. Although the figures are presented in terms of mutations per survivors, selection can be discounted as the cause of the effect since the same type of change was found for the absolute numbers of mutants scored. Survival was very high in all the experiments.

Table 1. The ability of cells treated with 0.005 M DEB, with and without actidione added, to display (a) a mutagenic after-effect (b) interaction with 20 seconds UV (dose rate approximately 20 ergs/mm^2 per second). Figures are $ad^+/10^6$ survivors, each estimate based on between 5×10^7 and 2×10^8 conidia. Figures in brackets are the factors of increase i.e. indications of the magnitude of the after-effect and the extent to which the observed interaction exceeds the expected value based on adding UV and DEB effects

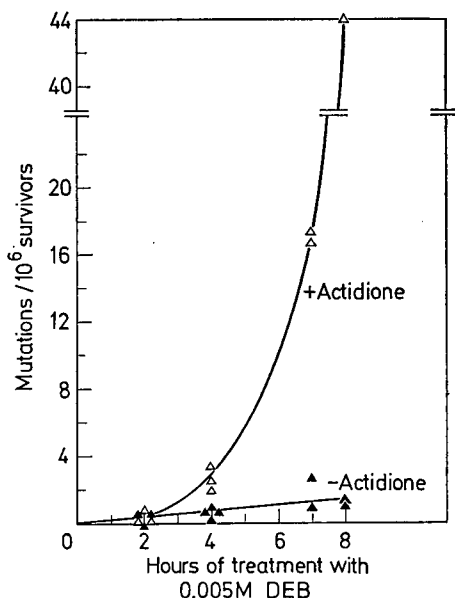| Expt.	DEB 0.005 M ± Actidione	DEB	After effect	ΣDEB, UV	Inter-action
I	−	0.64	1.45 ($\times 2.2$)	2.73	3.30 ($\times 1.2$)
	+	6.00	20.16 ($\times 3.4$)	8.09	35.40 ($\times 4.4$)
II	−	0.74	2.19 ($\times 2.9$)	2.28	2.41 ($\times 1.06$)
	+	0.63	4.00 ($\times 6.35$)	2.17	6.24 ($\times 2.9$)
II	−	0.73	1.37 ($\times 1.8$)	1.49	1.98 ($\times 1.3$)
	+	1.13	7.01 ($\times 6.2$)	2.89	10.1 ($\times 3.5$)

Fig. 1. The effect of actidione on the induction of mutations by DEB at low molarities

These results would appear to confirm very effectively the original suggestion that DEB mutation-induction kinetics is detemined by the balance between repair inactivation and replacement. However, in order to emphasise the point, two further tests were made. If the interpretation is the correct one, it is to be expected that conidia treated in the absence of actidione should show little if any sensitisation towards other mutagenic treatments. In contrast, those treated in the presence of the inhibitor should show sensitisation. To test this, conidia were treated for six hours with DEB in the presence or the absence of actidione. Each suspension was then washed and tested for its ability to interact with UV on the one hand, and to display a mutagenic after-effect on the other. The results are presented in Table 1. It is apparent that, in agreement with the expectation, cells incubated with DEB in the absence of actidione are far less capable of interacting with UV or displaying an after-effect than those incubated in its presence.

These results completely support the interpretation of DEB mutation induction kinetics proposed earlier. To the best of our knowledge this is the first occasion in which it has been possible to manipulate the dose-effect curves of a mutagenic chemical in this way. The findings emphasise the importance of considering not simply the effects of repair inactivation as a contributory factor in determining mutation-induction kinetics. Replacement must also be borne in mind as a possibility. In the present case the balance between the two events has proved to be the important determinant in deciding whether a linear or an upward bending curve is obtained.

References

Kilbey, B. J.: The stabilisation of a transient mutagen-sensitive state in Neurospora by the protein synthesis inhibitor Actidione. Molec. gen. Genet. **123**, 67–72 (1973).

Kølmark, H. G., Kilbey, B. J.: An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa* Z. Vererbungsl. **93**, 356–365 (1962).

Kølmark, H. G., Kilbey, B. J.: Kinetic studies of mutation induction by epoxides in *Neurospora crassa* Molec. gen. Genet. **101**, 89–98 (1968).

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THE ANALYSIS OF A DOSE-RATE EFFECT FOUND WITH A MUTAGENIC CHEMICAL[*][a]

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SUMMARY

Although much time has been devoted to studies of the effects of dose rate on mutational yields from ionising radiation, comparable information is generally lacking for mutagenic chemicals. Yet, dose-rate effects and interaction effects are among the more important problems for consideration by those evaluating the risks from environmental mutagens. In this contribution a description will be given of the steps in the analysis of a mutagenic dose-rate effect found with diepoxybutane (DEB). All the findings can be accounted for by postulating a mutation limiting process which is readily damaged by DEB and slowly replaced by the cells following treatment. It will be shown how this realisation has accounted for several interesting features of its mutagenic action; its after-effect and its interaction with radiation.

INTRODUCTION

During the past 15 to 20 years several interesting and important studies have been made of the dose-rate effects found with ionizing radiation. Possibly the best known of these is the study undertaken by the RUSSELLS which, among other things, showed that, in mouse spermatogonia, a reduction in dose rate from 90 rad/min to 0.009 rad/min reduced the yield of specific locus mutations to approximately one quarter[13]. In the opinion of these investigators, the effect of dose rate on mutational yield is related to an increase in effectiveness of cellular repair at lowered dose rates. Studies have also been undertaken with other experimental material[10,16] with varying degrees of success but, to date, almost all of them have been concerned with ionising radiation; very little at all is known about possible dose-rate effects with chemical mutagens. One major reason for this is undoubtedly the greater complexity of the situations encountered. A chemical may be unstable, or metabolised and it may be very difficult to control the dose reaching the DNA let alone measure it. However, just

as the findings with the mouse are important in the evaluation of the risks from
ionizing radiation, so are the studies of possible dose-rate effects with chemicals. This
is particularly so since we are often forced to conduct our laboratory tests under much
higher dose-rate regimes than are encountered in nature.

The object of this communication is to describe the analysis of one instance of a
dose-rate effect found by us for the mutagen DEB. I am not suggesting that the con-
clusions reached are widely applicable but they may serve as a starting point for
further studies using systems of greater complexity.

FUNGAL MATERIAL

Most of this work was done with the colonial strain of Neurospora, K3/17
(ref. 8). Although this strain carries two mutant alleles determining requirements for
both adenine and inositol, the reversion of only one of them was the subject of the
study; the *adenine 3A allele 38701*. Genetic studies have shown that the majority
of the events causing adenine reversions are point-mutational changes at, or near, the
site of the original mutation.

RESULTS

The kinetics of DEB-induced reversion

Fig. 1 shows the effect of varying the dose rate of DEB treatment on the re-
sponse of the adenine allele. Dose rate was varied by using solutions of DEB at
different molarities to treat conidia from the K3/17 strain (ref. 9). The time of ex-
posure was varied inversely with the concentration. Exposure at the highest molarity
used, 0.3 M, lasted only a few minutes while exposure at the lowest molarity 0.01 M,
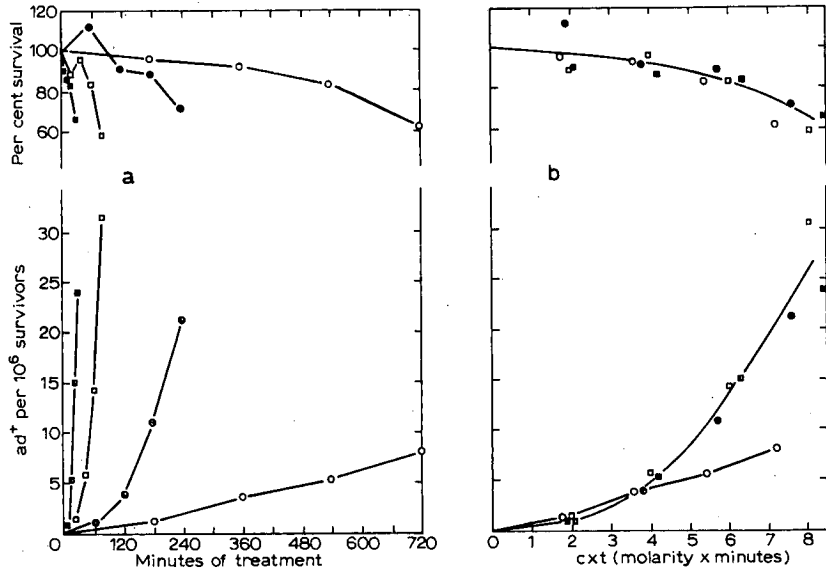
Fig. 1. The response of *ad-3A 38701* to treatment with DEB at different dose-rates. ○, 0.01 M
DEB; ●, 0.03 M DEB; □, 0.10 M DEB; ■, 0.31 M DEB (Reprinted with permission, from ref. 9).

lasted for some hours. However, even the longest treatment time was short in comparison with the known half-life of the chemical under these conditions. The effect shown here is thus unlikely to be the result of chemical degradation, and, as we shall see, the later results also make this unlikely. At the three highest molarities used, upward-bending dose–response curves were obtained. Dose-rate variations in this range seem to have little effect on the response of the system. At the lowest dose rate, however, a linear relationship may be observed for mutation *versus* dose. This is more clearly seen in panel b of Fig. 1. By multiplying the molarity by the time of treatment to give total dose, it is possible to plot all these data on the same scale. When this is done it is clear that the yield of mutations produced by similar doses at low dose rates falls below the levels achieved at higher dose rates. Furthermore, because of the differences in kinetics of induction, the difference becomes more marked as the dose increases.

At first sight, the upward-bending kinetics of mutation induction observed at the three highest dose rates might lead one to suspect that the cooperation of two independent events is required for an adenine reversion to take place. However, this interpretation seems unlikely for several reasons. In particular, repeated observations have shown that the mutations accumulate not in proportion to the square of the dose but in proportion to a higher power; 2.3 to 2.7. Furthermore, the superimposability of the curves obtained at the three highest dose rates suggests that the mutational yield is determined solely as a function of the number of molecules entering the cell and that, over a range of times, the rate of entry is not important. This also makes an understanding of the process in terms of cooperative events difficult without some additional assumptions, especially in view of the change in kinetics at low dose rates —now a well established observation.

The mutation-limiting process

As an alternative explanation, we have suggested that, besides producing reversional events, DEB also progressively interferes with the function of a process which limits its capacity as a mutagen in Neurospora. This might simply be a repair process or it might, for example, be a co-factor required for the accurate replication of DNA. Whatever it may eventually prove to be, its destruction should lead to a progressive increase in the mutagenic effectiveness of successive dose increments of DEB and an upward bending of the mutation-induction curves can be expected. The linear relationship between mutations induced and dose, found at the low dose rates, is easily accounted for by the assumption that conidia slowly continue to resynthesise the damaged components of the mutation-limiting process. Short intense exposures to the chemical provide insufficient time for significant replacement of these components to occur, but, over the extended times necessary for the low dose-rate experiments, replacement can match destruction and the maximal limitation of mutations is achieved.

Expectations and their verification

Several expectations follow from this interpretation which have the advantage of being testable and for the rest of the time I shall review these and try to show to what extent they have been fulfilled.

First of all it is to be expected that cells which have been treated with DEB, and

thus are supposed to have sustained damage to the mutation-limiting process, should show enhanced mutational responsiveness to subsequent exposures to the chemical. It is also possible that this enhancement might extend to other mutagens. AUERBACH has presented data which show clearly that this is indeed the case. Cells which are treated with DEB display an increased responsiveness to the mutagenic effects of ancillary doses of DEB so small that they have scarcely any detectable activity in their own right[1]. Several experiments have also been conducted which show that this increase in sensitivity extends to the mutagenic effects of UV[2,4]. RANNUG working in the author's laboratory has lent weight to this conclusion by showing that the extra mutations produced when UV follows DEB are UV-like in their photoreactivational properties[12].

A second expectation from the interpretation offered is that cells which have sustained damage to the mutation-limiting process, and are consequently more sensitive than normal to the mutagenic effects of ancillary treatments with DEB and other mutagens, should lose this sensitivity on storage because the components are being slowly renewed; *i.e.*, we should find we are dealing with a *transient* mutagen-sensitive state. Evidence has also been obtained for this: fractionation of a dose of DEB into two or more parts, even though the intervening periods are only minutes long, reduced the total yield of mutations significantly[3]. Other experiments, in which UV was the second mutagen, also show that sensitivity decreases with storage time. Slight differences in the time taken for complete loss of sensitivity exist from experiment to experiment but two to four hours appear necessary for complete loss— that is for complete replacement of the damaged mutation-limiting system.

These two expectations are concerned with situations in which the mutation-limiting process can be slowly renewed. It does not require much imagination to predict the results of preventing the renewal. One obvious consequence should be a perpetuation or stabilisation of the mutagen-sensitive state. Another, more exciting possibility, is that control of the resynthesis events should permit the manipulation of mutation induction kinetics. In particular, it should be possible to convert the linear low dose-rate response to the type of response found with high dose-rate treatments, if our interpretation of the kinetic differences is correct.

Working on the assumption that renewal probably requires protein synthesis, especially if an enzymatic repair system is involved, attempts were made to block the replacement using actidione (cycloheximide). This agent is known to inhibit protein synthesis in Neurospora[11] and the evidence available from a number of other studies shows that it apparently prevents the transfer of activated amino acids from the tRNA to the growing polypeptides. Consequently the nascent polypeptides remain attached to the ribosomes[5,14,15,17].

The effect of actidione on the retention of the sensitive state was tested as follows: DEB-treated and washed conidia of K3/17 were tested for their sensitivity to a second, barely mutagenic, dose of DEB. The second dose was applied either immediately after the first or after an intervening period of 4 h during which the cells were shaken in either water alone, or in water containing 20 μg per ml actidione.

Table I shows the result of this type of experiment. Immediately after the first exposure both samples show an enhanced sensitivity towards the mutagenic effects of the ancillary treatment. In water alone this sensitivity is lost after 4 h but, in the presence of actidione, the sensitivity remains as high at the end of the experiment as

TABLE I

THE STABILISATION BY ACTIDIONE OF THE MUTAGEN-SENSITIVE STATE INDUCED BY DEB AND THE RELATIONSHIP BETWEEN THE MUTAGENIC AFTER-EFFECT AND THE SENSITIVE STATE

The figures in brackets represent the factors of increase in the observed results of the interaction compared with the expected values based on additivity. All other figures are the frequencies of ad^+ revertants per 10^6 survivors. $5 \cdot 10^7$–10^8 cells formed the basis of each estimate and survival ranged between 60–100%. Figures in italics are those from Expt. 2. Adapted from Table I in ref. 6.

Time of incubation following DEB treatment	0 h (H₂O)		4 h (H₂O)		0 h (Actidione)		4 h (Actidione)	
DEB baseline response	1.35		1.35		2.16		2.80	
	2.33		*1.61*		*3.60*		*4.00*	
Ancillary DEB		0.48				0.15		
		0.92				*0.35*		
Σ DEB, ancillary DEB (expected)	1.83		1.83		2.31		2.95	
	3.25		*2.53*		*3.95*		*4.35*	
Interaction (observed)	6.43	(3.5)	2.09	(1.2)	10.23	(4.5)	9.5	(3.2)
	11.64	*(3.6)*	*1.69*	*(0.7)*	*12.65*	*(3.2)*	*16.57*	*(3.6)*
DEB after-effect (Compare with 1st line)	5.70		2.16		7.55		2.16	
	11.90		*1.22*		*14.59*		*4.3*	

it was at the beginning. We believe that this experiment demonstrates that renewal of the mutation-limiting process can be inhibited, and that renewal probably requires protein synthesis[6].

The mutagenic after-effect

The data presented in Table I also clarify another interesting facet of the mutagenic effect of DEB—its mutagenic after-effect. When DEB-treated conidia are stored after washing, they continue to accumulate adenine reversions for some hours at room temperature. Subsequent examination showed that mutant accumulation only occurred if the conidia were allowed to sediment. In suspensions, whether maintained by simple stirring, shaking or bubbling with air or nitrogen, mutations did not accumulate and the stirred cells rapidly lost their capacity to display the after-effect[8].

The simple explanation offered for this finding was that traces of the chemical are retained by the cells when washed. In the sedimented pellet of cells these traces cannot easily diffuse away and they remain to cause mutations. In suspension, diffusion is facilitated and mutation induction does not occur. One puzzling aspect of this interpretation was that no mutagenic activity could be detected in supernatants from treated, washed suspensions. If such small quantities of mutagen are involved how can they produce such marked mutagenic effects? The realisation that cells treated with DEB become temporarily sensitive to small traces of the chemical provides an explanation for the findings. However, the question must now be asked whether the loss of the ability to display an after-effect in shaken cells arises from loss of mutagen or simply from loss of sensitivity.

With the successful demonstration that the sensitive state could be stabilised in the presence of actidione, it became possible to approach the answer to this question. It is clear from Table I that retention of sensitivity alone is not sufficient for an after-effect; chemical traces must also be present. It is not clear at present whether

loss of the ability of DEB-treated cells to display an after-effect is occasioned pri-
marily by loss of the chemical rather than by loss of sensitivity, but it is clear that the
presence of the chemical traces is not a requirement for the maintenance of sensitivity.

The manipulation of mutation-induction kinetics

It follows directly from the interpretation given for the action of actidione that
it should be possible to prevent renewal of the mutation limiting process *during* DEB
treatments at low dose rates and to convert the linear relationship into an upward
bending one. Cells were treated with DEB at a molarity of 0.005 in the presence and
absence of actidione. In these experiments the survival of the treated populations was
very high and it is unlikely that the results are affected by selection. Fig. 2 gives the
results of three similar experiments.
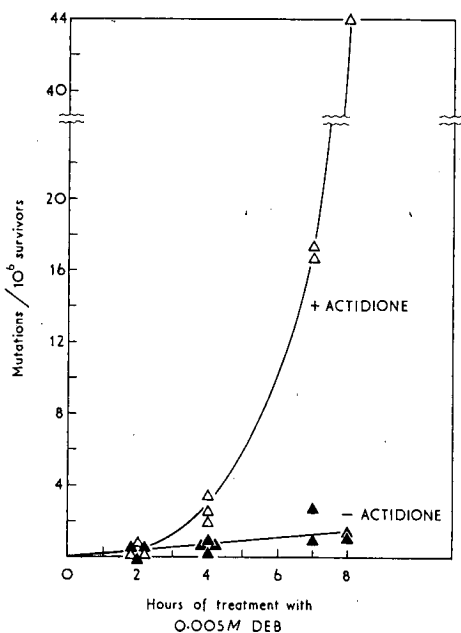
Fig. 2. The effect of actidione on the low dose-rate response of *ad-3A 38701* to DEB. (Reprinted
with permission, from ref. 7.)

There is no doubt at all, that in the presence of actidione, the linear relationship
is modified drastically to give the upward-bending curve normally typical of the high
dose-rate experiments but, as a final attempt to ensure that we were not being misled,
cells were taken from two populations both treated at this low dose-rate, but in one
case with actidione present and in the other with it absent. Each suspension was washed
and divided into two parts. The first was exposed to UV, the second was sedimented
and incubated to determine the mutagenic after-effect of DEB. From what has been
already said it should be clear that the extent of the mutagenic after-effect can be
used as a measure of the degree of sensitisation experienced by the cells during the
main exposure to DEB. It was possible to show (Table II) that cells initially treated
in the absence of actidione show little increased sensitivity to UV or to DEB (as

TABLE II

THE ABILITY OF POPULATIONS TO INTERACT WITH UV AND TO SHOW AN AFTER-EFFECT WHEN FIRST EXPOSED TO DEB AT LOW DOSE RATE IN THE PRESENCE AND ABSENCE OF ACTIDIONE

Figures are $ad^+/10^6$ survivors, each estimate based on between $5 \cdot 10^7$ and $2 \cdot 10^8$ conidia. Figures in brackets are the factors of increase *i.e.* indications of the magnitude of the after-effect and the extent to which the observed interaction exceeds the expected value based on adding UV and DEB effects. (Reprinted with permission, from ref. 7.)

Expt.	DEB 0.005 M ± actidione	DEB	After-effect	Σ DEB, UV	Interaction
I	—	0.64	1.45 (2.2)	2.73	3.30 (1.2)
	+	6.00	20.16 (3.4)	8.09	35.40 (4.4)
II	—	0.74	2.19 (2.9)	2.28	2.41 (1.06)
	+	0.63	4.00 (6.35)	2.17	6.24 (2.9)
III	—	0.73	1.37 (1.8)	1.49	1.98 (1.3)
	+	1.13	7.01 (6.2)	2.89	10.10 (3.5)

measured by the after-effect) presumably because their repair processes are being continuously renewed during the prolonged treatment. Cells treated in the presence of actidione on the other hand are highly sensitive to both supplementary treatments[7].

CONCLUSIONS

The evidence I have tried to present here, seems to indicate that, in the case of DEB, there is an effect of dose rate on the yield of chemically induced mutants. Furthermore our experiments strongly suggest that the precise level of mutations found for a given dose, or put another way, the kinetics with which mutants accumulate as the dose increases is explicable on the basis of two competing events; the destruction and renewal of a mutation-limiting process. Of course there are deficiencies in the information: it would be of considerable advantage to have some direct estimate of the extent to which equalisation of the external dose at different dose rates produced the same extent of alkylation of the DNA. It would also be useful to determine the extent to which sensitisation affects different parts of the genome and different types of genetic damage. Hopefully these and other questions will be answered in the near future. The point to note here, however, is that these data show that we may expect dose-rate effects from chemical mutagens which do not arise from trivial causes and that, in the evaluation of risks from environmental chemicals, dose rates of exposure must be borne in mind.

REFERENCES

1 AUERBACH, C., in *CIBA Symposium on Mutation as a Cellular Process*, Churchill, London, 1969, pp. 66–86.
2 AUERBACH, C., AND D. P. RAMSAY, Analysis of a case of mutagen specificity in *Neurospora crassa*, II. Interaction between treatments with diepoxybutane (DEB) and ultraviolet light, *Mol. Gen. Genet.*, 109 (1970) 1–14.
3 AUERBACH, C., AND D. P. RAMSAY, Analysis of a case of mutagen specificity, III. Fractionated treatment with diepoxybutane (DEB), *Mol. Gen. Genet.*, 109 (1970) 285–291.
4 AUERBACH, C., B. J. KILBEY AND H. G. KØLMARK, Response of two loci to interaction treatment, *Neurospora Newsletter*, 2 (1962) 4.
5 COLOMBO, B., K. FILICETTI AND C. BAGLIONI, Inhibition of protein synthesis in reticulocytes by antibiotics, I. Effects on polysomes, *Biochim. Biophys. Acta*, 119 (1966) 109–119.

6 KILBEY, B. J., Stabilisation of a transient mutagen-sensitive state in Neurospora by the protein synthesis inhibitor actidione, *Mol. Gen. Genet.*, 123 (1973) 67–72.

7 KILBEY, B. J., The manipulation of mutation-induction kinetics in *Neurospora crassa, Mol. Gen. Genet.*, 123 (1973) 73–76.

8 KØLMARK, H. G., AND B. J. KILBEY, An investigation into the mutagenic after-effect of butadiene-diepoxide using *Neurospora crassa, Z. Vererbungslehre*, 93 (1962) 356–365.

9 KØLMARK, H. G., AND B. J. KILBEY, Kinetic studies of mutation induction by epoxides in *Neurospora crassa, Mol. Gen. Genet.*, 101 (1968) 89–98.

10 MULLER, H. J., I. I. OSTER AND S. ZIMMERING, in F. H. SOBELS (Ed.), *Repair from Genetic Radiation Damage*, Pergamon, Oxford, 1962, pp. 275–311.

11 PALL, M. L., The use of cycloheximide as an inhibitor of protein synthesis in Neurospora, *Neurospora Newsletter*, 9 (1966) 16–17.

12 RANNUG, J. U., Photoreactivation of mutational damage produced by the interaction between DEB and UV in Neurospora, *Mol. Gen. Genet.*, 111 (1971) 194–196.

13 RUSSELL, W. L., in: *Proc. Conf. on Mechanisms of the Dose-rate Effect of Radiation at the Genetic and Cellular Levels, Suppl. Jap. J. Genet.*, 40 (1965) 129–140.

14 SIEGEL, M. R., AND H. D. SISLER, Site of action of cycloheximide in cells of *Saccharomyces pastorianus*, I. Effect of the antibiotic on cellular metabolism, *Biochim. Biophys. Acta*, 87 (1964) 70–82.

15 SIEGEL, M. R., AND H. D. SISLER, Site of action of cycloheximide in cells of *Saccharomyces pastoriamus*, II. The nature of inhibition of protein synthesis in a cell free system, *Biochim. Biophys. Acta*, 87 (1964) 83–89.

16 TAZIMA, U., S. KONDO AND T. SADO, Two types of dose-rate dependence of radiation-induced mutation rates in spermatogonia and oogonia of the silkworm, *Genetics*, 46 (1961) 1335–1345.

17 WETTSTEIN, F. O., H. NOLL AND S. PENMAN, Effect of cycloheximide on ribosomal aggregates engaged in protein synthesis *in vitro, Biochim. Biophys. Acta*, 87 (1964) 525–528.

DIEPOXYBUTANE PRETREATMENT EFFECTS ON UV-INACTIVATION AND PHOTOREACTIVATION OF *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Experiments are described in which the effects of diepoxybutane pretreatment on UV response were tested in a strain of yeast requiring adenine for growth. Pretreatment abolishes the sigmoid character of the UV-inactivation curve and also increases its slope. The pattern of photoreactivation is also altered by pretreatment. Diepoxybutane was found to produce a lethal after-effect in this strain which could be destroyed by extended washing. Extended washing also removed the effects of diepoxybutane pretreatment on UV inactivation and photoreactivation. The after-effect and the effect of pretreatment disappeared simultaneously. The possible significance of these findings is discussed.

INTRODUCTION

The UV-inactivation curves of haploid uninucleate micro-organisms are often sigmoid in shape. One explanation of this is that the treatment itself inactivates the cell's dark-repair processes[3]. Thus, at low doses, with repair operating, a given increment of dose is less effective than at higher doses when repair is inhibited or inactivated. The results of interaction experiments have been used to support this idea. HAYNES AND INCH[4], using haploid *Saccharomyces cerevisiae*, were able to show that pretreatment with nitrogen mustard modified the subsequent pattern of UV inactivation: the prominent shoulder was reduced and the inactivation became exponential at very low UV doses. In this case the interpretation of the results would be that nitrogen mustard interfered with the subsequent repair of UV lesions.

As a result of experiments in which the effect of dose-rate on the mutagenic activity of DEB was studied, KØLMARK AND KILBEY[8] suggested that this compound also might act as a dark repair inhibitor in *Neurospora crassa*. Later, this was also suggested for mutations by AUERBACH AND RAMSAY[1]. The present short report describes experiments made to investigate this possibility. The effects of DEB pre-

Abbreviations: DEB, diepoxybutane; PR, photoreactivation; PRS, photoreactivable sector.

treatment on UV inactivation were studied in a yeast system similar to that of
HAYNES AND INCH[4]. In addition, the photoreactivability of the lesions produced by
the interaction treatment was investigated in the hope of obtaining more insight into
the nature of the interaction.

MATERIALS AND METHODS

Strain. The yeast strain used throughout the study was kindly supplied by Dr.
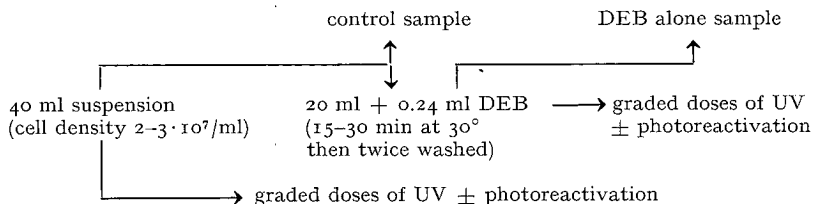Robin Woods. It requires adenine for growth and carries the allele $ad_{2.0}$. Its mating
type is α.

Media. Cultures were grown for 3 days at 25° in Glucose Nutrient Broth. Plat-
ings were done on Yeast Extract Agar supplemented with 20 μg adenine sulphate per
ml.

Treatment. Details of the UV-irradiation procedure and dosimetry have been
given elsewhere[5] together with details of the photoreactivation procedure. The latter
was modified since it was found that at 30° this strain of yeast required 30 min for
maximum photoreactivation. Accordingly 60 min were allowed for photoreactivation
as a standard procedure. The washing and suspending liquid used throughout was a
0.067 M KH$_2$PO$_4$ solution.

RESULTS

DEB pretreatment experiments

These experiments had the following protocol:

```
                              control sample            DEB alone sample
                                    ↑                          ↑
        ┌─────────────────────────┘    ┌──────────────────────┘
  40 ml suspension            20 ml + 0.24 ml DEB  ──→ graded doses of UV
  (cell density 2–3·10⁷/ml)   (15–30 min at 30°          ± photoreactivation
        │                     then twice washed)
        │
        └─────────────→ graded doses of UV ± photoreactivation
```

In the first experiments survival after DEB was 3–30%. All these gave closely
similar results, an example of which is shown in Fig. 1. Each point represents the
average of duplicate platings chosen from dilutions giving, as nearly as possible, 100
colonies per plate.

Pretreatment with DEB has an effect which is essentially similar to the one
described by HAYNES AND INCH[4] for nitrogen mustard. The subsequent UV-inactiva-
tion curve has lost its shoulder and, from the start, appears to be exponential. There
is also an indication that the slope of the curve is slightly steeper than the correspond-
ing portion of the curve produced by UV alone.

PR after pretreatment is also altered. With UV alone a constant PRS* of

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* The photoreactivable sector, or the photoreactivability of the UV damage is given by the term
(1 — dose reduction factor). The dose reduction factor is expressed by the ratio of

$$\frac{\text{dose required for given biological effect in absence of repair}}{\text{dose required for same effect with repair operating}}$$

For discussion of the photoreactivable sector see DULBECCO[2].

about 0.5 is obtained. After pretreatment the PRS is not constant. With low doses of UV following pretreatment there is little or no PR in spite of marked killing. As the UV dose is increased the PRS also increases. At 300 erg/mm² it reaches 0.5 and at higher UV doses the PRS becomes greater than 0.5. This can be seen in Fig. 1 by comparing the actual values of survival after PR with those expected on a basis of a PRS of 0.5 (dotted line). The excess of PR was a constant feature of these results.
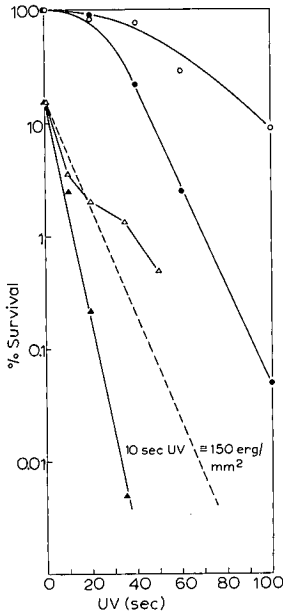

Fig. 1. DEB–UV interaction. O——O, UV alone: △—△, UV following DEB treatment. Open symbols, with photoreactivation; filled symbols, no photoreactivation. ————, expected survival based on a PRS of 0.5.

Lower DEB doses have also been used as pretreatments. Doses giving nearly 100% survival do not affect the UV response but an increase in the DEB dose diminishes the UV shoulder and, as this occurs, the new pattern of PR becomes established.

This modification of PR by DEB could conceivably result from competitive inhibition. This would occur if DEB lesions could bind the PR enzyme even though they are not repaired by it. Competitive inhibition of this type would be most effective at low UV doses. At higher doses the competition should be relieved and PR become apparent; however, its extent should never exceed the expected value. Since the latter always occurs, other factors must also be important in determining the pattern of photoreactivation.

Competitive inhibition has been excluded by the results of experiments in which a series of high DEB pretreatments were combined with a dose of 300 erg/mm² UV. Reference to Fig. 1 will show that this dose is just twice that giving little or no PR following pretreatment with DEB. If competition is important, photoreactivation of the damage induced by this UV dose should become inhibited at high DEB doses. This is not so. Even after DEB doses giving <0.01% survivors photoreactivation is apparently undiminished.

Besides excluding competitive inhibition, these experiments also make it unlikely that the unusual pattern of PR in DEB-treated cells results from a modification of the PR system itself.

The relationship between the interaction response and the DEB-after-effect

It has been known for some time that conidia of Neurospora treated with DEB or ethylene oxide[6,7] and subjected to standard washing procedures continue to mutate for some time. This is true for reversions of the allele *ad-3A 38701* and occurs when the cells are sedimented. By extending the time available for the after-effect, it has been possible to demonstrate that DEB can also promote a lethal after-effect. E.O. was not tested. These after-effects appear to be the result of the retention of traces of the chemicals by the cells. Continuous washing (*e.g.* by stirring) can abolish the after-effect. In the case of DEB the time taken to achieve this is 4 h. The possibility that this might occur in yeast also, and that it might be of significance in promoting the interaction, led to a similar set of experiments with yeast. Fig. 2 shows that also in yeast DEB promotes a lethal after-effect when the cells are sedimented by centrifugation. Continuous washing abolishes the after-effect but, in contrast to Neurospora, the time taken to achieve this is 10 h or more.

By interpolating various periods of washing between DEB treatment and subsequent exposure to UV, it was possible to investigate the relationship, if any, between the DEB after-effect and the DEB–UV interaction. In a series of experiments washing out times of 4, 8 and 12 h were used. Fig. 3 shows one set of results in which washing out times of 0, 8 and 12 h were used. Slight differences in initial survival
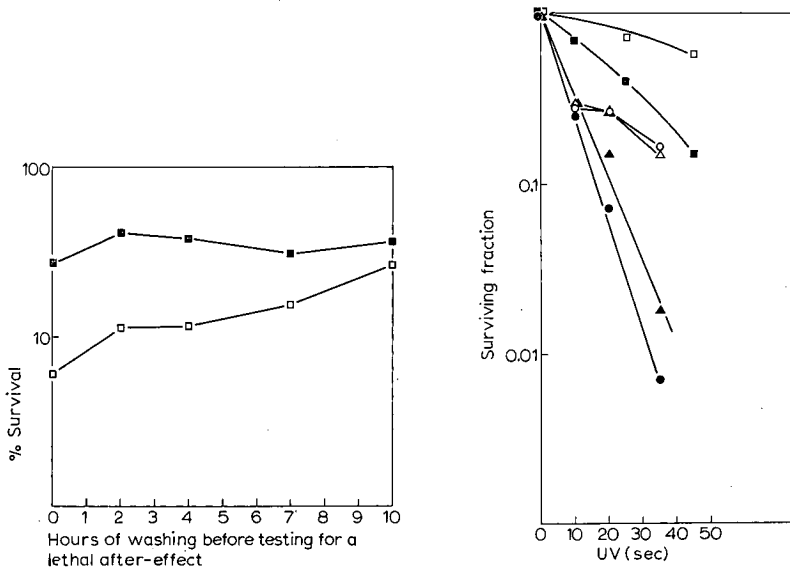
Fig. 2. Evidence for a lethal after-effect in yeast. Cells treated with DEB were washed and resuspended in 0.067 M KH_2PO_4 solution. The suspension was stirred for 10 h at 30°. During this time samples were removed and either immediately plated (■———■) or sedimented by centrifugation and allowed to stand at 30° for a further 24 h before plating them (□———□).

Fig. 3. The effect of extended washing on the DEB–UV interaction. Closed symbols, without PR; open symbols, with PR. ●, ○, no washing; ▲, △, 8-h washing; ■, □, 12-h washing.

have been eliminated in order to superimpose the 3 curves. No change in the pattern of interaction was found after 4 and 8 h of washing, but after 12 h the interaction response had disappeared and the normal UV response was produced.

These experiments cannot be considered wholly conclusive, since the length of time required for washing out is sufficient for other events to occur, which may be of greater significance in restoring the normal UV response. It is, however, of interest to note that these results are in agreement with some earlier unpublished data (KILBEY, 1963) which showed that the mutagenic interaction between DEB and UV in Neurospora was also abolished by prolonged washing between the treatments. In this case only 4 h washing was necessary to abolish both the after-effect and the interaction response.

DISCUSSION

The results presented here are concerned with the effects of DEB pretreatment on UV inactivation kinetics. They are broadly similar to those of HAYNES AND INCH[4]: DEB pretreatment abolishes the shoulder of the UV-inactivation curve which become immediately exponential. There also appears to be a slight increase in the slope of the inactivation curve. DEB pretreatment modifies the pattern of PR: Instead of a constant PRS there is little or no PR at low UV doses while at higher UV doses PR is greater than expected on a basis of a PRS of 0.5.

This pattern of inactivation and photoreactivation is not destroyed when 4- or 8-h periods of continuous washing are interpolated between the DEB and UV treatments. However, washing for 12 h destroys the interaction response and it is replaced by a normal response to UV and PR. This period corresponds closely to the washing-out time necessary for complete abolition of the lethal after-effect of DEB.

Inhibition of dark repair by DEB can account for several of these findings: Abolition of the shoulder on the UV survival curve and an increase in its steepness both suggest dark repair inhibition. Furthermore, by releasing more UV damage for PR, the abolition of dark repair might be expected to bring about an increase in the PRS as observed at higher doses of UV. It is hard to see, however, how abolition of dark repair alone could account for the failure of photoreactivation found at low UV doses.

A possible interpretation is provided by the finding that both the interaction response and the after-effect disappear simultaneously in washed suspensions. This suggests that the interaction depends on the presence of traces of DEB in the cells *during UV irradiation*. Normally, these traces react to produce a lethal after-effect over a period of several hours. It seems possible that, under the influence of small UV doses, molecules of the retained chemical react very rapidly to produce lethal lesions. The UV effect is probably not a direct one on DEB since this has been excluded in the case of the mutagenic interaction in Neurospora (AUERBACH, personal communication). UV is known to break hydrogen bonds causing strand separation within the DNA molecule[9]. Possibly events of this sort could facilitate chemical attack. Whatever the precise mechanism is, the result expected would be a rapid decrease in survival in response to a small UV dose. Since the lesions mainly responsible arise from chemical attack their non-photoreactivability is to be expected. DEB is probably bound to various cell structures producing lethality in a number of ways. This suggests

that the effects described are restricted to cells which contain DEB molecules in the vicinity of the UV induced changes. These cells are selectively inactivated at low UV doses leaving survivors which can respond to UV and PR in the normal way. This surviving population should exhibit the effects of reduced dark repair in the form of an increased sensitivity and an elevated PRS.

This interpretation is of course very speculative. However, if it obtains further experimental support, it implies that the exponential UV-inactivation curve obtained after DEB pretreatment is an artifact arising from the combination of two or more different modes of killing. Whether this may also be the case for other interaction treatments obviously needs to be tested.

These results support the suggestion made earlier that one aspect of the activity of DEB may be repair inactivation. This interpretation may also be a valid one for the mutational interaction between DEB and UV in Neurospora. The greater than additive yield of adenine reversions obtained when DEB and UV treatments are combined could result from repair inactivation. Two points may be made here, however; the first is that the *ad* allele studied almost exclusively in Neurospora is *ad-3A 38701*. The allele responds well to both DEB and UV. A more meaningful demonstration of the involvement of repair inactivation in the DEB–UV interaction would be obtained if it could be shown that DEB enhances reversion in a mutant responding only to UV. The second point to note is that no data are yet available concerning PR following the mutational DEB–UV interaction. Experiments are at present in progress to investigate both questions.

REFERENCES

1 AUERBACH, C., AND D. RAMSAY, Analysis of a case of mutagen specificity in *Neurospora crassa*, I. Dose–reponse curves, *Mol. Gen. Genet.*, 103 (1968) 72–104.
2 DULBECCO, R., A. HOLLAENDER (Ed.), *Photoreactivation in Radiation Biology*, Vol. II, McGraw Hill, New York, 1955, p. 455.
3 HAYNES, R. H., The interpretation of microbial inactivation and recovery phenomena, *Radiation Res.*, Suppl. 6 (1966) 1–29.
4 HAYNES, R. H., AND W. R. INCH, Synergistic action of nitrogen mustard and radiation in microorganisms, *Proc. Natl. Acad. Sci. (U.S.)*, 50 (1963) 839–846.
5 KILBEY, B. J., Specificity in the photoreactivation of premutational damage induced in *Neurospora crassa* by ultraviolet, *Mol. Gen. Genet.*, 100 (1967) 159–165.
6 KILBEY, B. J., AND H. G. KØLMARK, A mutagenic after-effect associated with ethylene oxide in *Neurospora crassa*, *Mol. Gen. Genet.*, 101 (1968) 185–188.
7 KØLMARK, H. G., AND B. J. KILBEY, An investigation into the mutagenic after-effect of butadiene diepoxide using *Neurospora crassa*, *Z. Vererbungsl.*, 93 (1962) 356–365.
8 KØLMARK, H. G., AND B. J. KILBEY, Kinetic studies of mutation induction by epoxides in *Neurospora crassa*, *Mol. Gen. Genet.*, 101 (1968) 89–98.
9 MARMUR, J., W. F. ANDERSON, L. MATTHEWS, K. BERNS, E. GAJEWSKA, D. LANE AND P. DOTY, The effects of ultraviolet light on the biological and physical chemical properties of deoxyribonucleic acid, *J. Cell Comp. Physiol.*, Suppl. 1, 58 (1961) 33–55.

THE INTERACTION BETWEEN DIEPOXYBUTANE AND ULTRAVIOLET LIGHT IN YEAST

II. EXPERIMENTS WITH UV-SENSITIVE AND RESISTANT STRAINS*

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SUMMARY

Earlier experiments in which the effects of diepoxybutane (DEB) pretreatment on UV inactivation were studied in wild-type yeast, have been extended to include DEB pretreatment effects in the strain *uvs-1* which is sensitive to both UV and DEB. The effects of post-UV treatment with DEB have also been studied in both strains.

DEB pretreatment produces a similar result in *uvs-1* as the effect described earlier for wild-type in spite of the necessary reduction in doses of both agents. Post-treatment with DEB fails to affect photoreactivation in both resistant and sensitive strains but in the resistant strain the shoulder on the UV curve is obliterated. There is no such effect in the UV-sensitive material.

INTRODUCTION

Earlier experiments showed that cells of *S. cerevisiae* treated with DEB respond differently from untreated cells to subsequent UV treatment[4]. The shoulder, normally present on the UV-survival curves, is absent after pretreatment and inactivation is exponential. In addition, the pattern of photoreactivation is altered: at low doses of UV little or no photoreactivation occurs in spite of marked inactivation. At higher UV doses the extent of photoreactivation increases until it reaches or even exceeds the value expected from the control samples.

DEB was also found to produce a lethal after-effect in yeast similar to that reported for Neurospora[6]. Continuous washing for several hours destroyed the after-effect and at the same time removed the effects of DEB pretreatment on the UV-inactivation kinetics and photoreactivation.

These observations led to the suggestion that traces of DEB or some derivative of it must be present in the cells at the time of irradiation for the interaction to occur.

The experiments described here were designed to test this further and to pro-

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vide evidence concerning the relationship between the interaction and the inactivation of cellular processes such as repair (cf. HAYNES AND INCH[3]). Two approaches were made: In the first place the order of the treatments was reversed. In this way DEB was not introduced into the cells until UV treatment was complete. Secondly, the interaction was examined in an UV-sensitive strain of yeast which is also DEB-sensitive. In order to operate at survival levels comparable with wild-type in this material, the doses of both agents were reduced to a fraction of those used with the wild-type strain. It seems reasonable to conclude that, in the event of an interaction response in the sensitive strain, (a) the cellular system which is lost in the UV-sensitive strain is not required for the appearance of the interaction, (b) cellular events such as enzyme inactivation are probably not involved either, unless they too are sensitised by the uvs-1 mutation to the very low treatment doses used.

MATERIALS AND METHODS

Strains. The yeast strains used were ad2-2.0α with normal UV sensitivity (often referred to as "wild-type") and the uvs-1 strain described first by NAKAI AND MATSUMOTO[7].

Media. Cultures were grown for 2–3 days in Glucose Nutrient Broth (20 μg adenine sulphate per ml). Platings were made on Oxoid Yeast Extract Agar supplemented with 20 μg adenine sulphate per ml.

Treatments. Full details of the treatments both with UV and DEB have been given in earlier papers[4,6]. All operations were made under yellow room light from Atlas "Gold" fluorescent tubes. In these experiments the chemical was removed and the cells were washed on Oxoid membrane filters in order to achieve the desired precision in timing the treatments. Throughout the work the solution used for washing the cells and diluting them was 0.067 M potassium dihydrogen phosphate.

Incubation. Plates were incubated for 4 days at 30° before scoring. Dilutions were selected which gave between 100 and 200 colonies per plate and each plating was made in triplicate.

RESULTS

(1) Treatment reversal in wild-type yeast

The effects of DEB pretreatment have already been described. In the experiments described here, samples were treated with graded UV doses and, within 20 sec, DEB was added to half of each irradiated suspension. The other half was kept under the same conditions but without DEB to provide an estimate of survival after UV alone. Samples were also prepared which either received no treatment at all or were only exposed to DEB. After termination of the DEB treatment, all the samples which received UV were divided once more into two parts and one of the two halves photoreactivated while the other was kept dark for the same time. These samples provided estimates of survival and photoreactivation after various UV treatments with or without post-treatment with DEB.

Fig. 1 shows the combined results of two typical experiments which differed in the DEB treatments. In the first, survival after DEB was 30%, in the second it was 1%. Post-treatment with DEB clearly has no obvious effect on photoreactiva-

tion although there is some indication that, at the higher DEB dose, the shoulder on the UV-inactivation curve is reduced. This point was examined in more detail. Dark survival only was followed and the UV doses were chosen to provide a more satisfactory distribution of the experimental points. DEB post-treatments were used yielding 20% and 7% survival. Both completely abolished the shoulder on the UV-inactivation curve reducing the extrapolation number[8] from 3 (without post-treatment) to 1 (with post-treatment). One of these experiments is presented in Fig. 2.
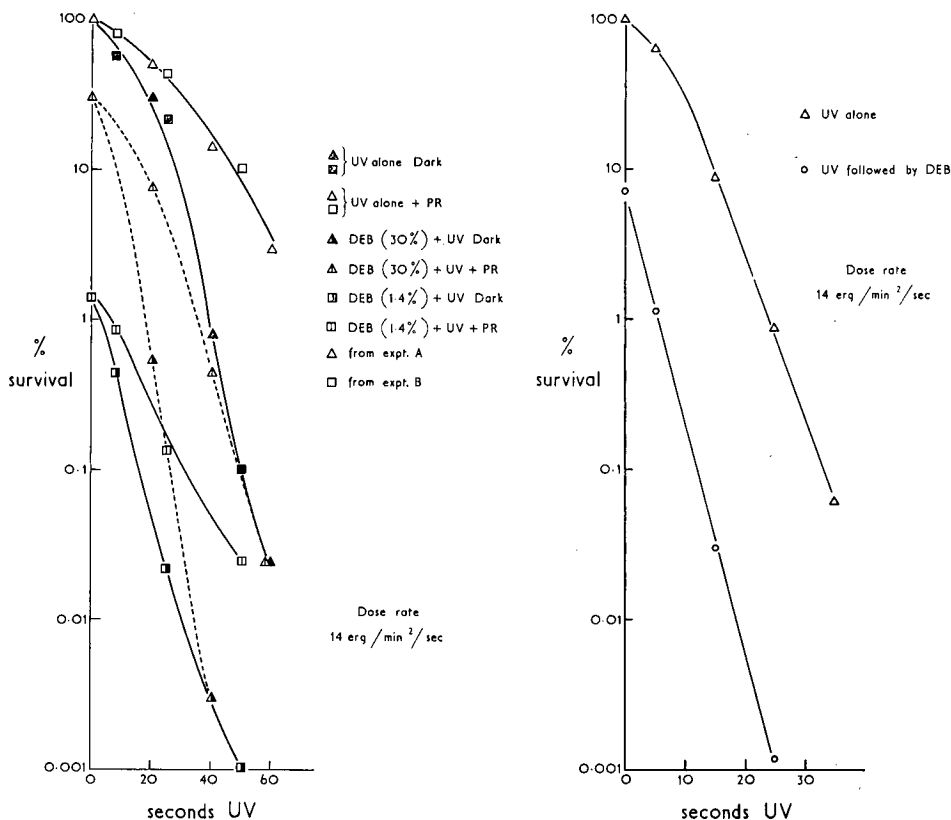
Fig. 1. The effect of post-treatment with DEB on UV inactivation and photoreactivation in wild-type yeast. The results of two independent experiments A and B are combined.

Fig. 2. Effect of DEB post-treatment on the kinetics of UV inactivation of $ad2$-2.0α in the dark.

(2) Experiments with the sensitive strain

In order to achieve similar survival levels in the sensitive strain the doses of UV were reduced to approximately 5% of the doses given to the wild-type strain. The DEB doses were also reduced to 20% of the concentration and reduced in duration from 15 to 10 min. Otherwise the protocols for these experiments were the same as described previously and above.

Fig. 3 gives the effect of pretreating cells of the sensitive strain with DEB on subsequent UV inactivation. In every experiment the pattern of interaction was indistinguishable from that found in the wild-type at higher doses.

Fig. 4 shows the effect of adding DEB after the conclusion of the UV treat-
ment. As with the wild-type strain, photoreactivation is unaffected by post-treatment.
Furthermore, post-treatment had little effect on the course of UV inactivation. Repe-
tition of the dark-inactivation parts of the experiments showed clearly that the ex-
trapolation number of 3 obtained without post-treatment was unchanged with it.
This is in contrast to the results reported above for the wild-type strain where post-
treatment reduced the extrapolation number from 3 to 1.
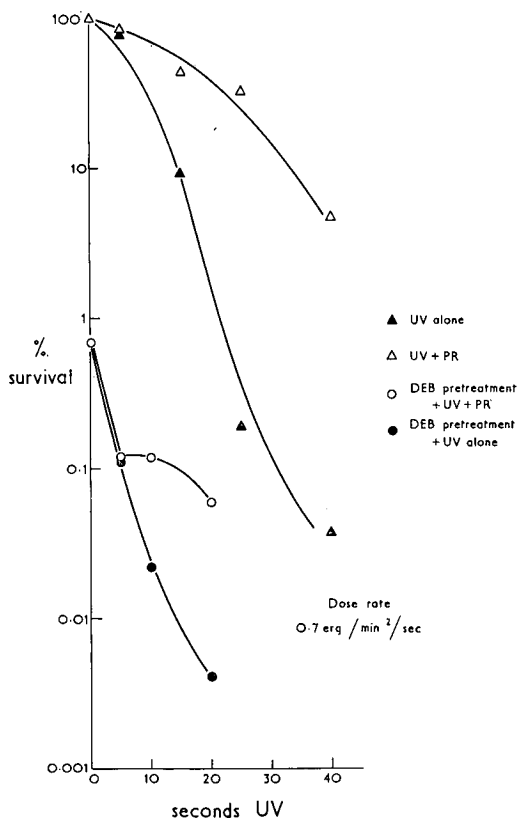
Fig. 3. The effect of DEB pretreatment on UV inactivation and photoreactivation in the UV
sensitive strain *uvs-1*.

DISCUSSION

Photoreactivation was unaffected by post-UV treatments with DEB. This was
true whether the sensitive mutant or the non-sensitive mutant was used. However,
the higher doses of DEB used as post-treatments in the non-sensitive strain elimina-
ted the shoulder on the UV-inactivation curve. Doses of DEB which produced the
same levels of survival in the sensitive mutant failed to modify the course of UV inac-
tivation. Pretreatment with DEB in the sensitive strain elicited a response very
similar to that described in the first paper in this series for wild-type yeast.
    In the earlier paper it was suggested that traces of the chemical retained by

the cells after washing, play an important part in the DEB–UV interaction. Under the influence of low doses of UV these may react rapidly with cellular targets to produce lethal damage which, although apparently the direct result of the UV, is in fact produced by chemical attack. This is consistent with its failure to respond to photoreactivation. If this interpretation is correct it is, to be expected that introduction of the chemical after the termination of the UV treatment should lead to a failure of this mechanism. The data presented here are in agreement with this expectation
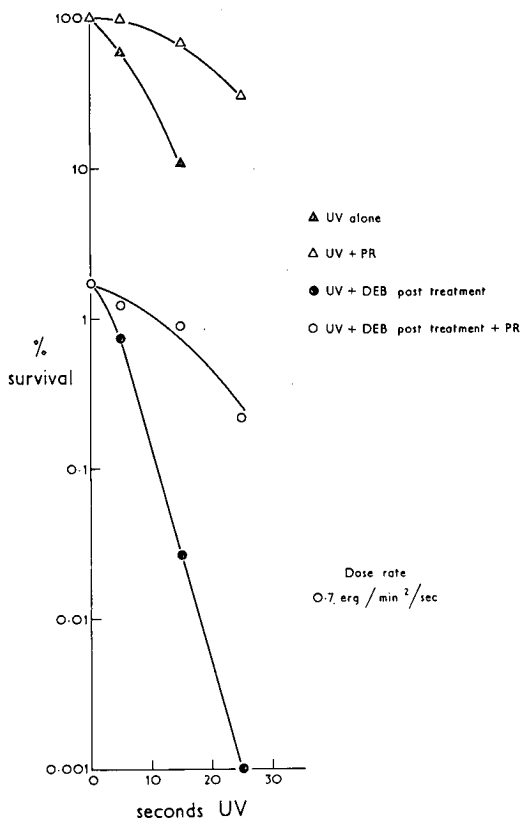
Fig. 4. The effect of DEB post-treatment on UV inactivation and photoreactivation in the UV-sensitive strain *uvs-1*.

since in neither strain is the pattern of photoreactivation disturbed by post-treatment with DEB. Furthermore, because the interval between the two treatments was always less than 20 sec, it may be concluded that the UV-induced lesions which interact with the chemical are rather short-lived.

It was also suggested in the earlier paper that DEB might act concurrently on systems of dark repair resulting in an enhanced sensitivity to UV. Such an effect might be expected whether DEB was administered before or after the UV treatments. As was shown above, the higher doses of DEB used as UV post-treatments in the resistant strain eliminated the shoulder on the UV-inactivation curve. The inactivation curve was also rather steeper than that obtained with UV alone.

The results obtained with the sensitive strain provide further evidence concerning the mechanism of the interaction. This mutant has been shown to possess several characteristics in common with excision-deficient bacterial mutants[5]. In spite of the repair deficiency and the consequent necessity to reduce the doses of the two agents, the typical interaction pattern was found in DEB-pretreatment experiments. The repair system is clearly unnecessary for the interaction between DEB and UV when the chemical precedes the irradiation. This suggests that the events concerned occur at the level of the DNA and that they are subject to excision repair in the wild-type strain. In contrast, post-treatment with DEB fails to affect either the pattern of photoreactivation or the kinetics of inactivation in the *uvs-1* strain. This suggests that in the *uvs-1* mutant the DEB dose cannot be brought to the level required for the elimination of the shoulder on the UV curve without killing the cells. These findings agree with the supposition that DEB has at least two effects when used in combination with UV in yeast. The first occurs at the DNA level and can only take place if the chemical is present at the time of irradiation. The effect can be recognised principally from its interference with photoreactivation. The second effect can only be recognised when DEB follows UV. It only occurs at higher doses of DEB and, as a result, cannot be obtained at the treatment levels used with the sensitive strain. When it operates, however, it results in the elimination of the shoulder on the UV-inactivation curve but does not interfere with photoreactivation. The second effect possibly concerns the inactivation or interference with some cellular repair system, but not the one lost in *uvs-1* since here too the inactivation curve is sigmoidal.

Other workers have studied the interaction between chemical and physical agents in yeast[3], and were able to show that pretreatment with nitrogen mustard reduced the shoulder on the UV-survival curve for haploid yeast. They interpreted their results as indicating interference with dark repair by lesions introduced by the chemical. Unfortunately, no comparable data for photoreactivation were obtained in these experiments. BRIDGES *et al.*[1,2] working with *E. coli* studied the mutational interaction between UV and γ radiations. They showed that interaction mutants were produced independently of both the order of treatment and the presence or absence of an excision repair system. The effect of post-treatment conditions on the recovery of the interaction mutants suggested that they originated from UV-induced lesions.

The results of the experiments described here suggest that the interaction lesions originate at least in part from the action of the chemical although at present the data are limited to inactivation. Experiments have been performed in Neurospora in which the mutagenic interactions between these agents has been studied (AUERBACH AND RAMSAY, *Mol. Gen. Genet.*, in the press). These are at present being extended to include experiments using photoreactivation and will form the subject of a later publication (RANNUG, in preparation).

REFERENCES

1 BRIDGES, B. A., R. J. MUNSON, C. F. ARLETT AND D. R. DAVIES, Interaction between ultraviolet light and γ-radiation damage in the induction of mutants of *Escherichia coli*, The effect of some modifying treatments, *J. Gen. Microbiol.*, 46 (1967) 339–346.
2 DAVIES, D. R., C. F. ARLETT, R. J. MUNSON·AND B. A. BRIDGES, Interaction between ultraviolet light and γ-radiation damage in the induction of mutants of *Escherichia coli*, The response in strains with normal and reduced ability to repair ultraviolet damage, *J. Gen. Microbiol.*, 46 (1967) 329–338.
3 HAYNES, R. H., AND W. R. INCH, Synergistic action of nitrogen mustard and radiation in microorganisms, *Proc. Natl. Acad. Sci. (U.S.)*, 50 (1963) 839–846.
4 KILBEY, B. J., Diepoxybutane pretreatment effects on UV inactivation and photoreactivation of *Saccharomyces cerevisiae*, *Mutation Res.*, 8 (1969) 73–78.
5 KILBEY, B. J., AND S. M. SMITH, Similarities between a UV-sensitive mutant of yeast and bacterial mutants lacking excision-repair ability, *Mol. Gen. Genet.*, 104 (1969) 253–257.
6 KØLMARK, H. G., AND B. J. KILBEY, An investigation into the mutagenic after-effect of butadiene dioxide using *Neurospora crassa*, *Z. Vererbungslehre*, 93 (1963) 356–365.
7 NAKAI, S., AND S. MATSUMOTO, Two types of radiation sensitive mutant in yeast, *Mutation Res.*, 4 (1967) 129–136.
8 NORMAN, A., Inactivation of Neurospora conidia by ultraviolet radiation, *Exptl. Cell Res.*, 2 (1951) 454–473.

Similarities between a UV-Sensitive Mutant of Yeast and Bacterial Mutants Lacking Excision-Repair Ability

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Summary. A UV-sensitive and a wild-type strain of *Saccharomyces cerevisiae* have been compared with respect to their responses to photoreactivation, retention of the capacity to photoreactivate when stored at 32°C in buffer, and sensitivity to diepoxybutane and nitroso-guanidine. In all these tests the behaviour of the sensitive mutant paralleled bacterial strains lacking excision repair ability. We may tentatively attribute the UV sensitivity in this mutant to a loss of some element of a repair system analogous to excision repair in bacteria.

Introduction

Although a large number of ultraviolet-sensitive fungal mutants are now available (COX and PARRY, 1968; HAEFNER and HOWREY, 1967; HOLLIDAY, 1965; CHANG and TUVESON, 1967), further work with them is greatly hampered by a lack of knowledge concerning the precise basis of the sensitivity in each case. Unfortunately, a direct application of the biochemical tests used with bacteria cannot be made for fungi and, until alternatives are found, only indirect methods can be used in an attempt to characterise UV[1]-sensitive mutants.

In the present report we describe an attempt to determine the basis for the sensitivity of a particular UV-sensitive mutant of *Saccharomyces cerevisiae*. The rationale is a simple one; we have studied the behaviour of the mutant under a series of experimental conditions which evoke apparently characteristic responses from bacterial mutants lacking excision-repair ability. It will be seen that in all the tests, there is a close correspondence between the behaviour of the yeast mutant tested and the behaviour of this type of bacterial mutant. Our data are thus consistent with the conclusion that this yeast mutant lacks some element of a dark repair system analogous to excision-repair in bacteria. However, this conclusion is reached with caution in the absence of direct biochemical information.

Materials and Methods

Strains. The ultraviolet sensitive yeast mutant used was first described by NAKAI and MATSUMOTO (1967). It is prototrophic and haploid and has the symbol $UV_1^S \alpha$. We are grateful to Dr. NAKAI for making the strain available to us.

The non-sensitive strain is the $ad_{2,0}\alpha$ strain of yeast supplied by Dr. R. WOODS and referred to in an earlier publication (KILBEY, 1969). This strain requires adenine for growth but has the UV-sensitivity of prototrophic wild-type haploid yeast.

Media. Cultures were grown for three days at 25°C with gentle shaking in Glucose Nutrient Broth. Platings were done on Yeast Extract Agar supplemented with 20 µg/ml adenine sulphate.

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1. *Abbreviations:* UV = Ultraviolet; PRS = Photoreactivable Sector (DULBECCO, 1955); DEB = Diepoxybutane; NTG = N-methyl-N'-nitro-N-nitrosoguanidine; PRL = Photoreactivating Light.

Treatments. Cultures were harvested, and washed suspensions of cells prepared in 0.067 M KH_2PO_4 solution. The cell density used was $1—3 \times 10^7$ cells/ml.

UV treatment and photoreactivation conditions were as previously described (Kilbey, 1969). Incident UV doses were measured using a "Jagger meter" (Jagger, 1961).

For treatment with DEB, 0.05 ml of the mutagen were added to 40 ml of a cell suspension and the mixture incubated at 30°C. At various times samples were withdrawn and washed on membrane filters before resuspending in 0.067 M KH_2PO_4 solution and plating.

Treatment with NTG was performed as follows. NTG was dissolved in pH 7 phosphate buffer in the proportions of 0.058 g NTG/80 ml buffer immediately before use. 1 ml of the solution was added to 9 ml of a cell suspension in pH 7 phosphate buffer at 25°C. Treatment was terminated when samples were diluted into 0.067 M KH_2PO_4 solution before plating.

Incubation

Plates spread with treated or untreated suspensions were incubated at 30°C for 4 days before scoring. Each experiment was performed at least three times. The points on the graphs are the averages of platings in triplicate. Dilutions were chosen which gave 100—200 colonies per plate.

Results and Discussion

In addition to ultraviolet sensitivity, four criteria were chosen for an evaluation of the UV_1^S mutant. They were (a) photoreactivability (b) retention of photoreactivability when UV-treated cells are stored in buffer. (c) Sensitivity to DEB and (d) sensitivity to NTG. In addition Nakai and Matsumoto (1967) have shown that the UV_1^S gene does not confer sensitivity to X-rays.

a) Ultraviolet Light and Photoreactivability. Nakai and Matsumoto were able to show that the UV_1^S mutation increases the sensitivity of the cells to UV approximately 27 times. Fig. 1 shows that the response of this strain in our hands is very similar to the results reported by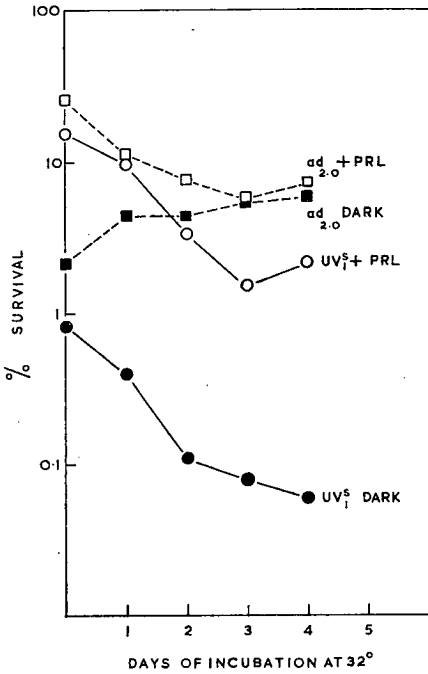 these workers. After photoreactivation there is enhanced survival in this mutant. The extent of photoreactivation is greater than for the wild-type strain. The PRS for $ad_{2.0}$ and wild-type yeast is invariably 0.5 (Kilbey, 1969) while in the mutant it is 0.68—0.70. A similar enhancement of photoreactivability has also been noted in bacterial strains which lack excision-repair (Hill and Simpson, 1961; Harm, 1968) and a simple explanation is that damage normally erased in the dark in the wild-type material remains available for photorepair in the mutant. However, it is clear that a substantial proportion of damage which remains in the mutant is not amenable to photoreactivation since, even after maximum PR the survival level in the mutant is still considerably less than wild-type at the same dose.

Fig. 1. UV-inactivation and photoreactivation in UV_1^S compared with UV-inactivation in $ad_{2.0}$. 10 sec UV = 7.3 ergs/mm²

DAYS OF INCUBATION AT 32°

Fig. 2

Fig. 2. Changes in photoreactivability in stored suspensions of UV_1^S and $ad_{2.0}$

Fig. 3. a Inactivation of UV_1^S and $ad_{2.0}$ by DEB. b Inactivation of UV_1^S and $ad_{2.0}$ by NTG
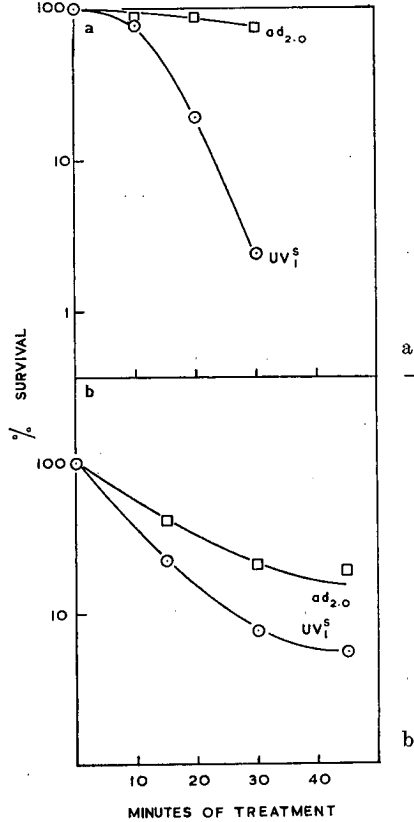
MINUTES OF TREATMENT

Fig. 3

b) Retention of Photoreactivability upon Liquid Holding. Photoreactivability can be measured either directly, as the proportion of pyrimidine dimers mono-merised in the cell by visible light (SETLOW and CARRIER, 1964) or indirectly, by measuring the increased viability of the UV'd suspension after PRL illumina-tion. When either technique is used, it has been shown that suspensions of excision-deficient bacteria retain their capacity for photoreactivation whereas excision-sufficient bacteria lose this capacity in a few hours (HARM, 1968). This result has been interpreted as an indication of the state of the intracellular pyrimidine dimers. In the excision-sufficient bacteria, it is found that dimers pass into the acid soluble fraction. Here they become inaccessible to the PR enzyme which cannot act on dimers in oligonucleotides. In the excision deficient strain, on the other hand, dimers are retained in the DNA and photoreactivability does not fall with time.

Fig. 2 shows the results of one of three experiments made with the UV_1^S yeast mutant and $ad_{2.0}$ to test for the retention of photoreactivability in cells incubated in buffer after UV. It will be seen that the $ad_{2.0}$ strain loses its capacity for photo-reactivation in about four days when incubated at 32°C in 0.067 M KH_2PO_4. In spite of an overall reduction in viability, the UV_1^S strain retains its photoreactiv-

ability during storage. The reason for the decrease in viability upon storing is not known but it is reminiscent of the "negative liquid holding effect" reported recently by HARM and HAEFNER (1968) for *Schizosaccharomyces pombe*. It is interesting to note that in their case the UV *sensitive* mutant of *S. pombe* they used displayed liquid-holding recovery under conditions in which the wild-type showed the "negative liquid holding effect", a result completely opposite from our findings with *S. cerevisiae*.

Retention of photoreactivability by the UV_1^S mutant and its loss in the wild-type material suggests strongly to us that a situation exists in yeast analogous to the one proposed for bacteria by HARM (1968) which implicates excision of dimers from DNA as the basis for a loss in photorepair ability.

c) Sensitivity to DEB and NTG. The wild-type and UV_1^S strains were also compared on a basis of their sensitivity to DEB and NTG. The former has not been used extensively in experiments with bacteria but it was recently shown that the WP2 *hcr⁻* strain of E. coli is markedly more sensitive to DEB than the *hcr⁺* derivative (SHANKEL and KILBEY, in preparation). DEB is a bifunctional agent which has been shown to be capable of cross linking the two strands of a DNA double helix (Ross, 1962). In contrast, WITKIN has shown that the *hcr⁻* genotype does not enhance the sensitivity of the cells to NTG (WITKIN, 1967). She has used a test of this type as one of the criteria for the characterisation of a series of UV sensitive mutants of E. coli. Fig. 3a and b show the response of UV_1^S and wild type to both DEB and NTG. Whereas the UV sensitive strain is markedly sensitive to DEB there is little increased sensitivity to NTG.

Conclusions

A parallel clearly exists between the UV_1^S mutant of yeast and excision defective bacterial mutants in their response to the tests used in this study. In the absence of biochemical information it would be unwise to be too dogmatic in any conclusions drawn from these facts. However the similarities in behaviour strongly suggest that the UV_1^S mutant lacks some element of a repair system present in wild-type yeast which is analogous to excision repair in bacteria.

References

CHANG, L. T., and R. W. TUVESON: U.V.-sensitive mutants in *Neurospora crassa*. Genetics 56, 801—810 (1967).

COX, B. S., and J. M. PARRY: The isolation, genetics and survival characteristics of ultra-violet light-sensitive mutants in yeast. Mutation Res. 6, 37—55 (1968).

DULBECCO, R.: Photoreactivation. In: Radiation biology, p. 455 et seq. vol. II (ed. A. HOLLAENDER). New York: McGraw Hill Book Co. 1955.

HAEFNER, K., and L. HOWREY: Gene controlled U.V.-sensitivity in *Schizosaccharomyces pombe*. Mutation Res. 4, 219—221 (1967).

HARM, W.: Dark repair of photorepairable U.V. lesions in *Escherichia coli*. Mutation Res. 6, 25—35 (1968).

—, and K. HAEFNER: Decreased survival resulting from liquid holding recovery of U.V. irradiated *Escherichia coli* C and *Schizosaccharomyces pombe*. Mutation Res. 8, 179—192 (1968).

HILL, R. F., and E. SIMPSON: A study of radiosensitive and radioresistant mutants of *Escherichia coli* strain B. J. gen. Microbiol. 24, 1—14 (1961).

HOLLIDAY, R.: Radiation sensitive mutants of *Ustilago maydis*. Mutation Res. **2**, 557—599 (1965).

JAGGER, J.: A small and inexpensive U.V. dose-rate meter useful in biological experiments. Radiat. Res. **14**, 394—403 (1961).

KILBEY, B. J.: Diepoxybutane pretreatment effects on U.V.-inactivation and photoreactivation of *Saccharomyces cerevisiae*. Mutation Res. (1969) (in press).

NAKAI, S., and S. MATSUMOTO: Two types of radiation sensitive mutant in yeast. Mutation Res. **4**, 129—136 (1967).

ROSS, W. C. J.: Biological alkylating agents. London: Butterworths 1962.

SETLOW, R. B., and W. L. CARRIER: The disappearance of thymine dimers from DNA; an error correcting mechanism. Proc. nat. Acad. Sci. (Wash.) **57**, 226—231 (1964).

SNOW, R.: Mutants of yeast sensitive to ultraviolet light. J. Bact. **94**, 571—575 (1967).

WITKIN, E., in: Proceedings of Symposium on "Recovery repair mechanisms in radiobiology". Brookhaven Nat. Lab. 1968.

<p style="text-align:center">Communicated by H. BÖHME</p>

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The Mutagenic Activity of Diepoxybutane in Yeast

Diepoxybutane is an interesting mutagenic agent. Studies of its ability to induce adenine reversions in Neurospora have shown that besides producing premutational damage, it also inactivates a cellular activity which limits mutation production - possibly a repair process. Evidence for this came first from studies of mutation induction kinetics which showed that the curves relating mutations produced to dose were markedly upward bending over a wide range of concentrations[5]. Additional evidence was also provided from experiments which demonstrated that conidia treated with DEB become more sensitive to further doses of the chemical[1]. The increased sensitivity extends to UV irradiation and it has been shown that the extra mutations produced when UV follows a pretreatment with DEB are indeed UV-like in their response to photoreactivation[6].

The kinetic studies also showed that if the treatment period is extended to several hours and the concentration diminished so that the total doses remain the same, the upward-bending kinetics is replaced by a linear relationship. This can be explained simply by making the assumption that the cells are slowly able to replace the damaged repair system during the extended treatment periods. An explanation of this sort is in line with a further observation that an extension of the period separating two DEB doses or a DEB and a UV dose leads to a loss of sensitivity towards the second dose[2]. Finally, we were able to show that cycloheximide, presumably acting as a protein synthesis inhibitor, prevents this decline in sensitivity with storage and, predictably, converts the linear relationship described for low dose-rate treatments into the upward-bending curves normally found at high dose-rates[3,4].

These observations are of practical as well as academic interest and it seemed important to try to determine the extent to which similar mechanisms exist in other organisms as well as examining their effects on genetic damage other than point mutations and on chemicals other than DEB. The present short communication describes our attempts to find a similar mechanism in Saccharomyces which limits genetic damage and which is susceptible to inactivation by mutagenic agents. The mutagen used has again been diepoxybutane, but the mutations scored were from isoleucine-valine requirement to independence. The strain used was kindly supplied by Dr. F.K. Zimmermann and is a diploid homozygous for the mutant ilv 1-92. Four aspects of DEB mutagenesis were selected as indicators of a mutation limiting process sensitive to the chemical. They were (a) the mutation induction kinetics, (b) enhancement of mutagen sensitivity, (c) the nature of the mutants produced by the interaction between DEB and UV, and (d) the decline in sensitivity upon storage of DEB-treated cells. The three criteria (a), (b) and (c), if satisfied, should indicate the existence of a DEB-sensitive process analagous to the one described for Neurospora while the criterion (d) should provide useful evidence concerning the abilities of the cells to replace the elements of the system when damaged.

(a)   Kinetics

Figure 1a gives the result of one of the three experiments made to obtain kinetic data for mutation production. The results obtained with yeast are very similar to those described earlier for Neurospora. For treatments with 0.1M DEB at $25^{\circ}C$ upward-bending curves were obtained for treatment times of up to twenty minutes. Beyond this point the mutation induction became erratic and the viability dropped rapidly. Our attentions have thus been focussed on the lower dose ranges.

Although the mutation-induction curves are similar in shape to those described for Neurospora, the rate at which the slope increases is somewhat less. A log-log plot of the data gives a straight line with a slope of less than 2 (Figure 1b) whereas for Neurospora conidia the values ranged from 2.1 to 2.4 [5].

(b)   Sensitisation

As expected from the findings of an upward-bending mutation-induction curve it has been possible to establish that cells treated with DEB are made more sensitive to second DEB treatments administered shortly after the first. To a lesser extent this is also true if the second treatment is with UV.

The experimental protocol is shown in figure 2. A cell suspension containing approximately 2.10^7 cells ml^{-1} was divided into two parts, A and B. A was further subdivided into two parts one of which formed the untreated control while the other was exposed to the ancillary treatment with either DEB or UV. Part B was exposed to the main DEB dose and washed on a membrane filter. The washed cells were then divided into two parts one of which served as an estimate of the main DEB dose. The other part was exposed to the second ancillary DEB or UV dose before washing and plating. (a) in figure 2 represents the time separating the two treatments and in these experiments it was never more than a few minutes.

Figure 3 gives histograms which show the extent of the interaction between 2 DEB doses. It should be pointed out that the best results from this type of interaction experiment are obtained when the doses are low and survival, even after two treatments, never falls below 50%. The reason for this is almost certainly connected with the observation already noted that above these dose levels the continued

rise in mutant production is interrupted. The nearer to this limit the two separate treatments come, therefore, the smaller can be the possibility of observing reliable increases. In both experiments shown in Fig. 3 there was not only an increase in mutations among survivors but also an absolute excess of ilv^+ mutants which shows that the enhanced response occurs independently of any selective forces.

Similar findings have been obtained for UV as described in the next section.

(c)   The interaction between DEB and UV

The sensitisation of Neurospora conidia to further mutagenic treatment by DEB has been shown to extend also to UV[2]. Conidia given a DEB pretreatment show an enhanced response to UV and the total number of mutants produced by the double treatments is significantly greater than the sum of the two single treatments. It has also been possible to show that the extra mutations produced by the interaction are the result of UV mutagenesis since they respond to photoreactivation[6].

Saccharomyces cerevisiae also displays an interaction between DEB and UV. It is less pronounced - never exceeding two times the expected value based on the addition of the effects of the single treatments. As in Neurospora it is very sensitive to the experimental conditions and is best demonstrated at rather low mutation frequencies and high viabilities.

It is possible to calculate the expected yield of mutants from an interaction followed by photoreactivation by assuming either that all the excess mutants are UV-like or that none of them are. Let u be the number of mutants produced by a particular dose of UV and d the corresponding figure for a particular dose of DEB (see figure 4). When the two treatments are combined and the cells treated with DEB

followed by UV the result is the production of extra mutants $\underline{e}$. If

the mutants corresponding to $\underline{e}$ are not typically UV-like in nature, they

will not be photoreactivated, however the UV-induced fraction of the

total mutants, $\underline{u}$, will be reduced to $\underline{u}_p$. The expected interaction

result is, therefore, given by histogram E in figure 4 and equals

$\underline{d} + \underline{e} + \underline{u}_p$. If, on the other hand, the $\underline{e}$ mutants are UV-like they

should be photoreactivated to approximately the same extent as the $\underline{u}$

mutants. $\underline{e}$, therefore, is reduced to a value which can be

approximated by the term $\underline{e} \cdot \dfrac{u_p}{u}$ or $\underline{e}_p$. The expected value for the

interaction now is given by histogram F in figure 4 and equals

$\underline{d} + \underline{u}_p + \underline{e}_p$. Obviously, the main assumption made here is that

photoreactivating enzyme is unaffected in its activities by DEB.

It should also be clear that the differentiation between the two

extreme alternatives represented here is made easier when the value

of $\underline{e}$ is large compared with $\underline{d}$ and $\underline{u}$. Unfortunately, this is not

always so in the present experiments. Table 1 presents values from

three determinations for $\underline{u}$, $\underline{u}_p$ and $\underline{d}$. $\underline{e}$ is also calculated, together

with the expected values for the interaction if (a) the $\underline{e}$ mutants are

all UV-like and (b) if they are not. In the final column the actual

value for the interaction after photoreactivation is given.

In each case, the value of $\underline{e}$ is small in comparison with $\underline{u}$

and $\underline{d}$ and the differences between the two expectations is smaller than

we might wish. However, in each case the observed value corresponds

well with the figure calculated assuming the $\underline{e}$ mutants to be UV-like

and, in the second case, there is, if anything, rather more

photoreactivation than would be expected on this basis alone.

(d)   The Persistence of Enhanced Sensitivity

A consequence of the suggested mechanism for the dose-rate

effect of DEB in Neurospora is the expectation that enhanced sensitivity
in the treated material will disappear with time of storage. The time
taken for this was found to be 2 to 4 hours. In yeast, the experiments
made to test for loss of enhanced sensitivity again followed the
protocol described in figure 2. In these experiments, however, the
interval between the main and ancillary treatments (a) was varied, and,
for its duration, the cells were left in suspension at $25^{\circ}C$. This
led to a complication since the stored samples showed a rise in mutation
numbers and frequency with time. It is possible that this increase
simply represents a limited mutagenic effect of chemical retained by
the treated cells after washing. Certainly the sedimentation of
parallel samples lead to an even greater mutagenic after-effect than
observed here (Olszewska, unpublished data) - an observation consistent
with the retention of mutagenic chemical by the cells. However, it
is also possible that other unspecified cellular processes are responsible
which operate during liquid holding. Because of the change in the
mutational yield with storage, the baseline (the sum of the main and the
ancillary doses) increased with time. However the enhanced sensitivity
also apparently declines, since the points for the interaction treat-
ment converge upon the baseline values after about six hours of
storage. Figure 5a provides an example of the type of result obtained
from such a test and in figure 5b the same data are plotted with the
base-line values subtracted. By doing this a picture emerges which
is very similar to the findings reported earlier for Neurospora.

Conclusions

The objectives of this study were to examine the possible
existence in yeast of mechanisms for limiting mutational yields as exist

for Neurospora and which are themselves mutagen sensitive.   Based on

the experience gained with Neurospora using epoxides, four approaches

were used.   The first and most obvious of these was to study the

kinetics of mutant production by DEB.   An upward-bending mutation

induction curve would be consistent with the inactivation of a

mutation limiting process by the chemical.   The second type of

experiment involved the direct test for increased mutagen sensitivity

in DEB-treated cells and represented a complementary test to the

kinetic examination.   Tests for sensitivity were next extended to

include UV as the second mutagen and attempts were made to determine

the nature of the excess mutants produced by the interaction on a

basis of their photoreactivability.   These three approaches concerned

themselves with the existence and inactivation of a mutation-limiting

process in yeast and the results obtained with them are in accord with

the conclusion that such a system exists for DEB-induced mutations in

yeast.   However, the evidence from the kinetic data suggests that its

sensitivity to DEB is lower than for/Neurospora/since the rate at which
                                 the   system

the slope of the mutation induction curve increases is less (slope of

the log/log curves in yeast $<$2 while in Neurospora they are between

2.4 and 2.1).   This agrees with the finding that although the cells

treated with DEB are sensitised to both DEB and UV the extent of the

sensitisation is generally less than for Neurospora.   Finally, although

the reliability of the photoreactivation experiments must be

diminished by the relatively small values of e, the results are again

in agreement with the conclusion that the extra mutants are UV-produced.

Only one type of experiment was conducted to examine the

cells' ability to replace the damaged components of the mutation-

limiting process.   The results obtained suggest that the enhanced

sensitivity is transitory, declining over a period of about six hours

following the first DEB treatment. However, the complications arising from the accumulation of mutations during storage must make such a conclusion tentative. Unfortunately, but possibly predictably in view of the liquid holding effects, it was impossible to demonstrate a reduction in mutant yield at low dose rates of treatment: The two processes would be expected to work in opposite directions the liquid holding effects increasing mutant frequencies, the low dose rate diminishing them. Because of these uncertainties no attempts were made to stabilise the mutagen sensitive condition using actidione: this aspect of the problem is receiving further attention.

Although minor differences may exist between the results described here for yeast and those described earlier for Neurospora, it is encouraging to find extensive similarities between the two organisms in terms of their responses to DEB. Similar repair systems, both DEB sensitive, appear to limit the mutagenic response of both organisms to UV and DEB and yeast, like Neurospora, is apparently capable of replacing the damaged components of the repair system following treatment. Limited though they are, these observations mark a useful step towards our ultimate aim of identifying and understanding those cellular processes which, by their very ubiquity , may be of general importance in the determination of mutational response.

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1. Auerbach, C., in CIBA Symposium on Mutation as a Cellular
    Process, Churchill, London, 1969, pp. 66-86.

2. Auerbach, C., and D.P. Ramsay, Analysis of a case of
    mutagen specificity in Neurospora crassa. II.
    Interaction between treatments with diepoxybutane
    (DEB) and ultraviolet light. Mol. Gen. Genet.,
    109 (1970) 1-14.

3. Kilbey, B.J., Stabilisation of a transient mutagen sensitive
    state in Neurospora by the protein synthesis inhibitor
    actidione. Mol. Gen. Genet., 123 (1973) 67-72.

4. Kilbey, B.J., The manipulation of mutation-induction kinetics
    in Neurospora crassa. Mol. Gen. Genet., 123 (1973)
    73-76.

5. Kølmark, H.G., and B.J. Kilbey, Kinetic studies of mutation
    induction by epoxides in Neurospora crassa. Mol.
    Gen. Genet., 101 (1968) 89-98.

6. Rannug, J.U., Photoreactivation of mutational damage
    produced by the interaction between DEB and UV in
    Neurospora. Mol. Gen. Genet., 111 (1971) 194-196.

Legends

Fig. 1   The kinetics of induction of ilv$^+$ mutants by DEB (0.1M, 25°C).

Each point represents 4 platings of 1.2 - 1.5 x 10^6 cells.

Survival fell from 100% to 60% at 20 minute exposure (a) linear

plot (b) log-log plot.

Fig. 2   The general protocol for experiments in which two successive

mutagenic treatments were employed.   Where a storage period

was interposed between the two treatments its position is in-

dicated by "a".

Fig. 3   The results of the interaction between two doses of DEB.   In

these experiments the second dose followed the first without a

storage break.

Fig. 4   Hypothetical mutant yields from the interaction between DEB and

UV $\pm$ photoreactivation (PR).

A = UV yield without PR   B = the DEB yield.

C = the UV yield when the same UV dose is followed by PR.

D = the result of the interaction between the DEB and the UV doses;

e = excess mutants over additive value.

E = D followed by PR assuming e are all non-UV-like

F is the same as E but assumes that e are all UV-like, ie. respond

      to PR.

Fig. 5   The decline in the DEB-UV interaction with time of storage.   In

panel (a) variations in the base-line with storage are shown but

in (b) they have been subtracted.

Table 1

Values for the DEB-UV interaction observed and expected after photoreactivation if excess mutants produced by the interaction are (a) not UV-like, (b) UV-like in nature.
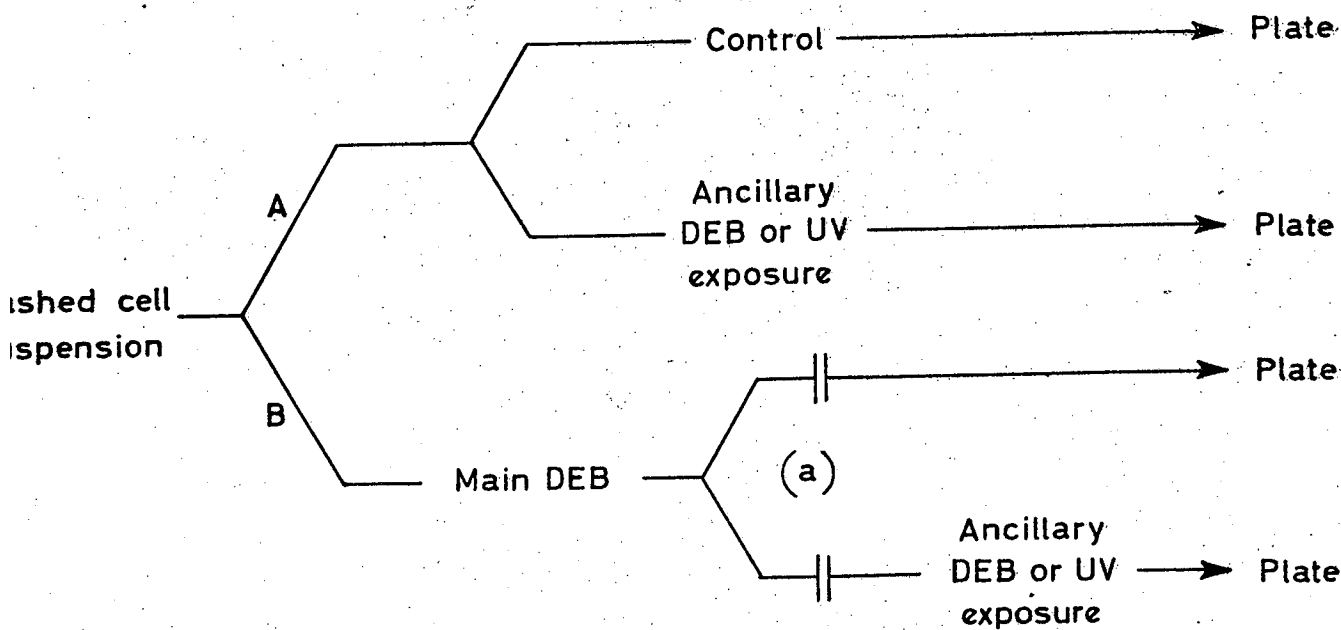
Expt	Observed values of u	up	d	Calculated value of e	Interaction Expected when e ≠ u	e ≡ u	Observed interaction value after P.R.
1	32.0	16.3	29.0	10.7	56.0	50.5	50.8
2	91.0	45.9	29.0	42.5	117.4	96.29	75.7
3	22.6	10.0	11.6	10.6	32.2	26.3	25.7

The figures are mutations per 10^6 survivors. Estimates are based on between 3 and 5 x 10^6 viable cells. In all cases viability exceeded 60%. e = observed interaction minus (u + d). For further explanation see text.
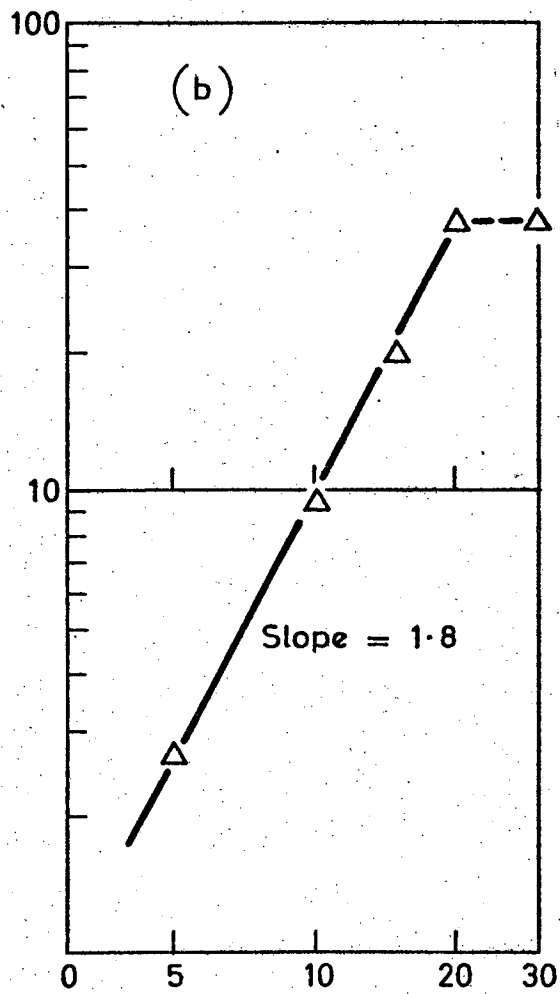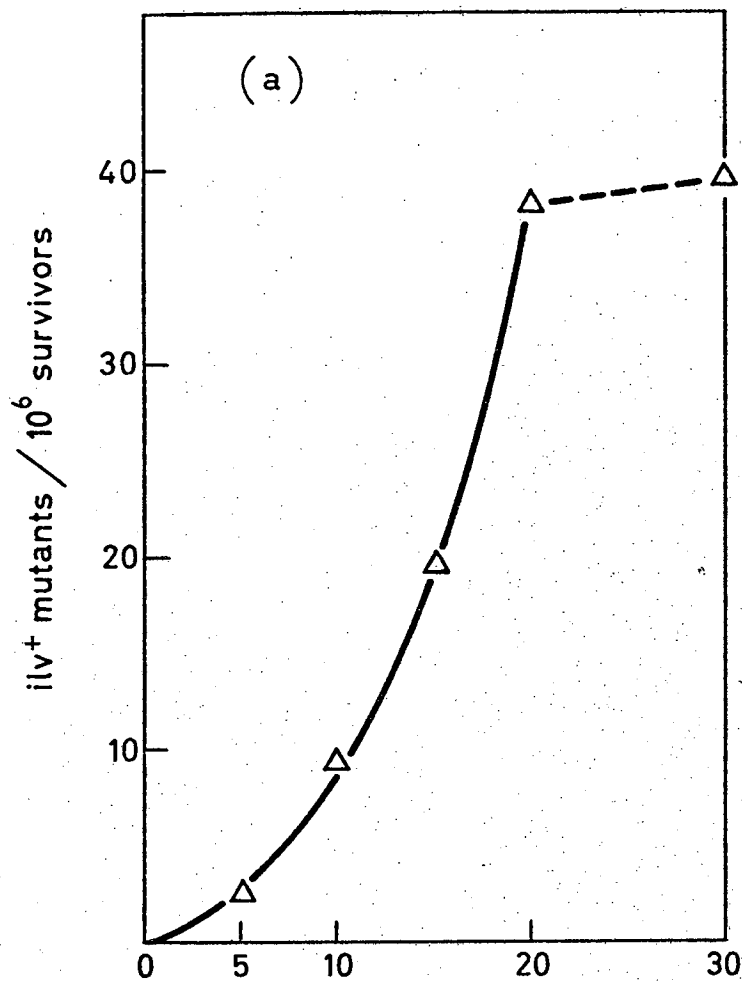
Plots showing ilv⁺ mutants / 10⁶ survivors versus DEB exposure time in minutes, with panels (a) linear scale and (b) log scale (Slope = 1·8), and a branching experimental scheme diagram below.

ilv$^+$ mutants / 10^6 survivors

Expt. 1
- 15' DEB
- 5' DEB
- 15' + 5' DEB

Expt. 2
- 15' DEB
- 5' DEB
- 15' + 5' DEB

Mutants produced (arbitrary scale)

A — u

B — d

C — u$_p$

D — d   u   e

E — d   u$_p$ e

F — d   u$_p$ e$_p$

$$e_p = e \cdot \frac{u_p}{u}$$

Fig. 5

2o
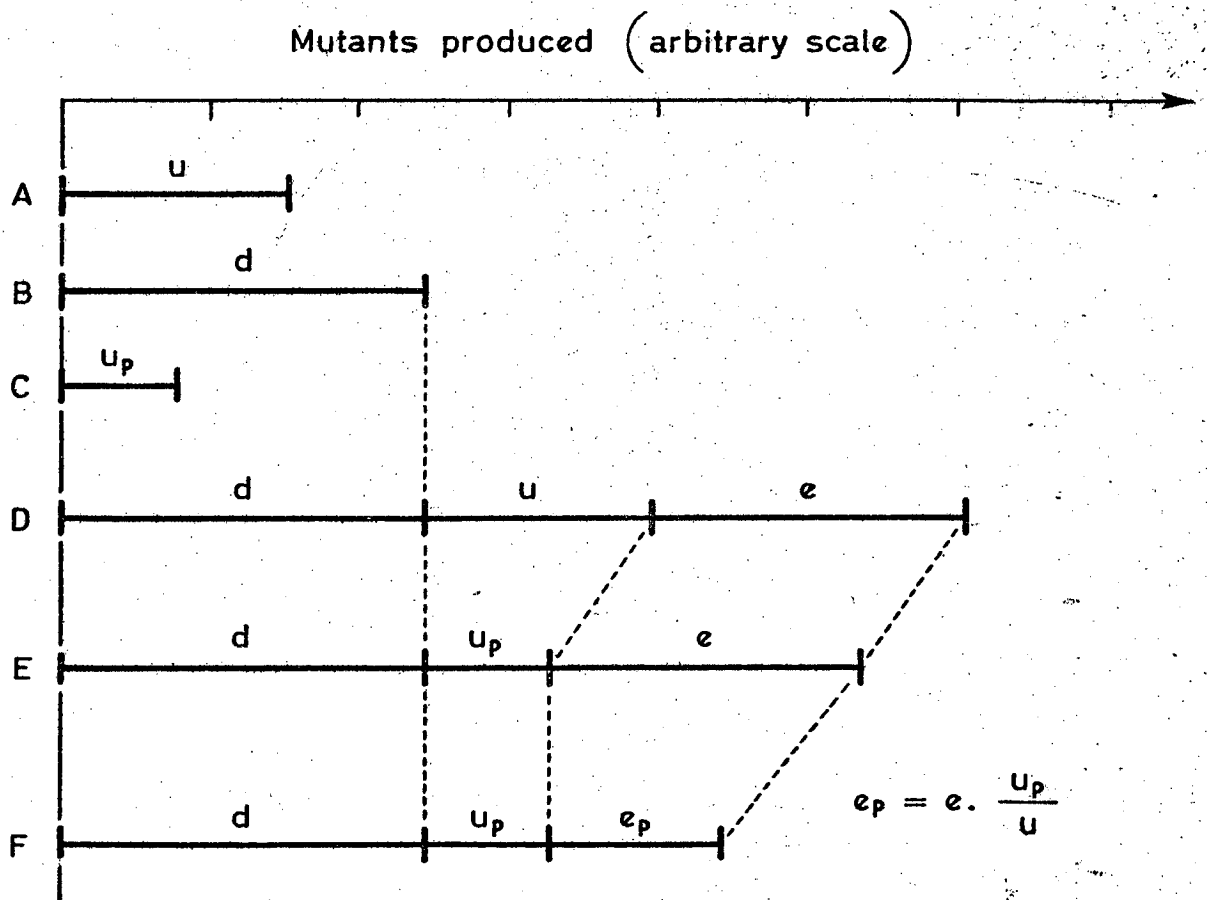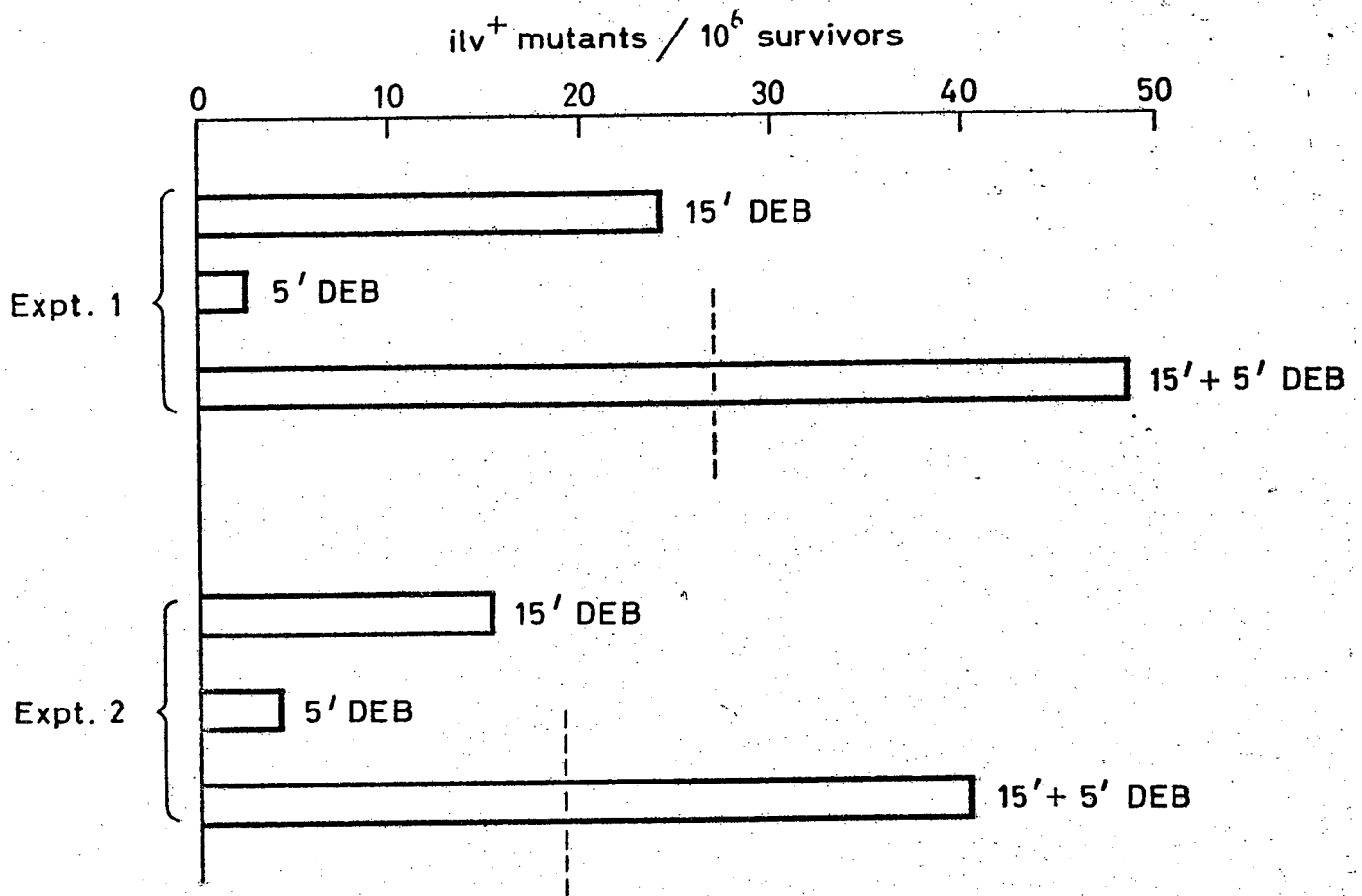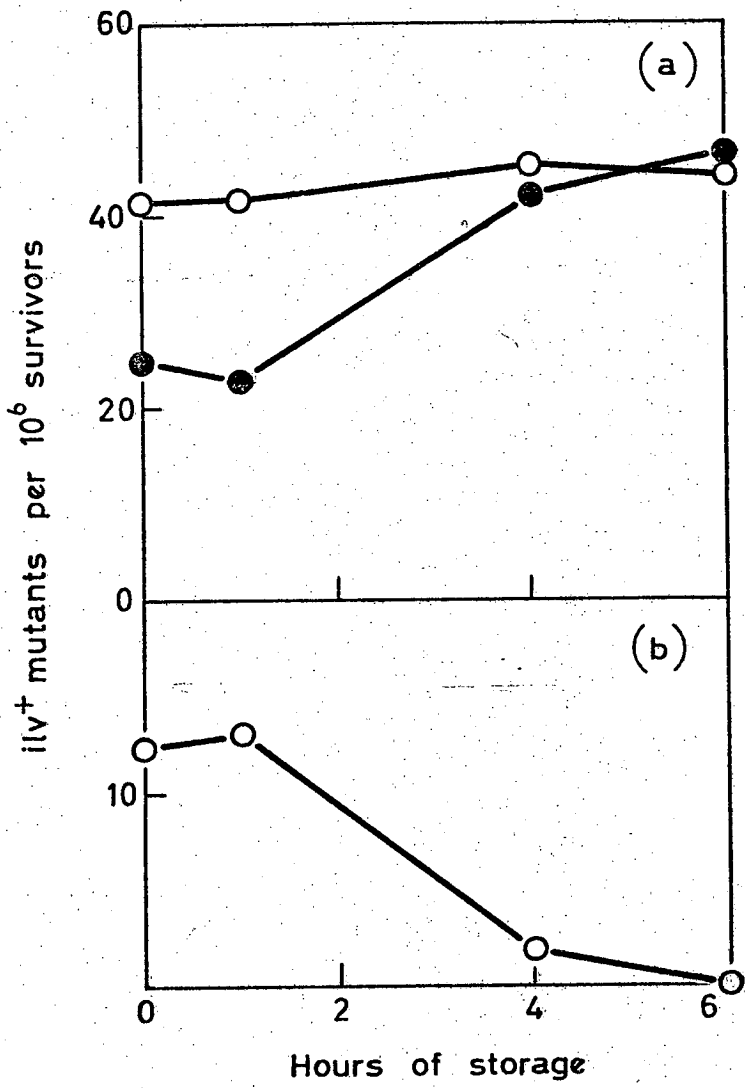
Hyper-UV-Sensitive Yeast I:
Isolation and Properties of Two such Mutants

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Summary. By using the UV-sensitive yeast mutant *uvs-1* (Nakai and Matsumoto, 1967) as starting material, a series of hypersensitive strains have been isolated. Two of these strains are described here. In addition to being hyper-UV-sensitive, they are also sensitive to gamma radiation and one is sensitive to visible light; a characteristic which was first observed as an apparent failure to photoreactivate. Both strains are cytoplasmic petites. The value of this method of isolating strains which have progressively lost their dispensible repair functions is discussed.

Introduction

Mutants of *Saccharomyces cerevisiae* which are sensitive to various chemical and physical agents have proved relatively simple to isolate (Cox and Parry, 1968; Snow, 1967; Nakai and Matsumoto, 1967; Zimmerman, 1968). In a few cases, similarities with UV-sensitive bacterial mutants have suggested that some of the yeast mutants may be deficient in systems of repair analogous to those studied extensively in bacteria (Brendel, Khan and Haynes, 1970; Kilbey and Smith, 1969). However, the variety of phenotypes exhibited by UV-sensitive yeast mutants suggest that causes of sensitivity exist which are unrelated to the repair of DNA damage. Indeed, in organisms with the complexity of eukaryotes, it would be surprising were this not the case. The present work was started as a conscious attempt to isolate mutants damaged in several cellular repair mechanisms. The rationale was simple: Mutations affecting one and the same system of repair are less likely to be additive in their effects than mutations affecting unrelated processes. Accordingly, rather than start with wild-type yeast, we chose a UV sensitive strain which has properties consistent with its lacking excision repair. Mutants with enhanced UV-sensitivity were isolated for study. Although these strains may, of course, affect processes of DNA repair other than excision, it is also possible that some may be sensitive by virtue of damage to systems unrelated to DNA repair.

This first paper deals with the phenotypic properties of two such hypersensitive strains. In a later paper we shall deal with the genetic analysis of their properties.

Materials and Methods

a) Strains ad-2.0. An adenine requiring strain with wild-type UV-sensitivity. *uvs-1.* This strain has been designated UV_1^s by Nakai and Matsumoto (1967) who first isolated it. It is prototrophic and sensitive to ultraviolet light.

b) Media. Suspensions were grown up in Glucose Nutrient Broth at $32°C$ until stationary phase was reached. Normally this was 48 hours but in the case of hyper-sensitive strains the slow rate of growth necessitated 5–6 days' incubation. Platings were done on Yeast Extract

Agar supplemented with 20 µg/ml adenine sulphate. Plates were incubated at 32°C for 4–7 days before counting depending on the strain being studied.

  c) *Mutation Induction and Detection.* A stationary phase culture of *uvs-1* was washed in 0.067 M KH_2PO_4 twice and treated with N-methyl-N-nitro-N-nitrosoguanidine (NTG) at a cell density of $2–3 \times 10^7$ cells/ml: 10 mg NTG were dissolved in 13.8 ml of sterile Phosphate Buffer at pH 7. 2 ml of the freshly prepared solution were added to 18 ml of cell suspension and shaken at 30°C for 30 minutes. Treatment was terminated by dilution into 0.067 M KH_2PO_4 before plating. Colonies growing after treatment were replicated twice onto two fresh plates of yeast extract agar plus adenine. One replica was exposed to 7 ergs of UV, a dose insufficient to kill *uvs-1* replicates. Colonies failing to form after irradiation and subsequent incubation were isolated using the non-irradiated replica or the master plate. These were retested and only those showing extra sensitivity were kept for further study.

  d) *UV-treatment and Photoreactivation.* Stirred samples were exposed to UV in open plastic petri-dishes. The source of UV in these experiments was a Philips TUV 15 watt medium pressure mercury vapour lamp. The dose rate used for wild-type yeast was 12 ergs/mm²/sec. For *uvs-1* it was 0.7 ergs/mm²/sec. For hyper-sensitive strains it was convenient to reduce the average dose rate to 0.07 erg/mm²/sec by means of a 36° sector. In practice this is a highly fractionated dose at a dose rate of 0.7 ergs/mm²/sec. Doses were monitored using a "Jagger Meter". Photoreactivating light was provided by a high pressure mercury arc enclosed in a glass envelope. Samples were exposed in glass bottles maintained at 30°C in a glass bottomed bath for one hour. Dark samples were maintained at the same temperature for a similar period.

  e) *Gamma Radiation.* The Gamma radiation source was a Cobalt 60 "Hot Spot". Samples of washed suspension (3.5 ml) were placed in a glass bottle through which oxygen was bubbled continuously. The dose rate was 8,500 Rad/min. 1 ml samples were withdrawn at various intervals and kept at 0°C before diluting and plating them.

Results

A. Isolation and UV Response of Hypersensitive Strains

A washed suspension of cells of *uvs-1* was treated with N.T.G. to give a survival of approximately 10%. 16,000 colonies arising when the treated suspension was plated were examined for their sensitivity to low doses of UV by the Replica Plating technique. Nine clones were finally isolated as being extremely UV sensitive.

The symbols used for these supersensitive strains are *uss* followed by the isolation number. In this report we shall be concerned exclusively with *uss-1* and *uss-6*.

Fig. 1 shows typical UV-response curves for *uss-1*, *uss-6*, and *uvs-1*. The response of the wild-type strain *ad-2.0* is also indicated for these doses.

From these and other experiments it is known that a shoulder exists on both the *uvs-1* and *ad-2.0* survival curves. In contrast, neither hypersensitive strain shows evidence of a shoulder; killing is exponential throughout the inactivation curve. For this reason the dose modifying factor relating the hypersensitive strains and *uvs-1* varies with dose. However where both curves are exponential, five times the UV dose is required to give the same survival levels in *uvs-1* as in the two hypersensitive strains. Both strains show a marked "tail" on the survival curve at about 0.01% survival. The reason for this is not known.

B. Sensitivity to Gamma Radiation

Fig. 2 shows the sensitivity of *uvs-1*, *uss-1* and *uss-6* to gamma radiation. Exposure to the radiation was done under aerobic conditions. All three strains exhibit
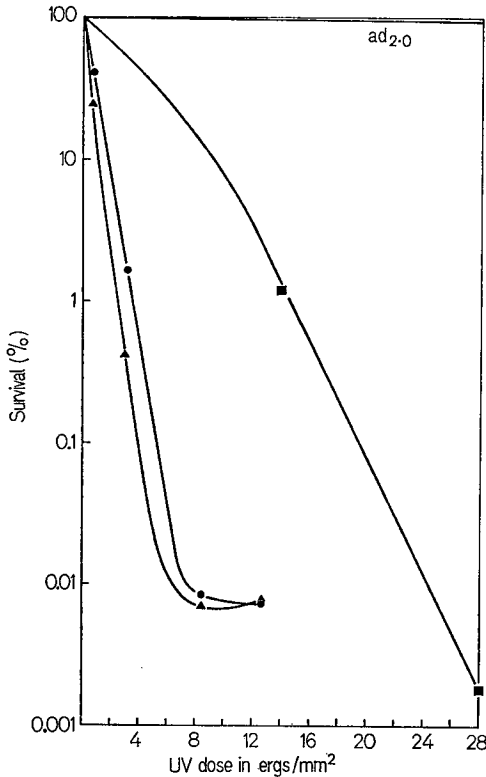
Fig. 1

Fig. 2

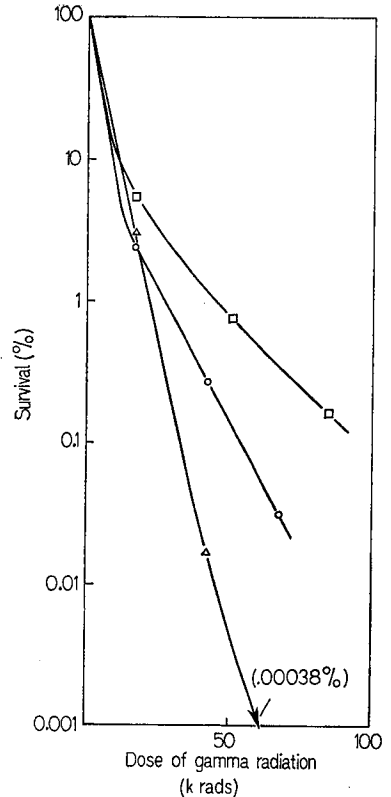Fig. 1. Response of *uvs-1* (■), *uss-1* (●), and *uss-6* (▲) to UV

Fig. 2. Response of *uvs-1* (□), *uss-1* (○), and *uss-6* (▲) to gamma radiation

similar gamma sensitivity at doses giving 10% survival. As the dose increases, *uvs-1* and *uss-1* become inactivated at ever decreasing rates. However even at these doses the rate of inactivation of *uss-1* is greater than for *uvs-1*. *uss-6*, in contrast, shows little change in the rate of inactivation over this dose range. Although not shown here, *ad-2.0* has a similar sensitivity to gamma radiation as *uvs-1*.

C. Photoreactivation and Light Sensitivity

We have studied the response of the hyper-UV-sensitive strains to photoreactivation in an attempt to obtain further information concerning the causes of the additional UV sensitivity.

If the photoreactivable sector for a sensitive mutant is significantly larger than that of the less sensitive parent material, it seems likely that the system of repair damaged in the mutant is capable of acting on pyrimidine dimers.

Photoreactivation using the standard conditions already described gave the results shown in Fig. 3a and b.
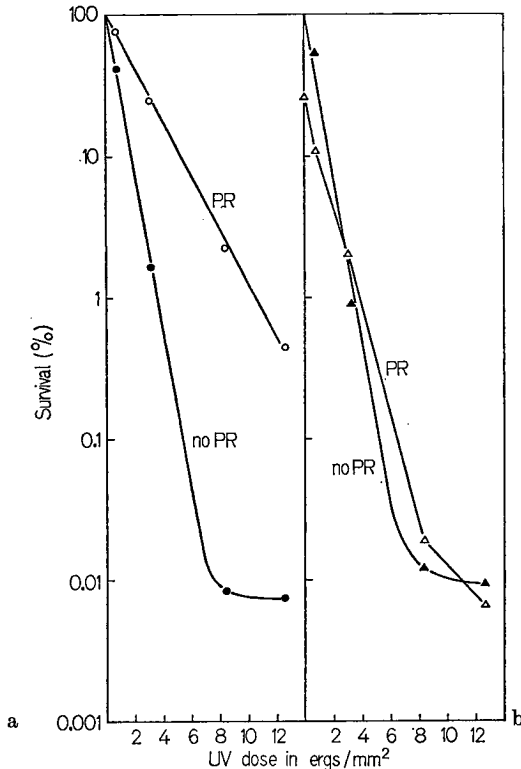
Fig. 3

Fig. 4

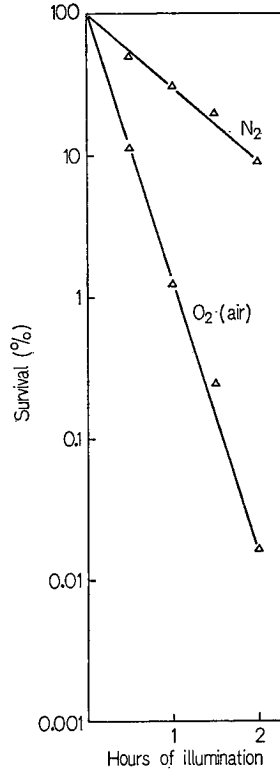Fig. 3a and b. Photoreactivation of *uss-1* (a), and *uss-6* (b)

Fig. 4. Light inactivation of *uss-6* in aerobic and anaerobic conditions

uss-1 displayed normal photoreactivation with a photoreactivable sector close to that of the parent strain (0.7). In contrast *uss-6* was apparently unable to photoreactivate UV-induced lethality. A common feature of the experiments with *uss-6*, however, was the reduction in survival of the control samples exposed to photoreactivating light. This suggested that the strain is sensitive to photo-reactivating light and that failure of photoreactivation is apparent rather than real, resulting from the opposing effects of reactivation and inactivation by the light. Fig. 4 shows the effects of visible light alone in *uss-6*. As expected, prolonged exposure rapidly inactivated the population. Inactivation was exponential. The results in Fig. 4 also demonstrate that the lethal effect of visible light can be modified by the oxygen tension during illumination. The strain is less light sensi-tive when kept anoxic. Although the dose modifying factor varied between experiments it was constant for all levels of survival within each experiment. The light wavelengths emitted by our photoreactivating source extend into the ultra-violet, and, although the light was filtered through three layers of glass before falling on the suspension, it seemed necessary to exclude any possibility that pyrimidine dimerisation was causing the lethal effect. Accordingly samples were
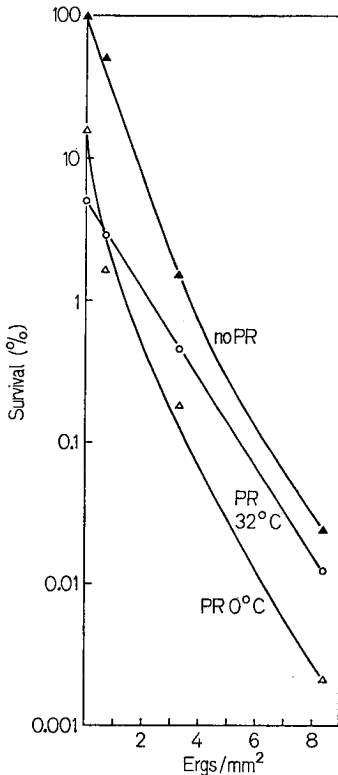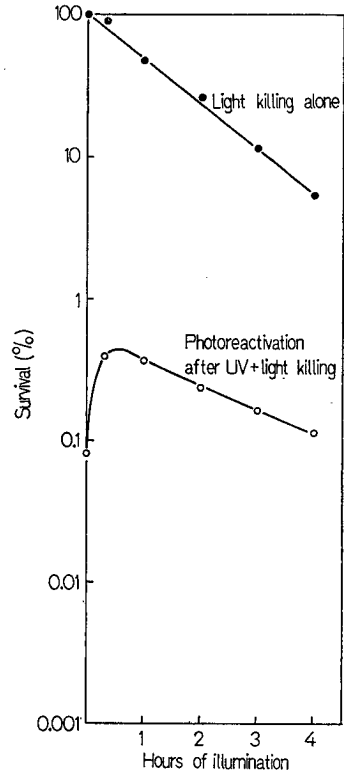
Fig. 5

Fig. 6

Fig. 5. Photoreactivation of *uss-6* at 0° and 32° C

Fig. 6. The time course of photoreactivation and light-induced killing in *uss-6*

exposed to light which had been passed through a filter excluding all wavelengths below 3,900 A. Inactivation occurred as before. It is unlikely, therefore, that pyrimidine dimers form a significant contribution to the lethal effect.

Two experiments were designed to demonstrate photoreactivation in *uss-6*. Both depend on the differential response of the two light effects to changes in physical conditions during photoreactivation.

a) Low Temperature Versus High Temperature of Photoreactivation. These experiments were made on the assumption that the lethal and reactivating effects of the light would have different temperature coefficients. At temperatures close to 0° C enzymatic photoreactivation should be severely curtailed and the lethal effect should predominate. At higher temperatures both processes should occur simultaneously. Samples were inactivated with a series of UV doses and exposed to photoreactivating light at either 2 or 32° C. The results are shown in Fig. 5.

At first sight the photoreactivation pattern obtained at 32° C with *uss-6* appears to be different from that shown in Fig. 3b. Closer inspection will show that the only difference lies in the extent to which the curve is displaced down the ordinate, in other words, the extent to which the cells are inactivated by the

photoreactivating light alone. As the UV dose increased the curves for inactivation with and without photoreactivation converged. At 2°C, as expected, the lethal effects of the photoreactivating light predominated and the curves ran parallel.

b) Time Course of Photoreactivation at Low Light Intensities. It is known that the photoreactivation conditions used routinely in our experiments are not limiting. The intensity of illumination can be considerably reduced without affecting the time taken to complete photoreactivation. In contrast cells of *uss-6* are killed rather slowly even at high light intensities. We therefore attempted a partial separation of the two light effects by lowering the intensity of the visible light, and following the course of photoreactivation with time. The results of a typical experiment are shown in Fig. 6.

Short exposures to photoreactivating light after UV inactivation do indeed increase the survival. A peak is reached after approximately one hour and thereafter a steady decline in viability ensues. This result clearly demonstrates that *uss-6* is capable of photoreactivation and that, subsequently, inactivation can cancel its effect completely. With the high intensity photoreactivating light we use routinely the two effects tend to cancel each other out.

D. Respiratory Competence

Growth tests have been made with glycerol as the sole carbon cource in the medium. Neither *uss-6* nor *uss-1* grow under these conditions. They are therefore respiratory deficient strains. In crosses to grande strains of yeast the deficiency disappears. They are therefore cytoplasmic petites. Loss of the petite character does not destroy the hyper-UV sensitivity nor the gamma sensitivity in the case of *uss-6*. The effects of the petite mutation on light sensitivity are complex and require further analysis.

Discussion

Two mutant strains have been obtained in Saccharomyces which show a considerable enhancement of UV sensitivity. Both are sensitive to gamma radiation although not to the same extent. Since they were both derived from an already UV-sensitive strain, *uvs-1*, they are at least double mutants and it may be expected that, in each strain, the two mutations affect different processes of repair. Genetic analyses at present being completed will provide further evidence on this point but it is already clear that in both strains, *uss-1* and *uss-6*, hyper-UV sensitivity behaves as if it is the result of a single second mutation. Sensitivity to gamma rays in *uss-6* also segregates as if it is the result of a second mutation although it is not yet certain that it is the same as that causing extra-UV sensitivity. These preliminary results do not permit us to decide whether the phenotypes observed are the product of an interaction between the *uvs-1* and the second mutations or whether their individual effects are simply additive. The effects of each mutation alone will form the subject of a further publication.

Gamma sensitivity differences between these strains develop only at higher doses of radiation. At a survival level of about 10% there is no detectable difference between them. However, as the dose increases the rates of inactivation fall. This is greatest for *uvs-1* and least for *uss-6* which continues to be inactivated at the same high rate over the whole dose range covered. *uss-1* is intermediate in

this respect. The appearance of resistant "tails" on the gamma-inactivation curves may indicate that the initial populations are heterogeneous with respect to their radiation sensitivity. However there is no evidence from these experiments that this is related to the cell stage reached at the time of irradiation. In contrast to their responses to gamma radiation, the two strains behave in a similar way towards UV. Here again there are "tails" on the inactivation curves. They occur at the same point on the survival curve for both mutants but, in contrast to the tails on the gamma inactivation curves they are very marked. In view of the prevailing uncertainty concerning the reasons for the appearance of a more radiation resistant fraction in yeast populations, further examination of these strains from this point of view might be worthwhile.

Besides being hyper-UV sensitive and sensitive to gamma radiation, *uss-6* is sensitive to visible light.

The emission spectrum of the mercury vapour lamp used for photoreactivation extends into the ultraviolet and it remained a possibility that at least part of the inactivation resulted from the formation of dimers between adjacent pyrimidine residues in the DNA. The fact that before the light reached the cells it passed through three layers of glass made this less likely and the finding that light of wavelengths in excess of 3,900 A also inactivated the suspension provided further support for the conclusion that dimerisation can only be a minor cause of lethality under the conditions described. A more probable cause of lethality is photodynamic inactivation. It has been shown that the lethal effect is considerably reduced by exposure under anoxic conditions, an observation which is consistant with a photo-oxidative step. A clearer picture may be obtained when the action spectrum for visible light-induced lethality is known. It is obvious, however, that the failure of this strain to photoreactivate UV damage is apparent and not real. By careful adjustment of the experimental conditions it is possible to separate the photoreactivating and inactivating effects of the light. It is therefore accidental that, under our normal experimental conditions, the two effects of the visible light tend to cancel each other out.

Elkind and Sutton (1957a, 1957b) described a petite strain of yeast which developed light sensitivity upon storage. Appearance of light sensitivity was dependent on the storage conditions. No attempt was made to determine the sensitivity of the strain to either UV or gamma radiation but it was shown that the sensitivity disappeared if the strain was irradiated under anoxic conditions.

The fact that *uss-6* is also petite in phenotype led us to examine its properties under similar conditions to those employed by Elkind and Sutton. In contrast to their findings, *uss-6* does not show enhanced light sensitivity if it is stored for several hours. Furthermore the lack of complete dependence upon oxygen for inactivation also suggests that the two strains are different. However, the fact that both *uss-6* and the strain studied by Elkind and Sutton are petites, raises the question of the involvement of the petite mutation in the expression of light sensitivity. At present our results reveal that, if any relationship exists, it is a complicated one. It is certain at this stage that petite-ness alone does not always result in light sensitivity since *uss-1* is light insensitive. On the other hand a petite has been isolated by acridine mutagenesis in *uvs-1* which has become

slightly light sensitive. It is hoped that further study will help to clarify the situation.

The experimental procedure described here provides the means of isolating yeast strains which are progressively more deficient in systems which reduce the effects of radiation. It is also apparent that, by this means, mutants may be obtained which might alone produce too small an effect on the phenotype for them to be detected in mutation experiments with wild-type strains. Provided genetic analysis can be accomplished at each stage in the process it should be possible to study the effects of each new mutation as it arises either alone or in combination with others. Just how far this can be pursued is not known, however it holds out the possibility of obtaining strains which have lost all their dispensable systems of repair. Such material should be of value in studying further the mechanisms of inactivation, mutagenesis and recombination.

Note added in proof: Because of a change in sensitivity in the UV-meter used, the dose rates given should all be multiplied by a factor of 4.

References

Brendel, M., Khan, N. A., Haynes, R. H.: Common steps in the repair of alkylation and radiation damage in yeast. Molec. Gen. Genetics **106**, 289–295 (1970).

Cox, B. S., Parry, J. M.: The isolation, genetics and survival characteristics of ultraviolet light-sensitive mutants in yeast. Mutat. Res. **6**, 37–55 (1968).

Elkind, M. M., Sutton, H.: Lethal effects of visible light in a mutant strain of haploid yeast I: General dependencies. Arch. Biochem. **72**, 84–95 (1957a).

— — Lethal effects of visible light in a mutant strain of haploid yeast II: Absorption and action spectra. Arch. Biochem. **72**, 96–111 (1957b).

Kilbey, B. J., Smith, S. M.: Similarities between a UV-sensitive mutant of yeast and bacterial mutants lacking excision-repair ability. Molec. Gen. Genetics **104**, 253–257 (1969).

Nakai, S., Matsumoto, S.: Two types of radiation sensitive mutants in yeast. Mutat. Res. **4**, 129–136 (1967).

Snow, R.: Mutants of yeast sensitive to ultraviolet light. J. Bact. **94**, 571–575 (1967).

Zimmerman, F. K.: Sensitivity to methylmethanesulphonate and nitrous acid of ultraviolet light-sensitive mutants in *Saccharomyces cerevisiae*. Molec. Gen. Genetics **102**, 247–256 (1968).

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