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Induction of Respiratory Deficiency in Yeast by Ultraviolet Irradiation

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ABSTRACT The present investigation was carried out to elucidate the nature of the nonchromosomal gene which controls respiratory competence in yeast (*Saccharomyces cerevisiae*). By irradiating diploid cells with ultraviolet light, its effect of inducing respiratory deficiency was compared with its killing effect. The results obtained were as follows.

1. The rates of killing and respiratory deficiency mutation due to ultraviolet light irradiation varied according to growth phase, but the ratio of the two rates did not.
2. The rate of recovery from respiratory deficiency-inducing effect was less than that of recovery from killing effect both in light and darkness, especially so in the latter.
3. Preculturing of cells in a 5-fluorouracil-containing medium raised cells' killing effect of ultraviolet light conspicuously, but not so much for respiratory deficiency mutation.
4. The nature of the nonchromosomal gene is discussed.

Introduction

Ephrussi classified respiratory deficient (RD) mutants of yeast into two groups; segregational and vegetative (1). The former is considered to be due to mutation of chromosomal genes, and the latter to be due to mutation of the nonchromosomal gene. The latter occurs with a much higher frequency than the former. Raut (2) and Raut & Simpson (3) observed using *Saccharomyces cerevisiae* that 1) RD mutants were obtained with a high frequency as a cosequence of the effect of ultraviolet (UV) on the gene, but not as a consequence of

selection of RD cells by UV, 2) the bulk of RD mutants were nonchromosomal gene mutants, and 3) the action spectrum of UV inducing such mutation fitted the light absorption spectrum of the nucleic acids. They assumed that the nonchromosomal gene had the nucleic acid nature, and UV damage on the nucleic acid caused the mutation.

Swann (4) studied into the killing activity of UV using another species of yeast, *Schizosaccharomyces pombe*, and concluded that at least at low irradiation doses killing was resulted from UV damage on the nucleic acid, but not from that on other cellular components. The UV sensitivity of chromosomal genes and the recovery from UV damage have been elucidated at least partly by the fact that they are DNA. However, the nature of the nonchromosomal gene is still obscure, e.g., as to whether it is DNA or RNA, and why its mutation does occur with unusually high frequencies.

The present paper will report some experiments using UV, conducted with the aim of characterizing the nonchromosomal gene of yeast in comparison with chromosomal genes. It was assumed that 1) the RD mutants obtained after UV irradiation of a diploid strain were nonchromosomal gene mutants, 2) under the experimental conditions UV exerted random damages both on chromosomal and on nonchromosomal genes to give biological effects, but not on other cellular components, and 3) the cellular death was caused by UV damage on chromosomal genes and RD mutation by that on the nonchromosomal gene.

Material and Method

A diploid strain *a* of *Saccharomyces cerevisiae* (5) was used. The diploid strain was used because the frequency of chromosomal RD mutants can be neglected and clumping of cells, which disturbs population analysis is reduced.

The frequency of spontaneous RD mutants was about 1%, and 3% at most. The medium used contained peptone 2g, sucrose 50g, KH_2PO_4 3g, $MgSO_4 \cdot 7H_2O$ 1g, $CaCl_2 \cdot 2H_2O$ 0.25g, and vitamin mixture (5) 10ml in 1000ml deionized water.

Air was passed through in liquid cultures including incubation for starvation.

Incubation temperature was 30° throughout the experiment.

The viable count and the frequency of RD mutants were determined as follows. Cell suspension appropriately diluted was mixed into 2.5ml of melted 1% agar medium at 45° and this was layered onto nutrient 1.5% agar plate. After 3 days, incubation melted 1% agar solution containing 0.06% triphenyltetrazolium chloride was overlayed. Wild type colonies turned red within 2 or 3 hours but RD mutant colonies did not (6). The frequency of RD mutants was represented as a ratio of the number of uncolored colonies to that of total colonies. The colony with a colored sector or sectors was counted as one wild type and one RD mutant colonies. For UV irradiation, yeast cells were washed twice with M/15 KH_2PO_4 buffer, and suspended in the buffer to a final concentration of about 10^7 cells/ml. The suspension, 1 cm thick, was irradiated in a 9 cm Petri dish, with agitation by a small magnetic stirrer.

The UV source was a Toshiba Germicidal Lamp (15W) located 73 cm above the dish. A safety lamp was used to prevent photoreactivation.

In photoreactivation experiment, test tubes containing UV-irradiated cell suspensions were held in a glass incubator and illuminated by two "Daylight" fluorescent lamps (10 W each) at a distance of 10 cm. In darkreactivation experiment, they were covered with aluminum foil.

The rate (r) of recovery from UV killing and that from RD mutation are defined by the following equations :

As regards killing,

$$r=(Sr - Su)/(So - Su) \qquad 1$$

and as regards RD mutation,

$$r=(Ru/Su - Rr/Sr)/(Ru/Su - Ro/So) \qquad 2^*$$

where So, Su and Sr represent total viable cell numbers before and after UV irradiation and after recovery period, respectively, and Ro, Ru and Rr represent numbers of RD cells before and after UV irradiation and after recovery period, respectively.

Result

1. Relation between killing and RD mutation induced by UV irradiation.

Giese *et al.* (7) observed in *Saccharomyces cerevisiae* that cells in the log phase were more resistant to UV irradiation than those in the past log phase. Swann (4) reported using *Schizosaccharomyces pombe* that the time of gene replication would be in accord with that of UV resistance. Hence it was examined whether the effect of UV irradiation inducing RD mutation would vary with stage of culture.

Cells were transferred from a 44 hr-preculture into a fresh medium and portions of the culture were removed at intervals for UV irradiation.

As shown in Fig. 1, effects of UV irradiation on killing and RD mutation were at the maximum in the stationary phase, and began to be decreased after transference into the fresh medium. The effects became smaller during the log phase, and were increased toward the stationary phase. Most cells in the log phase were budding and were large in size. In this phase many of the RD mutants appeared as sectors in colonies. For example, at a UV dose of 180 sec the ratios of sectored colonies to unsectored RD colonies were 2.5, 5.1, 22.3 and 8.7% for 0, 1.5, 5 and 10 hr-cultures, respectively. And cells which have duplicated chromosomes will not die even if defect occurs in one of the two.

The data presented in Fig. 1 are replotted in Fig. 2 to compare the effects of each dose of UV irradiation on the induction of RD mutation and that of killing.

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* See Appendix.

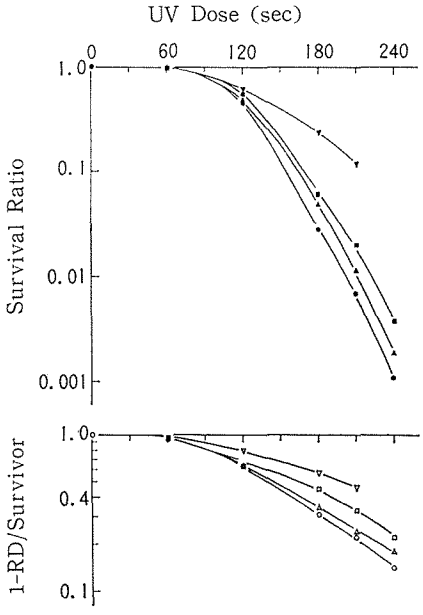

Fig. 1 Proportion of survivors and RD cells after UV irradiation from different stages of aerobic culture.

Cells were cultured for 44 hours, transferred into a fresh medium, sampled after 0 hr (—●—, —○—: stationary phase), 1.5 hr (—▲—, —△—: lag phase), 5 hr (—▼—, —▽—: logarithmic phase) and 10 hr (—■—, —□—: late logarithmic phase) and irradiated by UV. Ratio of survivors to original viable count (closed symbols) and ratio of non-RD mutants to survivors (open symbols) are plotted in logarithmic scale against UV dose.

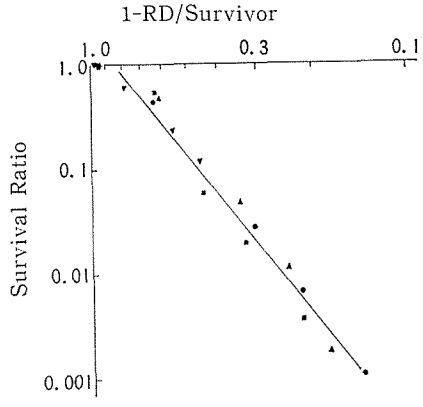

Fig. 2 Relation between killing and RD mutation induced by UV irradiation.

Ratio of survivors to original viable count is plotted against ratio of non-RD mutants to survivors, using the data presented in Fig. 1. Symbols correspond to those in Fig. 1, each pair of closed and open symbols in Fig. 1 making a point here.

All the points fell along a straight line, showing that two kinds of the effects of UV irradiation well correlated each other, and the relation between the two was independent of the culture age. This suggests that the molecular states and/or the molecular environments of chromosomal and nonchromosomal genes, as far as the UV effects are concerned, change in a way corresponding to each other as the growth stage of the culture proceeds.

2. Photo- and darkreactivation of UV-induced RD mutation.

It was reported that RD mutation could be photoreactivated by illuminating cells with visible light after UV irradiation in aerobically grown yeast, but not in anaerobically grown yeast (8), and that nonchromosomal RD mutation could not be phototeactivated in a segregational RD mutant (9). Hence mechanism (s) or enzyme (s) involved in the photoreactivation of UV effect seems to be different between chromosomal and nonchromosomal genes. Experiments were conducted to compare the reactivation of RD mutation and that of killing induced by UV irradiation.

Cells from a 24 hr-culture were collected by centrifugation, resuspended in M/15 KH_2PO_4 buffer (about $5x10^7$ cells/ml), and cells were starved for 24 hours to make cell stage uniform. Cells were then collected, subjected to UV irradiation and the recovery from the UV effects was pursued by keeping cells in the dark and in the light before plating.

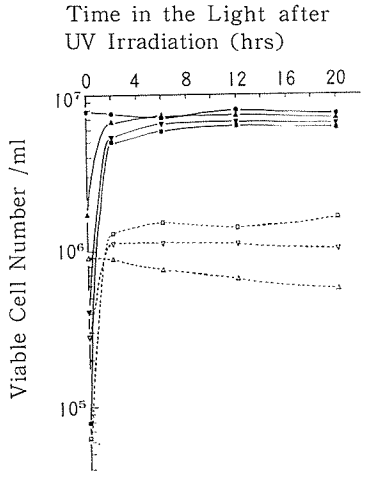

Fig. 3 Photoreactivation of UV-irradiated cells.
Cells from a 24 hr-culture were suspended in M/15 KH_2PO_4 buffer, and subjected to UV irradiation. The cell suspensions were then incubated in the light, samples were withdawn at intervals to be plated for determining survivors/ml (closed symbols) and RD cells/ml (open symbols). Cells were irradiated by UV for 0 sec (—●—, —○—), 140 sec (—▲—, —△—), 170 sec (—▼—, —▽—) and 200 sec (—■—, —□—).

The results obtained are shown in Figs. 3 and 4. The killing effect was recovered almost completely by 2 to 6 hours' illumination (Fig. 3). The rates of recovery as calculated by equation 1 were 0.95, 0.83 and 0.77 for cells irradiated by UV doses of 140, 170 and 200 sec, respectively. On the other hand, the recovery of viability in the dark (Fig. 4) was slow and incomplete, the rates of recovery after 20 hr-incubation being 0.56, 0.33 and 0.101 for UV doses of 140, 170 and 200 sec, respectively. In the dark recovery, the number of RD mutants was increased in parallel with the viable count. This means that the rate of recovery from RD mutation was very low. The ratio of RD mutants to survivors remained without remarkable change during the incubation in the

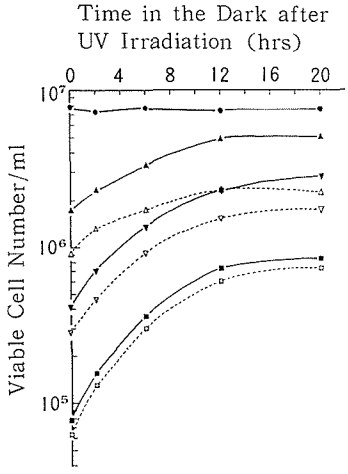

Fig. 4 Dark recovery of UV-irradiated cells. Just as in Fig. 3, except that cells were incubated in the dark.

dark, while it was decreased in the light (Fig. 5). Each point in Fig. 5 was calculated from the data represented in Figs. 3 and 4.

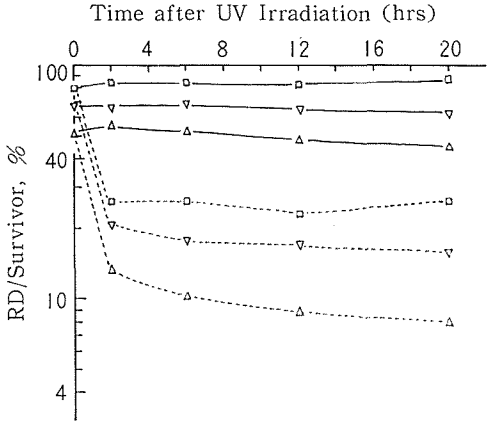

Fig. 5 Effects of incubation in the light and darkness following UV irradiation on the numbers of RD mutants in percentage of survivors.
Each point was calculated from the data given in Figs. 3 & 4.
Symbols as in Fig. 3. ·····, light incubation; ——, dark incubation.

Before reaching a conclusion that the activity of dark repair of RD mutation is much lower than that of killing, the following possibilities must be taken into consideration.

Firstly, the enzyme (s) needed for the dark recovery of RD mutation may have been degenerated owing to the starvation before UV irradiation. Hence the rates of recovery of killing and of RD mutation were determined using cells which had and had not been starved before UV irradiation.

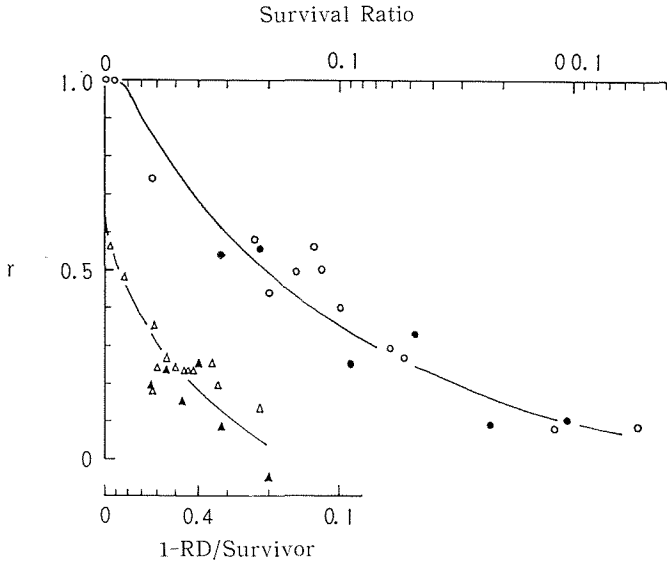

Fig. 6 Comparison of dark recovery starved and not starved cells.

Cells from a 24 hr-culture were irradiated by UV with and without previous starvation engendered by incubation in phosphate buffer for 24 hours. Immediately following UV irradiation, a portion of each cell suspension was plated to determine viable count and count of RD cells. Cells from another portion was suspended in phosphate buffer, incubated in the dark, and plated after 20 hours to determine dark recovery from killing and RD mutation. Rates of dark recovery (r; calculated with equations 1 and 2) are plotted against fraction of survivors (○, ●) and against non-RD mutants (△, ▲). Closed symbols: starved cells; open symbols: cells not starved.

In Fig. 6 the rates of recovery are plotted against the ratio of non-RD mutants to survivors and also against the proportion of survivors. Each rate (r) was calculated by equation 2. There was hardly any difference between starved and not starved cells both in killing and in RD mutation. Therefore, enzymes (if any) involved in the repair of killing and RD mutation do not seem to be degenerated considerably through the starvation.

Secondly, there is a possibility that the cells which had suffered from UV damage on the nonchromosomal gene had higher activities of recovering from lethality than those which had not. Hence the recovery from lethality was examined for the UV-induced RD mutants. The cells which have stabilized

as RD cells might not be quite the same as the cells in which disturbance due to UV irradiation has not yet settled down. But experiments using the former will tell something about the relation between the UV-induced change of the nonchromosomal gene and the recovery from the lethal change of chromosomal genes. Seven strains of UV-induced RD mutants were cultured for 24 hours and cells were irradiated by UV. Their survival ratios determined by plating immediately following UV irradiation (140 sec) were as follows :

Strain	U2	U4	U5	U6	U7	U9	U12	Wild type
Survival (%)	15.6	28	24	2.2	34	17.1	23	16.1

After UV irradiation cells were suspended in phosphate buffer and incubated for 20 hours in the dark. Thereafter, viable count was examined. It was not increased but was decreased during the incubation with the exception of U12 substrain and this was also observed when cells of these RD strains were incubated in the buffer without any UV irradiation.

Chloramphenicol (CM) inhibits formation of mitochondria and syntheses of some respiratory enzymes in yeast (10). Without any UV irradiation, decrease in viable count was also observed in wild type cells during the incubation in the buffer containing 4 mg/ml of CM, when they were precultured in a medium containing the same amount of CM.

Patrick & Haynes (11) reported that dark reactivation of UV killing was energy-requiring and oxidative metabolism was indispensable for the energy supply in *Saccharomyces cerevisiae*. Cells of wild strain and three UV-induced RD strains were cultured and a portion of each cell suspension was UV-irradiated, the other portion being not irradiated as control. Cells were then transferred to the buffer supplemented with sucrose at a final concentration of 1% and not supplemented. During 20 hours' incubation no budding cells were observed in either unirradiated or irradiated cells. By an addition of sucrose to the buffer, lethality was eliminated both in RD mutants and in CM-treated wild strain during the dark incubation.

Strain	Post-UV incubation	Killing		R D	
		%	r	%	r
Wild type	Buffer	88.9	0.45	56	0.23
Wild tvpe	Buffer + Sucrose	88 9	0.63	56	0.175
R D U 4	Buffer + Sucrose	72	0.34	—	—
R D U 6	Buffer + Sucrose	97.8	0.04	—	—
R D U12	Buffer + Sucrose	77	0.47	—	—

Table 1 Dark recovery from killing in wild type and RD mutant cells.

Cells from 24 hr-cultures were exposed to UV for 140 sec and incubated in the dark for 20 hours in phosphate buffer supplemented and not supplemented with sucrose (1%). Rates (r) of dark recovery from killing and RD mutation (in the case of wild type cells) were calculated according to equations 1 and 2.

From the data shown in Table 1, rates of dark recovery from UV killing in the RD strains were somewhat less than those in the wild strain. Therefore the possibility can be eliminated that the rate of dark recovery from UV killing in the UV-induced RD cells was higher than that in the wild type cells.

Since the above mentioned two alternative explanations can be rejected from these experimental results, it is very probable that the ability of dark recovery from RD mutation was very weak as compared with that from killing. As to the wild strain shown in Table 1, the recovery from RD mutation was less effective than that from killing during the dark incubation both in the presence and in the absence of sucrose. The addition of sucrose to the buffer raised the rate of recovery from UV killing but lowered that from RD mutation.

3. Effect of 5-fluorouracil on killing and RD mutation to be induced by UV irradiation.

It is conceivable that sensitivity of cells to UV damage and its reaction will be changed when pyrimidine analogs are incorporated in nucleic acids. For example, it was reported that, in bacteria, UV sensitivity was raised and no photoreactivation was manifested when 5-bromouracil was incorporated in DNA (12), and UV of longer wavelength became effective for cell inactivation when 5-bromouracil or 5-iodouracil was incorporated (13).

Yeast cells were cultured in a medium containing 5-bromouracil or 5-bromodeoxyuridine (100 or 200 μg/ml). But these analogs exhibited no effect on

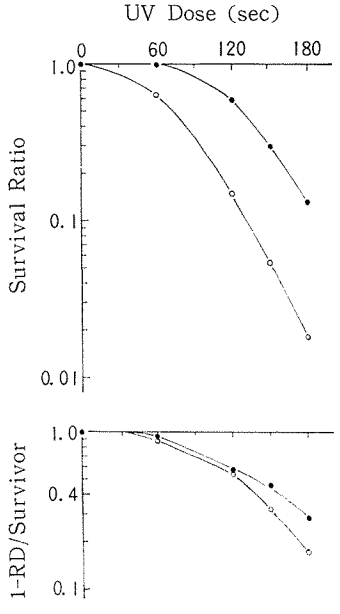

Fig. 7 Effect of FU on killing and RD mutation to be induced by UV irradiation.
Cells from a 24 hr-culture were inoculated in a fresh medium. After incubation for 2.5 hours, cells were collected, inoculated at a cell density of 10^5/ml in media containing and not containing 20μg/ml of FU and incubated for 2 hours. Cells were then starved for 24 hours and exposed to UV. Fractions of survivors and non-mutant cells per survivors plotted against UV dose. —○—, FU-treated cells; —●—, cells not treated with FU.

killing, RD mutation and growth of cells. The UV sensitivity was not apparently modified either.

As it has been known that 5-fluorouracil (FU) has some influences on yeast cells (14), effects of FU on UV-induced killing and RD mutation were studied. Cells were cultured for 24 hours, transferred in a fresh medium and incubated for 2.5 hours. These cells were inoculated at a cell density of 10^8/ml in media containing and not containing 20 μg/ml of FU, and incubated for 2 hours. After the incubation survival and RD mutant ratios were about 100 and 20%, respectively. Cells were then starved for 24 hours in M/15 KH_2PO_4 buffer and were irradiated by UV. During the starvation survival and RD mutant ratios remained unchanged.

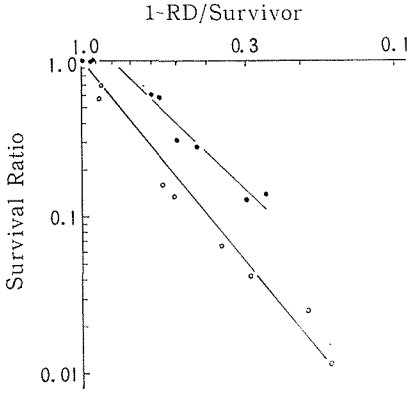

Fig. 8 Relation between the effects of UV on killing and RD mutation of cells treated with FU.
Plotting of the data obtained from two experiments in the same way as in Fig. 7. —○—, cells treated with FU; —●—, cells not treated with FU.

Fig. 7 shows that FU treatment made cells more sensitive to UV killing, but its effect was small for RD mutation. The frequency of RD mutants was represented in this case by the ratio: (Number of RD mutant cells after UV irradiation − Number of RD mutant cells before UV irradiation) / Number of wild type cells before UV irradiation. Fig. 8 shows the relation between the fractions of unmutated cells and of surviving cells, plotted by using the values obtained from two experiments. These figures show that at least within a range of UV doses used the fraction of RD mutants induced by UV irradiation was smaller in FU-treated cells than in control cells under a given fraction of UV killing. It suggests that FU affects chromosomal genes more remarkably than the nonchromosomal gene as regards UV sensitivity.

Discussion

Yotsuyanagi (15) observed with electron microscope that in aerobically grown yeast mitochondria were small in number and poor in inner organization during the log phase, to be increased in number and develope cristae toward the

stationary phase. DNA has been observed in yeast mitochondria (16) and the amount of the DNA is said to vary during cell growth (17). If loss or any alteration of mitochondrial DNA is responsible for RD mutation, the frequency of RD mutation induced by UV irradiation may vary according to the phase of growth. Fig. 1 shows that lethal effect and RD mutation inducing effect of a given UV dose vary with the stage of culture, but Fig. 2 shows that there is a definite relation between the two effects. This indicates that the amount of mitochondrial DNA and the number and organization of mitochondria do not correspond to the rate of RD mutation.

Ginsberg & Jagger (18) observad that cells of *Escherichia coli* in the stationary phase were more resistant to UV irradiation than those in the log phase. To explain this they pointed out that the apparent UV sensitivity may be influenced by the time available for repair and proposed that the hit number in the target theory would rather represent the ability of dark repair within the duration before onset of DNA replication. If their proposition is applied here, true hit number can not be obtained from Fig. 1. But the number of the nonchromosomal gene is considered to be a few per cell from the facts that the hit number was usually 2 to 6 and not more than 10 in RD mutation in contrast to a few hundreds in killing, and that the ability of dark recovery from RD mutation was very weak. Then, if most of mitochondria in an yeast cell carry DNA, it is doubtful that mitochondrial DNA is identified to be the nonchromosomal gene.

Biological effect of low doses of UV irradiation is considered to be due to hydration and dimerization of the pyrimidine bases in DNA (19). Since hydrated pyrimidines are readily restored, they may not play a major role in UV effect inducing lethality and RD mutation.

Thymine dimer is thought to be one of UV products which could induce some biological effects (20). In *E. coli* there should be dark repairing enzymes for UV-damaged DNA including thymine dimer and some UV-sensitive mutants lacked at least a portion of this repairing mechanism (12). The results in this paper show that UV damage on chromosomal genes can be repaired to some extent in the dark and that UV damage on the nonchromosomal gene is repaired significantly in the light but only little in the dark. It may be supposed that enzymes or mechanisms reactivating chromosomal genes are different from those reactivating the nonchromosomal gene in their nature and/or in the localization in yeast cell. This supposition may be supported by the finding of Sarachek (8) that in anaerobically grown yeast cells killing was recovered but RD mutation was not under the light condition, whereas both were recovered in aerodically grown cells, and by the finding of Pittman *et al.* (9) that a segregational RD mutant had no ability of photoreactivation only of nonchromosomal RD mutation.

The very weak ability of dark repair in the nonchromosomal gene of yeast may explain why nonchromosomal RD mutants are induced by UV irradiation with a very high frequency.

It was observed in the strain used that killing and RD mutation were induced by FU and 5-fluorodeoxyuridine, and a conclusion was proposed that

these effects were due to the thymineless state caused by the analogs (22). In a thymine requiring mutant of *E. coli*, the hit number of UV inactivation was decreased by thymine starvation (23). In another thymine requiring mutant nonconservative DNA synthesis was observed after thymine stavation and it was supposed that there might be a common repairing mechanism for UV damage and thymineless death (24). In combination with those observations, it is understandable that FU-treated cells became sensitive to UV irradiation both in killing and in RD mutation as shown in Fig. 7 because FU may restrict the amount of thymine in the cell needed for the repair. Fig. 8 indicates that the effect of FU was much more remarkable in killing than in RD mutation. This may be interpreted as a difference in the ability of dark repair between chromosomal and nonchromosomal genes. Repairing activity must be affected by the available amount of thymine. But as for RD mutation, lack of thymine does not affect dark repair as cells possess only inconsiderable repairing activity.

Appendix

Represent the probability of a cell to be killed by UV without causing RD mutation as p, and that to be killed with concomitant RD mutation as p', and the probability of RD mutation to be caused in viable cells by the same UV irradition as q, then

$Su/So=(1-q)\ (1-p)+q(1-p')$ and $Ru/So=q(1-p')$

Here it is assumed that no RD mutants would be involved in the population to avoid complexities. Again, expressing the probability of a normal cell to be recovered as v and that of an RD cell as v' and the probability of an RD cell to be recovered and simultaneously turned to be normal as m, then

$Sr/So=(1-q)\ (1-p+vp)+q(1-p'+v'p')$ and

$Rr/So=q(1-p'+v'p')\ (1-m)$

When p' is equal to p (ref. 2), then

$Su/So=(1-p)$

$Ru/So=q(1-p)$

$Sr/So=(1-q)\ (1-p+vp)+q(1-p+v'p)$

$Rr/So=q(1-p+v'p)\ (1-m)$

Solving above four equations for m gives

$m=(1-Rr/Sr)+(Rr/Sr)\ (1-Su/Ru)\ ((v+n)/(v'+n))$

where n represents $Su/(So-Su)$.

The equation indicates

$$\begin{matrix} m & > m & > m \\ v'>v & v'=v & v'<v \end{matrix}$$

namely, the real value of m is smaller than the apparent value when v' is smaller than v.

If v' is equal to v,

$m=(Ru/Su-Rr/Sr)/(Ru/Su)$

Since spontaneous RD mutants were involved in the population, the equation

must be revised by subtracting the fraction of spontaneous RD mutants from each fraction of them and it gives

$m=(Ru/Su-Rr/Sr)/(Ru/Su-Ro/So)$

This equation was used to determine the rate (r) of recovery of RD mutation.

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