MUTAGENESIS BY CYTOSTATIC ALKYLATING AGENTS IN YEAST STRAINS OF DIFFERING REPAIR CAPACITIES

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Manuscript received July 11, 1978
Revised copy received October 31, 1978

ABSTRACT

Reversion of two nuclear ochre nonsense alleles and cell inactivation induced by mono-, bi-, and tri-functional alkylating agents and by UV has been investigated in stationary-phase haploid cells of yeast strains with differing capacities for DNA repair. The ability to survive alkylation damage is correlated with UV repair capacity, a UV-resistant and UV-mutable strain (*RAD REV*) being least and a UV-sensitive and UV-nonmutable strain (*rad1 rev3*) most sensitive. Mutagenicity of alkylating agents is highest in the former and is abolished in the latter strain. Deficiency in excision repair (*rad1 rad2*) or in the *RAD18* function does not lead to enhanced mutability. Mutagenesis by the various agents is characterized by a common pattern of induction of locus-specific revertants and suppressor mutants. Induction kinetics are mostly linear, but UV-induced reversion in the *RAD REV* strain follows higher-than-linear (probably "quadratic") kinetics. The alkylating agent cyclophosphamide, usually considered inactive without metabolic conversion, reduces colony-forming ability and induces revertants in a manner similar but not identical to the other chemicals tested. These findings are taken to support the concept of mutagenesis by misrepair after alkylation, which albeit sharing common features with the mechanism of UV-induced reversion, can be distinguished therefrom.

AN efficient repair system allows the simple eucaryote *Saccharomyces cerevisiae* to overcome DNA damage induced by a variety of external agents, such as radiation, heat and chemicals. Experimental information related to this phenomenon has been recently reviewed or summarized by HAYNES (1975), MOUSTACCHI (1976) and PRAKASH (1976a). About 50 genes are known to control sensitivity to these agents (GAME 1975; PRAKASH and PRAKASH 1977; EVANS and PARRY 1974). LEMONTT (1971a, 1973, 1977a), using two different procedures of isolation, has found mutants with decreased UV mutability. Only one gene has been associated with photoreactivation (RESNICK 1969), and most of these genes seem to be involved in other forms of DNA repair. By indirect evidence, these repair activities have been structured into three pathways (BRENDEL and HAYNES 1973; GAME and COX 1973), some steps of which are already characterized at the molecular level: excision repair (UNRAU, WHEATCROFT and COX 1971; PRAKASH 1975, 1977a,b); repair of double- or single-strand breaks (RES-

NICK and MARTIN 1976; JACHYMCZYK *et al.* 1977); and apurinic site endonuclease (CHLEBOWITZ and JACHYMCZYK 1977).

The dependence of induced mutation on activities of the DNA repair system has been exemplified by LAWRENCE and CHRISTENSEN (1976): UV-induced reversion of *cyc1* alleles is controlled by *REV3*, *RAD6* and partially by *RAD18* and others. These gene functions are thought to belong to a repair pathway functionally comparable to the "REC" repair system in *E. coli* (HAYNES 1975). A similar conclusion, *i.e.*, dependence of mutability on a kind of "error-prone" repair (WITKIN 1967), has been arrived at by PRAKASH (1976b), using a series of chemical mutagens.

It seems that this body of knowledge not only justifies further research into yeast DNA repair, but also that this system may be useful as a tool for studying mechanisms of mutagenesis by genetically active agents. One class of such agents is comprised of alkylating chemicals, especially those with more than one functional group. In addition to their historic role in basic mutation research (AUERBACH and ROBSON 1947), their medical use as cytostatic drugs (*e.g.*, CARTER, BAKOWSKI and HELLMAN 1977) necessitates continued investigation on their interactions with biological systems. Biochemically analyzed reaction products of these agents include "functionally tolerable" DNA monoadducts, as well as DNA intra- and interstrand crosslinks, which may have great impact on cell survival (LAWLEY *et al.* 1969). Both mutagenesis and the formation of interstrand crosslinks in yeast DNA by bi- and tri-functional alkylating agents have recently been demonstrated in the same dose ranges (RUHLAND, FLEER and BRENDEL 1978). This report describes a study in greater depth of mutation induction by nitrogen mustard and its analogues that are or have been in medical use. UV reaction products in DNA (pyrimidine dimers) may also be seen as intrastrand crosslinks. Accordingly, similarities in the repair of UV and bifunctional alkylation damage have been noted in yeast (BRENDEL, KHAN and HAYNES 1970). Our study, therefore, centered on the following questions: (1) to what extent is the mutagenic effect of the above agents influenced by defects in excision repair or in the *RAD6 REV3 RAD18* pathway? (2) what can be said about mechanism of action, influence of functionality and similarity of UV and alkylation mutagenesis? and (3) in general, what can be learned about DNA repair and mechanisms of mutation by measurement of biological endpoints?

MATERIALS AND METHODS

Media and growth conditions: YEPD: see BRENDEL and HAYNES (1973). Synthetic complete medium (SC): 0.67% yeast nitrogen base without amino acids (DIFCO), 2% glucose, 2% agar, 20 μg per ml each of adenine, uracil, arginine, methionine and histidine, and 30 μg per ml each of leucine and lysine. Omission media lacked histidine or lysine. The incubation temperature was 30°.

Precautions were taken to avoid artifacts due to crowding on plates (GRIGG 1952) and related effects: (a) Colony formation by prototrophic cells on the omission media is not influenced by the presence of up to 8×10^7 nongrowing auxotrophic cells per plate. Actual cell numbers per plate were always lower. (b) No plates with more than 200 visible colonies were

used for quanitative determinations. Similarly, no replica plates (see below) giving rise to more than approximately 50 colonies were taken into account.

Yeast strains: Haploid strains of *S. cerevisiae* of similar genetic background were used.

MB1114–5D, **a** *RAD REV his5–2 lys1–1 arg4–17 met1–1 tum2* (*tum* marker: BRENDEL 1976).
MB1113–8C, **a** *rad1–18 rad2–16 REV his5–2 lys1–1 ade2–1 leu1–12.*
MB1114–16D, *α rad18–2 REV his5–2 lys1–1 arg4–17 leu1–12 ura1.*
MB1054–5A, **a** *rad1–18 rev3–1 his5–2 lys1–1 arg4–17 ade2–1 leu1–12 met1–1.*

Of these, MB1114–5D is a fully repair-proficient wild type; MB1114–16D a UV- and X-ray-sensitive mutant (BRENDEL and HAYNES 1973) epistatic to *rad6* (HAYNES 1975); MB1113–8C a mutant deficient in excision repair [for a discussion of these and related mutants see PRAKASH (1976a, 1977a,b) and REYNOLDS (1978)]; and MB1054–5A an excision-negative and UV-nonmutable strain (LEMONTT 1973). While the last is a double mutant blocked in two different pathways, the combination of the epistatic mutations *rad1–18* and *rad2–16* in MB1113–8C does not increase the radiation sensitivity above the level of the single mutants except for the abolition of a slight shoulder in the survival curve (loss of leakiness).

Mutational system: To study well-defined mutational events, the following procedure was used. After mutagen treatment, survival (on SC, after three days of incubation) and numbers of phenotypically HIS- or LYS-prototrophic colonies (on appropriate omission media, after five days of incubation) were scored. Mutant colonies were replicated onto the corresponding omission media (*i.e.*, HIS prototrophs onto SC-Lys and *vice versa*); the fraction of colonies able to grow on both types of omission media was determined after one to two days of incubation.

Both *his5–2* and *lys1–1* are suppressible UAA (ochre) nonsense mutants (HAWTHORNE and LEUPOLD 1974). The probability of simultaneous reversion can be expected to be extremely low ($< 10^{-8}$). Therefore, doubly prototrophic mutants induced in a *his5–2 lys1–1* strain are, with high probability, locus nonspecific intergenic revertants (suppressors). After correcting for this class of mutants, a relatively homogenous population of "true" locus-specific revertants can be examined. For brevity, the two types of revertants will be referred to as "suppressors" and "locus revertants," respectively. The mutational system described here is essentially the same as that used in reversion experiments by LEMONTT (1977a) and ECKARDT, KOWALSKI and LASKOWSKI (1975).

Mutagen treatment: Cells were grown for five days on solid YEPD, harvested, washed with phosphate buffer (0.067 M, pH7), stored for approximately 20 hr at 4°, washed again and resuspended at 4×10^8 (UV experiments: 2×10^8) cells per ml. The fraction of budding cells was always less than 10%, with the exception of MB1114–16D *rad18–2*.

Cell suspensions were UV-irradiated under constant agitation by an OSRAM HNS12 source (intensity maximum at 254 nm) with an output of 1.5 J m^{-2} sec^{-1}. All manipulations were carried out at room temperature in yellow light. Due to the shading effect, dose-response curves are not to be compared directly with values obtained at lower cell densities.

Cells were treated for a constant time (two hr if not noted otherwise) with different concentrations of chemicals at 36° (shaker water bath) and pH7 (0.067 M phosphate buffer). At the end of the exposure period, suspensions were mixed with an equal volume of 4% sodium thiosulfate. Mutagen solutions were prepared by suspending the required agent in ice-cold phosphate buffer and were added to cell suspensions (prewarmed to 36°) by means of cooled pipettes. These measures were aimed at diminishing the loss of alkylating activity due to premature hydrolysis (A. RUHLAND, unpublished data) and thereby increasing reproducibility by stabilizing the initial experimental conditions.

Mutagens used: Nitrogen mustard (HN2): 2,2′-Dichloro-N-methyl-diethylamine hydrochloride; chlorambucil: 4-(p((Bis(2-chloroethyl)amino)) phenyl)butyric acid; triaziquone: 2,3,5-Tris(1-aziridinyl)p-benzoquinone; nitrogen half mustard (HN1): 2-Dimethylaminoethylchloride; and cyclophosphamide: 2-(Bis(2-chloroethyl)amino)tetrahydro-2H-, 1,3,2-oxazaphosphorine 2-oxide.

HN2, chlorambucil and cyclophosphamide are bifunctional agents; triaziquone is trifunctional, HN1 monofunctional. Triaziquone and chlorambucil were dissolved in ice-cold acetone before suspending in phosphate buffer. Cyclophosphamide was dissolved at room temperature.

RESULTS

Our reasoning in the choice of strains used in the present study was that genetic effects should be tested in: (1) wild-type (*RAD REV*), *i.e.*, fully repair-proficient cells; (2) cells blocked in excision repair (*rad1 rad2 REV*), which are therefore unable to remove UV-induced pyrimidine dimers; (3) cells nearly nonmutable by UV (*rev3*), which are simultaneously blocked in excision repair (*rad1*) (the latter block probably forces many lesions into the presumably error-prone pathway controlled by genes *rad6* and *rev3*); and (4) cells harboring the *rad18* mutation which, although functionally related to *rev3* and *rad6* (HAYNES 1975), does not behave in the same way with respect to mutation (see results shown below).

In all strains, repair depending on genes *RAD50* to *RAD57* was fully operable. These genes are thought not to be involved in error-prone repair (HAYNES 1975).

To reduce inconsistencies brought about by population inhomogeneity (budding cells, etc.), mutagen doses were chosen in such a range that cell survival did not fall substantially below 10%. Mutagenic activities of mono-, bi-, and tri-functional agents and of UV in the various strains are described in the following sections. Cyclophosphamide activity has special characteristics and will be described separately.

Inactivation and locus reversion: Efficiency of cell killing by UV and alkylating agents was clearly dependent on *rad* and *rev* markers (Figure 1). In general, the *RAD REV* strain had the highest survival probability, the doubly mutant *rad1 rev3* strain the lowest (except for HN1 and HN2 acting on MB1114–16D *rad18*). Most survival curves were of the single-hit type often found with bifunctional agents (HAYNES and INCH 1963, BRENDEL and HAYNES 1973). The monofunctional HN1 led to inactivation curves with "shoulders," at least in wild-type and excision-deficient cells. The UV-inactivation curve in the *RAD REV* strain can be interpreted as the beginning of an extensive shoulder, which may be due to dose-dependent diminution of repair capacity (WHEATCROFT, COX and HAYNES 1975). Relative sensitivities of strains to the various agents can be quantified by comparing doses needed to produce the first lethal hit, *viz.*, the LD_{37} ratio of wild type to mutant (Table 1). UV and HN2 sensitivities are comparable to values obtained by BRENDEL and HAYNES (1973) with similar strains. The high sensitivity of the *rad18* strain to HN1 is in agreement with results of these authors describing the activity of the similarly monofunctional agent methyl methanesulfonate. Differences in sensitivity between wild type and the excision-deficient strain are greatest for UV.

Effective concentration ranges of alkylating agents (see Table 1 and Figure 2) varied from 5×10^{-8} M triaziquone to 5×10^{-2} M HN1 in the wild type and from 1.9×10^{-9} M triaziquone to 3.6×10^{-3} M HN1 in the most sensitive strain, *rad1 rev3*. Thus, HN2 was about four orders of magnitude less potent than triaziquone, with the other agents being even less effective.

Induction of locus-specific *HIS5* revertants is depicted in Figure 1. While all *REV* strains were mutable to different degrees, no mutation induction could be

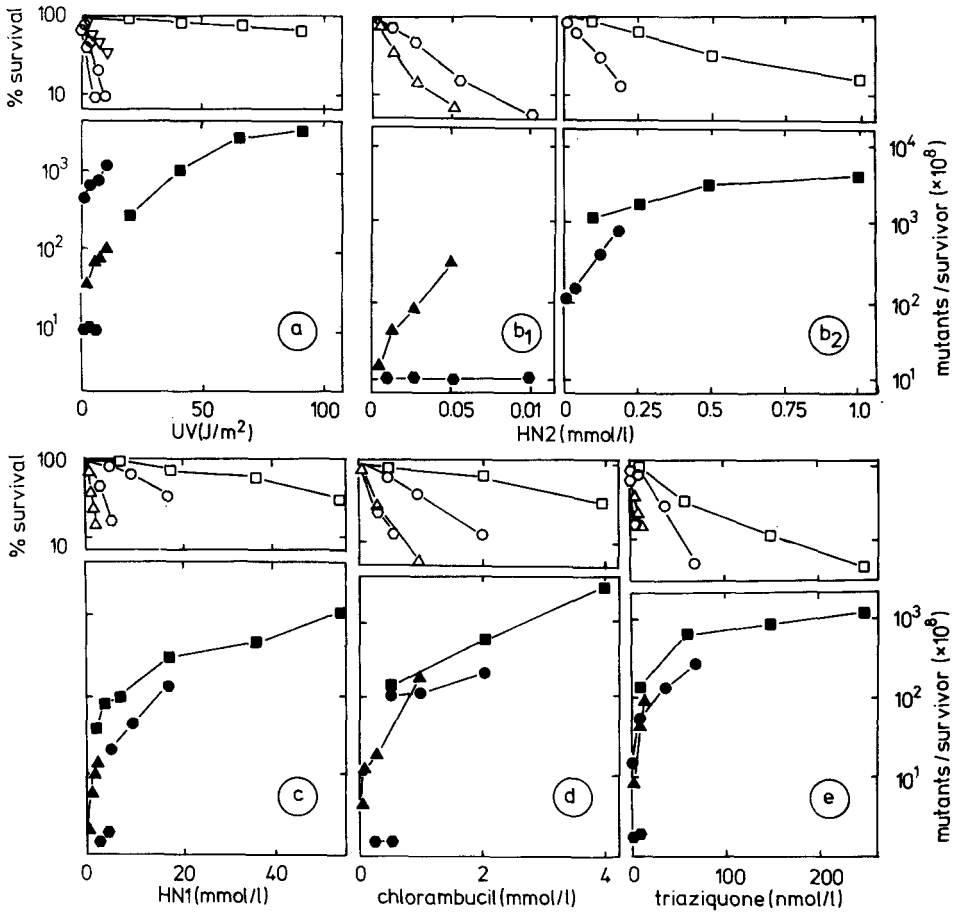

FIGURE 1.—Inactivation of colony-forming ability (open symbols) and mutation induction (filled symbols) in *RAD REV* (squares), *rad1 red2 REV* (circles), *rad18 REV* (triangles) and *rad1 rev3* (hexagons) by UV (a), HN2 (b), HN1 (c), chlorambucil (d) and triaziquone (e). Mutant frequencies are those of locus-specific *HIS5* revertants, corrected for spontaneous backgrounds.

detected in strain MB1054–5A *rad1 rev3*; a small number of locus-specific and nonspecific revertants, however, have been found in this strain to occur both spontaneously and after mutagen treatment. Mutant frequencies for this strain (Figure 1) should therefore be regarded as upper limits only.

If the mutational behavior of strain MB1114–5D (*RAD REV*) is set as standard, the induction curve in strain MB1114–16D (*rad18*) can be seen formally as the extrapolation of the wild-type curve to lower mutant frequencies. The only exception is the low mutability of this strain by HN1. The relation between frequencies of induced mutants in the excision-negative strain MB1113–8C (*rad1 rad2*) to those in MB1114–5D (*RAD REV*) depended on the nature of the mutagen: at equal doses of UV irradiation, the strain carrying *rad1 rad2* alleles

TABLE 1

Lethal and mutagenic activities of UV and alkylating agents

Strain	UV	HN2	Chlorambucil	Triaziquone	HN1
RAD REV	* 160	5.4×10^{-4}	3.7×10^{-3}	5.5×10^{-8}	5.0×10^{-2}
	‡ 1×10^{-4}‖	2.7×10^{-5}	2.0×10^{-5}	5.8×10^{-6}	8.6×10^{-6}
	§ 3.8×10^{-5}	2.0×10^{-6}	1.7×10^{-5}	1.6×10^{-6}	2.4×10^{-6}
rad1 rad2 REV	*†5.5(29)	9.7×10^{-5}(5.7)	1.1×10^{-3}(3.4)	3.0×10^{-8}(1.8)	1.7×10^{-2}(2.9)
	‡ 7.5×10^{-6}	3.7×10^{-6}	1.5×10^{-6}	1.0×10^{-6}	1.2×10^{-6}
	§ 8.0×10^{-7}	2.5×10^{-7}	4.5×10^{-7}	5.0×10^{-7}	5.0×10^{-7}
rad18 REV	*†9.5(17)	1.3×10^{-6}(430)	3.0×10^{-4}(12.3)	6.5×10^{-9}(8.5)	1.2×10^{-3}(41.6)
	‡ 8.5×10^{-7}	6.3×10^{-7}	2.0×10^{-7}	6.5×10^{-8}	6.0×10^{-8}
	§ 4.4×10^{-7}	1.6×10^{-7}	1.1×10^{-7}	2.0×10^{-8}¶	2.0×10^{-8}¶
rad1 rev3	*†2.5(64)	2.9×10^{-6}(193)	2.5×10^{-4}(15)	1.9×10^{-9}(28)	3.6×10^{-3}(13.8)
	‡ 1×10^{-7}¶	1×10^{-7}¶	2.0×10^{-8}¶	2.0×10^{-8}¶	2.0×10^{-8}¶
	§ 1.0×10^{-7}¶	1.0×10^{-7}¶	2.0×10^{-8}¶	2.0×10^{-8}¶	2.0×10^{-8}¶

* Dose producing 37% survival (J/m^2 for UV and molarity for alkylating agents).
† Wild-type/mutant LD_{37} ratio.
‡ Frequency of locus-specific *his5-2* → *HIS* revertants per survivor at 37% survival.
§ As ‡, but for *lys1-1* → *LYS* reversion.
‖ Value derived from extrapolation of data for survival above 37%.
¶ Upper limit of mutant frequency.

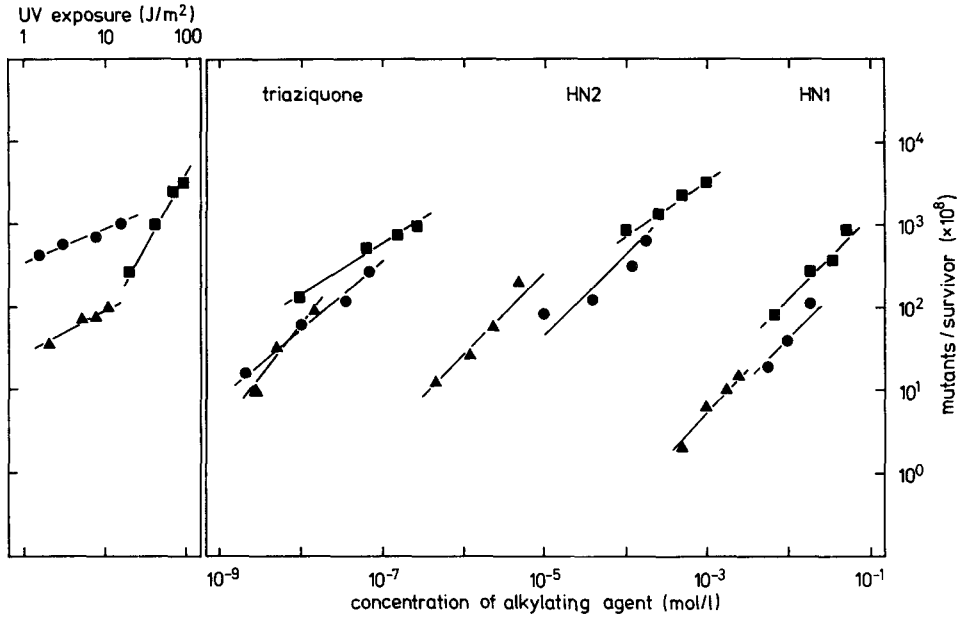

FIGURE 2.—Kinetics of mutation induction by UV and by tri-, bi- and monofunctional alkylating agents. Doubly logarithmic transformation, symbols as in Figure 1. In all cases straight lines were used for interpolation, irrespective of possible nonlinearity of some of the curves in a double-log plot. Lines were fitted by eye.

was more mutable than the wild type, while this was not the case at equal doses of chemical mutagens (Figure 1). When mutability of strains differing in repair capacity is compared at equal survival (Table 1), it is evident that wild-type cells always have the highest probability to mutate. This is also true for UV action.

Locus specificity: Two comparisons of *his5–2* reversion can be made: (1) with the mutational response of the analogous ochre mutant *lys1–1*, and (2) with the mutability of genes that control the suppressor character, presumably tRNA genes comprising class I and II suppressors (HAWTHORNE and LEUPOLD 1974). As for the first, a remarkable consistency was observed: in all cases *lys1–1* reversion paralleled that of *his5–2*, though at a lower level, *i.e.*, the frequency of *LYS1* revertants was always smaller by a factor that depended on strain and mutagen, but was constant for a given constellation. *LYS1* data have therefore been omitted from Figure 1; relevant information may be obtained from Table 1, where mutant frequencies are compared at 37% survival. *HIS5/LYS1* ratios were largest for HN2 in strain MB1114–5D *RAD REV* (ratio of mutant frequencies about 10) and lowest for chloroambucil in the same strain (ratio < 1.5). While the two ochre sites behaved qualitatively in the same way, no clear induction of supressor mutants was found (data not shown). Suppressor frequencies exceeded the spontaneous values only marginally; low suppressor mutability is

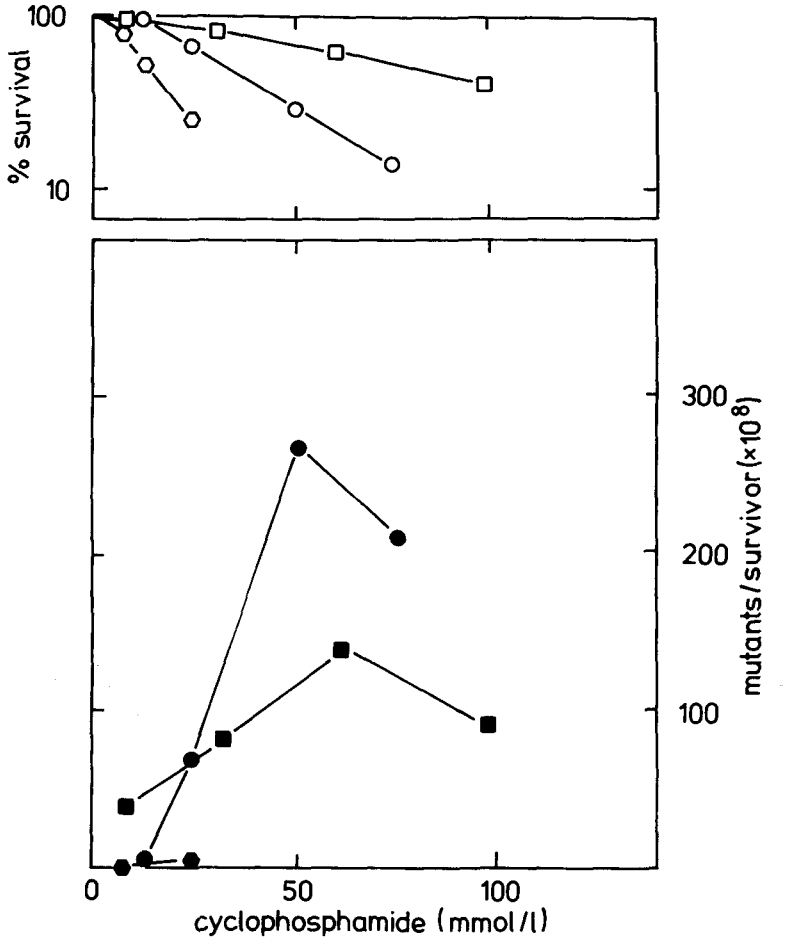

FIGURE 3.—Inactivation of colony-forming ability and mutation induction by cyclophosphamide. Symbols as in Figure 1. Mutant frequencies are depicted on a linear scale.

the more interesting as in all strains the spontaneous mutant population consisted of 90% or more of suppressors.

Kinetics of mutation induction: A double logarithmic transformation of mutation data, *i.e.*, log of mutant frequency *versus* log of mutagen exposure or concentration, is shown in Figure 2. While linear induction kinetics (dose proportionality) after such a transformation will lead to a line with a slope of 1, mutation induction proportional to higher or lesser powers of the dose will produce lines with accordingly changed slopes. Most of the curves in Figure 2 are similar (approximately linear kinetics). The single exception is UV mutagenesis in the *RAD REV* wild-type; the slope of the curve is definitely not 1 and is probably 2 ("quadratic" kinetics). This indicates (1) that mutation induction by UV in the wild type is different from mutagenesis in strains defective in DNA dark repair and also from mutagenesis by other agents, and (2) that there is no detectable difference caused by functionality of the alkylating agent.

Cyclophosphamide: This drug, while in principle a bifunctional agent of the HN2 type, is biologically inactive in its original form and must be converted to active reaction products (MONTGOMERY and STRUCK 1973). Genetic effects of cyclophosphamide in various systems have been reviewed by MOHN and ELLENBERGER (1976), who maintain that no genetic activity without prior activation has been reported in microbial systems. Recently, MAYER, HYBNER and BRUSICK (1976) could, without recourse to metabolic activation, demonstrate forward mutation and mitotic recombination induced by cyclophosphamide in some yeast strains, as well as "activation" of the drug by incubation in buffer. To detect effects of cyclophosphamide in our system, it was necessary to extend the treatment period; 24 hr was chosen as the standard time of reaction. The amount of cell killing was dependent on the *rad* and the *rev* genotypes of the strains, thereby indicating the involvement of repair functions and, implicitly, the role of DNA lesions as a cause of cell death (Figure 3).

As for mutagenesis, in this report we wish to state one point only, *viz.*, that, at least in our yeast system, the "nonactivated" drug is able to induce defined nuclear mutations in both wild-type and excision-deficient cells. Similar to other mutagens studied, it is not mutagenic in *rad1 rev3 cells*. The increase in mutant frequency per survivor, albeit small, cannot be solely due to selection of preexisting mutants since the mutant frequency per treated cell is clearly higher than the spontaneous values [9×10^{-7} for *HIS5* revertants of MB1114–5D (*RAD REV*) at a concentration of 6×10^{-2} M compared to 1×10^{-8} without mutagen treatment]. It should be noted that cyclophosphamide activity seems to be especially dependent on extrinsic factors such as certain properties of tester strains (MAYER, HYBNER and BRUSICK 1976) and conditions of treatment.

DISCUSSION

The ability of haploid stationary-phase cells of yeast to survive treatment by alkylating agents of different functionality is clearly dependent on the integrity of several genes involved in DNA dark repair: for each agent tested the *RAD REV strain* (wild-type; no block in known repair pathways) had the highest survival probability (highest LD_{37}, see Table 1) followed by the *rad1 rad2 REV* strain and—with exceptions to be discussed below—the *rad18 REV* strain (both strains are assumed to have at least two repair pathways operable); survival probability was lowest in the double mutant *rad1 rev3* in which two pathways of DNA dark repair are presumably inoperative (LEMONTT 1971b). HN2, chlorambucil and triaziquone, *i.e.*, bi- or trifunctional agents, exhibited single-hit inactivation kinetics (no detectable "shoulder") in all strains, indicating that one unrepaired lesion induced by these agents is sufficient to inactivate colony formation. Single-hit inactivation by bifunctional agents has also been found in procaryotic and viral systems (LAWLEY and BROOKS 1968; BRAKIER and VERLY 1970). Similarly, the shape of survival curves generated by UV irradiation in *rad* strains (Figure 1a) can be attributed to single-hit killing; the curve for the *RAD REV* strain represents the beginning of a large "shoulder," the form of

which has been explained by dose-dependent inactivation of repair (WHEATCROFT, COX and HAYNES 1975).

Like the chemicals noted above, cyclophosphamide has two groups capable of alkylating reactions; the slight shoulders found with this agent (Figure 3) may either be due to predominant monofunctional alkylations or may be connected with the proposed "metabolic activation" of the drug.

Treatment with the monofunctional HN1 likewise leads to somewhat "shouldered" inactivation curves (Figure 1); this may reflect a limited tolerance of the cell to monofunctional alkylation (PRAKASH and STRAUSS 1970).

For any given strain—irrespective of repair capacity—LD_{37} values increase in the order triaziquone, HN2, chlorambucil and HN1, *i.e.*, the efficiency ranking of agents is trifunctional > bifunctional > monofunctional. However, in view of the absolute values, spanning six orders of magnitude, it must be doubted whether these differences can be explained solely by the number of reactive groups per molecule. Other parameters such as solubility, permeability and stability in aqueous solution may have a great influence on activity; this illustrates the difficulty in deducing valid dose-effect relationships without additional biochemical data (see below).

When LD_{37} values of the wild type (*RAD REV*) and the excision-deficient strain (*rad1 rad2 REV*) are compared for all agents tested, survival differs most markedly after UV irradiation (Table 1). Excision repair, therefore, seems much better at handling UV- than alkylating agent-induced DNA lesions.

Of the alkylating agents, HN2 has the highest (wild type/excision deficient) LD_{37} ratio, and thus DNA lesions produced by it may be the most strongly affected by repair. Similarities of cellular response after UV and HN2 treatment of different yeast strains have led BRENDEL and HAYNES (1970) to conclude that repair of DNA lesions induced by these agents may share some common step(s). One aim of the present work was to see whether this also holds true for mutation induction.

The most general statement to be made about mutability is that, while all agents tested induced revertants in the wild type (*RAD REV*) in a dose-dependent manner (Figures 1 and 3), no such induction could be found in the double mutant harboring the *rad1 rev3* alleles. Since *REV3* is known to be involved in repair leading to UV-induced mutation (LEMONTT 1971a), failure to mutate in *rev3* strains may be due to blocked error-prone repair; thus the mutagenicity of all agents tested (including cyclophosphamide) may be due to misrepair mechanism(s). This underlines the finding of PRAKISH (1976b) that yeast requires a functional repair system for mutation induction by several chemicals. One counter-example may be hydrazine-induced mutation, whose division dependence suggests a mispairing mechanism (LEMONTT 1977b).

The *rev3* marker was initially isolated as UV nonmutable. Its involvement in mutagenesis induced by the alkylating agents tested supports the conclusion of BRENDEL, KHAN and HAYNES (1970) that repair of damage induced by UV and some alkylating agents has at least one step in common. However, the fol-

lowing facts reveal that there are also differences in parts of the mutational process:

(1) Induction of mutation by UV and by alkylating agents in the wild type (*RAD REV*) follows different kinetics (Figure 2). Reversion exhibiting approximately quadratic kinetics is usually thought to depend on the interaction of two independent events (see WITKINS 1975). Another explanation based on the assumption of differenial sensitivities of mutant and nonmutant cells, has been proposed by ECKARDT and HAYNES (1977). If the first hypothesis is correct, this implies a requirement of two hits for UV- but only one for alkylation-induced mutagenesis. The *rad* mutants did not exhibit quadratic kinetics (Figure 2). At higher UV doses than used here, mutation induction in the excision-deficient mutant *rad2* also followed nonlinear kinetics (ECKARDT and HAYNES 1977).

(2) A further discrepancy can be found by comparison of mutant frequencies between strains. If this is done on the basis of comparisons at equal doses (Figure 1), all *rad* strains (including the *rad1 rad2 REV* double mutant) are less mutable than the wild type (*RAD REV*): only UV irradiation leads to higher mutant frequencies in the excision-deficient strain (Figure 1a). This UV hypermutability is usually interpreted as the effect of increased activity of error-prone repair after blocking of (relatively error-proof) excision repair. Since there is no comparable hypermutability of the excision-deficient strain after treating with alkylating agents, cellular processing of lesions induced by UV or alkylation might follow different patterns. PRAKASH (1976b) has found that, in contrast to the UV-mimetic nitroquinoline oxide, EMS and HNO_2 do not trigger hypermutability in excision-deficient yeast. Similarly, KONDO *et al.* (1970) have shown that the cross-linking agent mitomycin C is mutagenic in a wild-type strain of *E. coli* but not in *uvrA*$^-$ strain that is extremely mutable by UV.

If mutability of the different strains is compared at equal survivals (Table 1), the picture is changed insofar as neither UV nor alkylating agents enhance mutant frequencies in any *rad* mutant over that of the wild type (*RAD REV*). In other words, the probability of mutation by treatment leading to a defined amount of cell killing is highest in the wild-type strain and not in a *rad* mutant. ECKARDT, KOWALSKI and LASOWSKI (1975) have shown that this is true for UV mutagenesis if survival is not lower than about 1%. The finding of UV hypermutability, therefore, depends on the methods chosen for interpretation of data, a problem recently discussed by SMITH (1976).

Comparison of the mutagenicity of the various agents (Table 1) shows that at equal levels of lethality the efficiency of mutation induction varies: UV irradiation is usually most effective, followed by HN2, chlorambucil, triaziquone and HN1. This can be most easily explained if it is assumed that these agents produce premutagenic and (pre)lethal damage in different ratios. In our case the nature of these kinds of damage is not known. Recently, SEKI, NOZU and KONDO (1978) could show that crosslinks induced in *E. coli* by psoralen plus light are the major cause of lethality, whereas monoadducts are premutational lesions.

The distinction between different types of lesions may also play a role in the two cases of mutagen specificity observed:

(1) The ratio of reversion frequency of *his5-2* to *lys1-1* is always > 1 (*cf.*, Table 1). *A priori*, there is no reason to expect different chances of colony formation for the respective mutants. Experiments aimed at the detection of possible influences of medium composition (lysine-arginine antagonism) did not reveal such an effect (data not shown). Possible mechanisms of mutagen specificity (AUERBACH 1969, KAPLAN and STOYE 1974) include (a) differential reactivity of mutagen towards different sections of the genome, (b) differing physiological states of genes (e.g., transcribed or non-transcribed), (c) different possibilities and mechanisms of repair, and (d) different chances for expression of mutated genes. In *Saccharomyces cerevisiae*, SHERMAN and STEWART (1972) have shown that mutability of ochre codons is dependent on the position of the codon in a cistron, the position of a base pair within a codon and the repair mechanism(s) (LAWRENCE *et al.* 1974). It is unlikely that the observed mutagen specificity is due to unequal reactivity of mutagen and mutational sites, since both UV and alkylating agents show at least qualitatively the same reversion pattern, in spite of very different modes of penetration into the cell and reaction with DNA. Furthermore, the physiological states of the *his5* and *lys1* genes in stationary-phase cells probably do not differ. This leads to the assumption that if expression probabilities are equal, the observed reversion spectrum may be related to nucleotide sequence-specific activity of repair (LAWRENCE and CHRISTENSEN 1975).

(2) Numbers of induced suppressor mutants detected by the plate assay were generally small in relation to the number of "locus" revertants, even though most spontaneous mutants are suppressors. Similar findings have been reported by LEMONTT (1971a) and ECKARDT, KOWALSKI and LASKOWSKI (1975). Due to the many possible mutation sites (see discussion of suppressor systems in yeast by HAWTHORNE and LEUPOLD 1974), induction of suppressors resembles a forward mutation process. Therefore, it is doubtful if low frequencies of induced suppressors found after mutagen application can be explained by low inducibility. One may suggest that suppressor mutants are induced by the various mutagens, but are not detected in adequate frequencies in the selective system employed. The dependence of the recovery of nonsense suppressors on at least one extrinsic factor, the growth medium, is already well documented in yeast (QUEIROZ 1973).

Taken together, these findings give a hint as to the extent to which the effects of DNA-mutagen reactions can be modified by genetical or physiological factors. This is exemplified by some special characteristics of the *rad18 REV* strain. The *RAD18* gene has been assigned by genetic analysis to a repair pathway, together with *REV3* and *RAD6*. It is involved in repair of both UV- and X-ray-induced lethal damage, as well as in UV-induced mutagenesis (LAWRENCE and CHRISTENSEN 1976) and in recombinational events (BORAM and ROMAN 1976). Our results show that a *rad18 REV* strain is mutable by bi- and trifunctional alkylating agents and by UV, indicating that the *RAD18* function is not identical with the error-prone activity. The monofunctional HN1 is only slightly mutagenic in this strain, but has a high wild type-mutant LD_{37} ratio (Table 1). This

is most easily explained if it is assumed that HN1-induced lesions are not well repaired in cells harboring the *rad18* allele (low mutagenicity due to lack of misrepair). High toxicity (BRENDEL and HAYNES 1973) and low mutability (A. RUHLAND, unpublished data) have also been observed with methyl methanesulfonate.

The LD_{37} ratio of HN2 is somewhat extreme and contrasts with earlier findings by BRENDEL and HAYNES (1973). Part of this discrepancy can be attributed to different treatment conditions: 36° *versus* 30° and preparation of alkylating agent (see MATERIAL AND METHODS). Nevertheless, the interaction of the *rad18* mutation and HN2 (two different commercial products were tested) seems to be quite specific. In view of the fact that a single strain may show aberrant behavior (LAWRENCE and CHRISTENSEN 1976), we tested several *rad18* strains, including strains not constructed in our laboratory. Results were similar to those described here. On the other hand, the HN2 analog bis(2-chloroethyl)sulfate (sulphur mustard) has a wild type/*rad18* LD_{37} ratio of about 10 (A. RUHLAND, unpublished data), similar to the other alkylating agents in this strain (Table 1).

In conclusion we should like to state that the investigations presented here show some of the merits, as well as the pitfalls, of mutation experiments based solely on the detection of biological endpoints. Measurement of mutagen dose at the cellular level and of mutagen-DNA reaction products should facilitate the interpretation of the mutation process.

We wish to thank Ms. G. POLZER for drawing the figures. This research was supported by a grant from the Deutsche Forschungsgemeinschaft.

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Corresponding editor: J. W. DRAKE