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Effect of Uracil Derivatives on Respiratory Deficiency Induction in Yeast

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ABSTRACT Effect of nucleic acid base analogs on the induction of respiratory deficiency mutation was examined under the assumption that the bulk of respiratory deficient mutants produced from diploid cells are nonchromosomal gene mutants, and the following results were obtained.

1. Fluorouracil and fluorodeoxyuridine were powerful inducers of respiratory deficiency mutation but bromouracil and bromodeoxyuridine showed no appreciable effect.

2. Fluorouracil and fluorodeoxyuridine are considered to induce respiratory deficiency mutation by causing thymineless state in yeast cells.

Introduction

Since the pioneering work of Ephrussi *et al.* (1) in 1949, various chemical and physical agents have been found, as reviewed by Nagai *et al.* (2), to induce with high frequencies respiratory deficiency in yeast transmitted by nonchromosomal gene distinct from chromosomal genes. Raut and Simpson (3) found that the action spectrum of ultraviolet (UV) irradiation inducing respiratory deficient (RD) mutants followed the light absorption of nucleic acids. Since DNA has been found to be contained in yeast mitochondria (4), the mitochondrial DNA is surmised to make a molecular basis of the nonchromosomal gene. Mounolou *et al.* (5) studied the DNA which was rich in mitochondrial fractions and found that its buoyant density was different between nonchromosomal RD mutants and normal strains. And Moustacchi & Williamson (6) found that nonchromosomal RD mutants lacked in a satellite DNA which was found in normal strains. The nature of the nonchromosomal gene, however, has yet to be studied from different sides. 5-Fluorouracil (FU), an analog

of uracil (U), is effectively incorporated into RNA in bacteria, yeasts, plants and animals, but not into DNA (7) probably due to that these organisms can convert FU to its ribosyl triphosphate but not to deoxyribosyl triphosphate, a DNA precursor. The incorporation of FU into RNA is competitively inhibited by administration of U (8). FU is converted by the cell into fluorodeoxyuridylate, which inhibits thymidylate synthetase noncompetitively in the phage-induced bacterial system (9). One may, therefore, be able to tell whether the nonchromosomal gene is RNA or DNA by testing whether FU and fluorodeoxyuridine (FDU) induce the nonchromosomal RD mutant and whether the induction is inhibited by U.

This paper will report killing effect and RD mutation inducing effect of the uracil derivatives in yeast, and will suggest the DNA nature of the nonchromosoal gene.

Material and Method

A diploid strain *a* (10) of *Saccharomyces cerevisiae* was studied. The diploid was used in order to make low the occurrence of RD cells due to mutation of chromosomal genes, since RD mutation on one of homologous chromosomes may keep cells respiration sufficient, and diploid cells are preferable to haploid cells for population analysis, as cellular clumping is far less in the former than in the latter.

The culture medium 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 (10) 10ml in 1000ml deionized water.

Air was passed through in liquid cultures. Incubation temperature was 30°.

Cells used were grown under the condition above mentioned for 24 hours and at 0 time they were transferred into a fresh medium as far as not mentioned at an inoculation size 1/15.

Hemocytometer and Coleman nephelometer were used for cell counting and for turbidity determination, respectively. A budding cell was calculated as two cells.

Viable count and frequency of RD mutants were determined as follows. Cell suspension appropriately diluted was mixed with 2.5ml of 1 % agar medium in fluid state at 45° and poured onto a solid agar (1.5%) plate. After 3 day-incubation at 30°, liquid agar (1%) solution containing 0.06% of triphenyltetrazolium chloride (TTC) was overlayed. Wild colonies turned red within 2 or 3 hours, but RD colonies did not (11). Frequency of RD mutants was represented as a ratio of the number of uncolored colonies to the total number of colonies. The colony which had sector was counted as one wild and one RD colonies.

For counting frequency of chromosomal gene mutation, mutations from galactose non-fermenter to fermenter (Gal^+) and from copper sensitivity to resistance (Cu^r) were assayed. These mutations are expected to be expressed in diploid cells, as dominant genes are concerned in them. For galactose

fermenters, colonies were counted which grew on the plates containing galactose (10g/l) instead of sucrose of the nutrient medium. For copper resistant mutants, colonies growing on the standard nutrient medium to which $CuSO_4$ was added at a final concentration of 1 mM were counted.

FU and FDU were gifts from Hoffman La Roche Co., N. J., U. S. A..

Result

1. *Effect of base analogs on cellular growth.*

Cellular growth was measured by means of hemocytometer and nephelometer. Yeast cells were cultured in the medium containing 5-bromouracil or 5-bromodeoxyuridine (100 or 200μg/ml). But these analogs exhibited no effect on killing, RD mutation and growth of cells. The UV sensitivity was not apparently modified either.

As for the effect of FU, cells did not double in number within at least 11 hours in the medium containing 1μg/ml of FU, and budding was completely inhibited by 10μg/ml of FU (Fig. 1). When U was added to the medium together with 1μg/ml of FU, growth was increased with the concentration of U (Fig. 2).

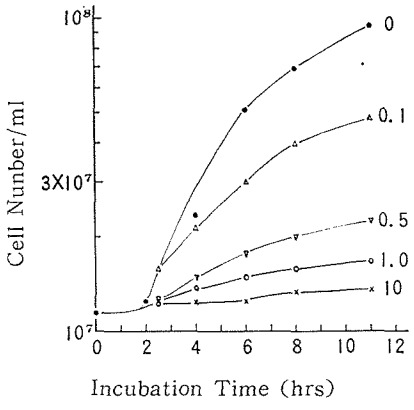

Fig. 1 Effect of FU on growth.

One volume from a 24 hr-culture was transferred to 14 volumes of a fresh medium containing FU, and culture was sampled at intervals to count cells. FU concentration in μg/ml is indicated on each curve.

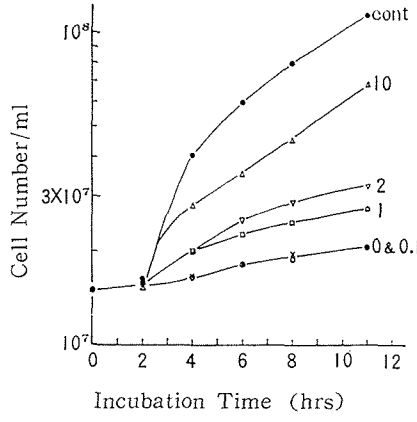

Fig. 2 Effect of U on growth inhibition by FU.

Cells were inoculated in the way as in the legend for Fig. 1 in media to which 1μg/ml of FU and various concentrations of U were added. Concentration of U in μg/ml is indicated on each curve. Control (—●—) contains no FU and U.

On the other hand, turbidity was increased about two fold in 6–7 hours' incubation in the presence of 1 μg/ml of FU. But no increase of turbidity was observed thereafter. When 20 μg/ml of U or 40 μg/ml of uridine (UD) was added to the culture containing 10 μg/ml of FU, turbidity kept to increase with incubation time (Fig. 3).

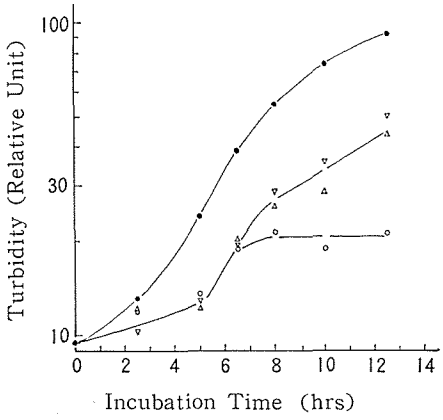

Fig. 3 Effect of U and UD on growth inhibition by FU.

Cells were inoculated in the way as for Fig. 1 in a medium containing 20μg/ml of U or 40μg/ml of UD in addition to 10μg/ml of FU. Culture was sampled at intervals and growth was measured turbidometrically. —●—, control; —▽—, FU+UD; —△—, FU+U; —○—, FU.

It has been reported that the incorporation of FU was inhibited by an addition of U in *Candida* (8). To avoid such an effect of U, if any in the present case, U was added to the culture 2 hours after the exposure of cells to FU (1μg /ml), and cellular growth was followed. As shown in Fig. 4 the growth restoration by U reached its maximum at a concentration of 5 μg/ml and no further improvement was observed with 10 μg/ml as far as the growth was observed in 9 hours of incubation. Growth inhibition of FU could not be completely restored by U.

It is known that additions of both U and thymidine (TD) are required to restore the inhibition by FU in *Escherichia coli* infected and uninfected with a bacteriophage (12). Hence U and thymine (T) were added to the FU-containing culture which had been incubated for 2 hours. Fig. 5 shows that T improved no more.

2. *Killing and induction of RD mutation by FU.*

Typical results representing the effects of FU on killing and RD mutation are shown in Fig. 6. With both 1 and 10 μg/ml of FU the number of RD mutants was increased almost in parallel with that of non-viable cells. The rates of both killing and RD mutation did not change when U (20μg/ml) was added to the nutrient agar plates.

From the plates used in these determinations 57 colonies not colored by TTC were picked up at random and streaked on glycerol agar plates. No growth occurred on these plates. The RD nature of samples was also confirmed by manometric determination of respiration. And the RD clones isolated did not produce wild type cells. It was concluded that stable RD mutants could be

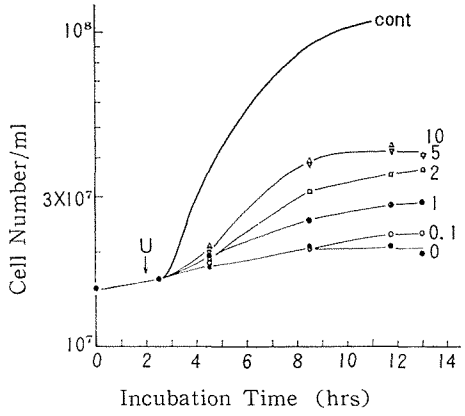

Fig. 4 Effect of U on growth inhibition by FU.

Cells were inoclulated in the same way as for Fig. 1 to a medium containing FU (1μg/ml). After 2 hours' incubation various amounts of U were added. Culture was sampled at intervals and cells were counted. Concentration of U in μg/ml is indicated on each curve. Control (—) contains no FU and U.

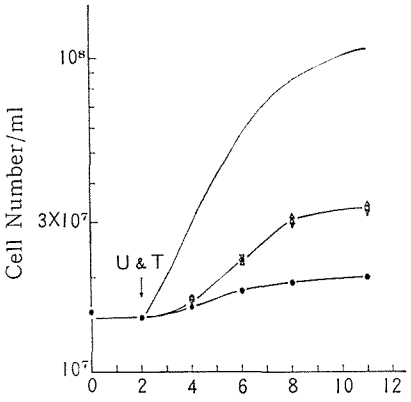

Fig. 5 Effect of T on growth inhibition by FU.

At 2 hours of FU-containing (1μg/ml) culture U (5μg/ml) and different amounts of T were added. Samples were withdrawn at intervals and cells were counted. —△—, U+T (160μg/ml); —▽—, U+T (40μg/ml); —○—, U without T; —●—, FU only; ——, control.

obtained by the action of FU. Cells were grown for 11 hours in media containing 1 μg/ml of FU supplemented with U in various concentrations, and were plated. The results shown in Table 1 indicate that the effects of FU on killing and RD production were reduced by U.

The following experiments were performed to see whether RD mutants occurred through the induction by FU, instead of being increased in proportion by selective killing of wild type cells.

Firstly, whether RD mutants would appear under a condition where no killing occurred was examined.

Rate of killing was apt to fluctuate form experiment to experiment as seen

Conc. of U (μg/ml)	Percentage of Killing	RD
0	44	29
0.5	44	22
1.0	39	15.8
2.0	8	4.5
10.0	3	0.7

Table 1 Effect of U on killing and RD induction by FU.

One volume of a 24 hr-culture was inoculated in 14 volumes of fresh media containing 1μg/ml of FU and various concentrations of U. After 11 hours of incubation cells were plated to determine viable and RD mutant counts.

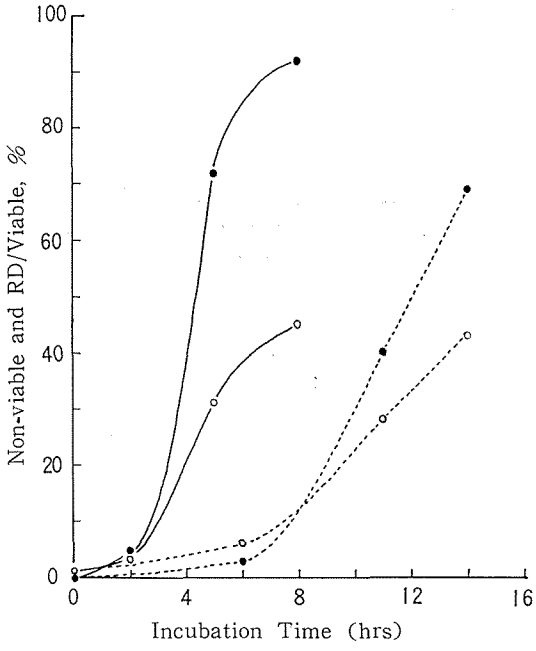

Fig. 6 Lethal and RD inducing effects of FU.

One volume from a 24 hr-culture was inoculated in 14 volumes of media containing 1μg/ml (···) and 10μg/ml (—) of FU. During incubation cells were sampled at intervals and plated. (●), difference, viable count before FU treatment minus that after FU treatment, in percentage of the former; (○), number of RD colonies in percentage of viable count.

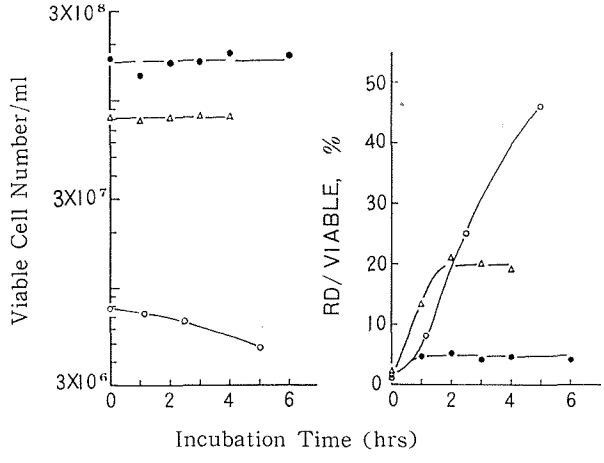

Fig. 7 RD induction by FU.

One volume from a 24 hr-culture was inoculated in three volumes of a fresh medium. Two hours later cells were collected by centrifugation and reinoculated at cell densities of 7.7×10^6 (—○—), 8.2×10^7 (—△—) and 1.8×10^8 (—●—) per ml in fresh media cotaining 10μg/ml (○, ●) and 20μg/ml (△) of FU. Cells were sampled at intervals and plated.

in Table 2, when cells in the stationary phase were directly inoculated in the medium containing FU. Cells were grown for 24 hours, inoculated at an inoculation size of 1/4 in a fresh medium and incubated for 2 hours. These cells

in the late lag phase were collected by centrifugation, inoculated at several cell densities in fresh media containing 10 and 20 μg/ml of FU, and cells were sampled at intervals for plating.

As shown in Fig. 7, both killing and RD mutation were increased with incubation time when the initial cell density was 10^7/ml and the FU-concentration was 10 μg/ml. When the inoculation size was larger, on the other hand, no killing occurred within a range of the incubation time observed. With an FU-concentration of 20 μg/ml and a cell density of 10^8/ml at the time of inoculation, the frequency of RD mutants was increased while no killing effect was observed at least in 4 hours. RD mutants were increased up to 20% in 2 hours, but no further increase was observed.

Secondly, it was examined whether the killing effect of FU would differ in wild type cells from that in RD cells. Eleven wild type colonies, five spontaneous RD colonies and seven FU-induced RD colonies were picked up. Cells from each clone were inoculated in a medium containing 10 μg/ml of FU, and after 5 hours of incubation viable count was determined. The variance analysis of the values shown in Table 2 tells that there are no significant differences among the three types of substrains with respect to the effect of FU on killing. It can be concluded that FU for the most part induces RD mutants.

	Percentage of Killing						
Wild type	45	51	60	61	62	67	77
	77	78	86	96			
FU-induced RD	53	56	61	63	68	69	88
Spontaneous RD	59	62	67	69	72		

Table 2 Killing effect of FU on wild type and RD cells.

Wild type clones and clones of spontaneous and FU-induced RD mutants were cultured for 24 hours, and one volume from each culture was transferred into 14 volumes of a fresh medium containing 10 μg/ml of FU. At 5 hours of incubation cells were plated. Differences, viable count before FU treatment minus that after FU treatment, in percentage of the former during the incubation of parallel cultures are presented.

3. *Effect of FU on gene mutation.*

Observations mentioned above show that FU is a powerful inducer of RD mutation. To see whether FU mainly influences the nonchromosomal gene as acriflavin (13) or heat (14) does, or whether it is also a powerful inducer of

chromosomal gene mutation, two kinds of chromosomal gene mutation were studied. One is a forward mutation to copper resistance, which is under the control of dominant genes (10), and the other is a back mutation from galactose non-fermenter to fermenter.

Cells in the late lag phase, obtained as in the above experiment, were incubated for 2 hours in the culture media with and without addition of 20 μg/ml of FU, and plated on agar plates containing 10 g/l of galactose in place of sucrose or $CuSO_4$ at a final concentration of 1 mM.

	Survivor	RD/Sur.	Cu^+/Sur.	Gal^+/Sur.
Control	1.00	2.3×10^{-2}	3.6×10^{-7}	1.74×10^{-6}
FU-treated	0.92	2.4×10^{-1}	12.3×10^{-7}	2.3×10^{-6}

Table 3 Effect of fluorouracil on chromosomal gene mutation.
One volume from a 24 hr-culture was inoculated in 3 volumes of a fresh medium. After 2 hours of incubation cells were collected by centrifugation and inoculated at a cell density of about 10^8/ml in media containing and not containing 20 μg/ml of fluorouracil. After 2 hours of incubation cells were plated to determine frequencies of galactose fermenters and copper resistant mutants, as well as respiratory deficient mutants.

TABLE 3 indicates that relative frequencies of copper resistant mutants and galactose fermenters were about 3.5 and 1.3 times, respectively, as high in FU culture as in control. Under the same conditions, relative frequency of RD mutants was 10 times as high in FU culture as in control.

4. *Effect of FU on cell wall synthesis.*

It has been reported that FU inhibits cell wall synthesis of *Staphylococcus aureus* through its incoporation into UD coenzymes (15). The killing effect is lessened in *E. coli* K-12 by osmotic stablization (16). Although the cell wall of yeast differs from that of bacteria in chemical composition, one may suppose that FU inhibits syntheses of some components of cell wall in yeast leading to cellular death or permanent loss of respiratory activity.

To examine this possibility, the following experiments were carried out. Cells were incubated in a medium containing 20 μg/ml of FU for 2 hours, collected by centrifugation, and inoculated at a cell density of about 4×10^6/ml in two kinds of media : one containing the same amount of FU as before and the other which was supplemented with mannitol at a final concentration of 1 M in addition to FU. At the steps indicated in Table 4 cells were plated, and decreases in viable count and fractions of RD mutants in viable cells were determined.

The table shows that the effects of FU on killing and RD mutation were not affected by the osmotic stabilization.

It is known that 2-deoxyglucose inhibits cell wall synthesis in yeast (17).

Sample	Percentage of Killing	Percentage of RD
A. Before FU treatment	0	2.9
B. 2hr FU treatment	35	10.5
Further FU treatment		
C. with mannitol	95.0	56
D. without mannitol	95.7	55

Table 4 Effect of osmotic stabilizer on killing and RD induction by FU.

Cells were grown for 24 hours (sample A). A part of sample A was inoculated in 14 volumes of a medium containing 20 μg/ml of FU, and cells were collected by centrifugation after 2 hours of incubation (sample B). A part of sample B was inoculated at a cell density of 4×10^5/ml in FU-containing (20 μg/ml) media with (sample C) and without (sample D) mannitol at a final concentration of 1M and cells were plated after 3 hours of incubation.

When cells were incubated for 24 hours in a medium containing 0.4% glucose and 160μg/ml of 2-deoxyglucose in place of sucrose, viable count was decreased to 67% of the initial value but the frequency of RD mutants remained unchanged.

Therefore the possibility is eliminated that FU induces RD mutants and kills cells by inhibiting syntheses of cell wall components.

5. *Effect of FDU.*

FU was converted to its deoxyribonucleotide and this compound was a non-competitive inhibitor of thymidylate synthetase in *E. coli*-phage complex (9). This inhibition of DNA synthesis was not restored by an addition of U (12). On the other hand, FU interferes with cellular metabolism by being incorporated into RNA (8) and UD coenzymes (15). This can be reversed by U. Therefore FDU may have some effects on growth and viability of cells, assuming that the effects of FU inhibition of thymidylate synthetase in yeast. It is also expected that nonchromosomal RD mutation will be induced by FDU if the non-chromosomal gene is composed of DNA.

One volume from a preculture was inoculated in 49 volumes of a medium containing 80 μg/ml of FDU. The inhibition of FDU on the growth was remarkably less than that of FU, hardly any inhibition being noticed turbidometrically. But it was microscopically observed that daughter cells which appeared in the FDU medium were elongated in shape and hardly separable from their parental cells. Fig. 8A shows viable count per ml of a medium containing 80 μg/ml of FDU and another containing 40 μg/ml of U in addition to FDU. Since in this case one cluster of cells was counted as if it were one viable cell, the

recorded count must be less than the true viable cell numbers. Therefore, killing effect of FDU was less than that of FU. Fig. 8B shows the frequency of RD mutants in the same cultures used for Fig. 8A. Apparent increase in RD mutants occurred after 12 hours of incubation. This may perhaps be due to low permeability of cells to FDU or low incorporation of it into cellular components, considering that the effects of FDU on cellular growth and killing were very weak.

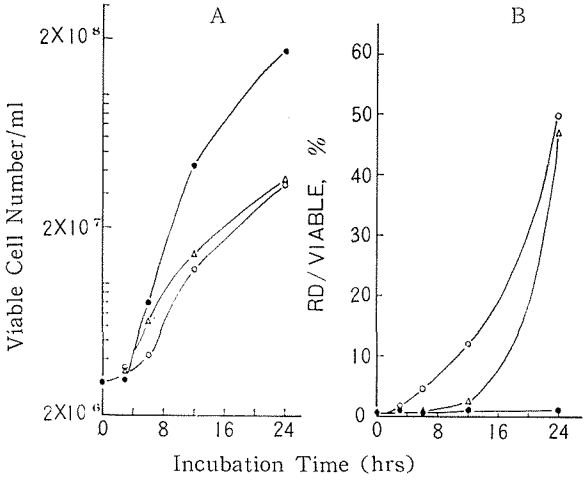

Fig. 8 Growth inhibition and RD induction by FDU.

One volume from a 24 hr-culture was inoculated in 49 volumes of fresh media containing FDU with and without supplement of U. At intervals cells were sampled and plated —●—, control;—○—, FDU (80 μg/ml); —△—, FDU (80 μg/ml)+U(40 μg/ml).

By an addition of U, the frequency of RD mutants due to FDU effect was lowered and viable count was a little increased during the first 12 hours of incubation. FDU is known to be split into FU (18). It may be supposed that the FU so derived is counteracted by the presence of U, the growth thus being restored. Intracellular concentration of FDU may become lower as a consequence of growth restoration. An explanation is thus found for the fact that the induction of RD mutants by FDU was alleviated by U in an early period of incubation.

At a later period of incubation, namely in the assay at 24 hours of incubation, U did not modify the effects of FDU both on growth and on RD mutation induction. In due time, gradually incorporated FDU may possibly reach a concentration in cells sufficient to induce RD mutation even with an addition of U as well as without it. This is conceivable also from the facts represented in Table 5 that the addition of U to the FDU-containing cultures at 12 hours or to the testing plates had no effect on the RD mutation induction, and that supplement of TD had no effect either.

Addition to FDU culture at		Experiment I		Experiment II	
		RD (%) at		RD (%) at	
0^h	12^h	12^h	24^h	12^h	24^h
none	none	11.3 (13.7)	58 (59)	15.0	60
U	none	3.8 (2.6)	57 (55)	5.5	56
U, TD	none	-	-	6.8	58
none	U	-	58 (58)	-	-
U	U	-	49 (48)	-	-
none	TD	-	-	-	60
U	TD	-	-	-	57
U, TD	TD	-	-	-	56

Table 5 Effect of U and/or TD on RD induction by FDU.

One volume of a 24 hr-culture was inoculated in 49 volumes of media containing FDU (80 μg/ml) supplemented with U (200 μg/ml), U (200 μg/ml) plus TD (80 μg/ml), and without the supplements. At 12 hours of incubation each culture was divided into two portions, and cells of one of them were plated. Cells of the other portion to which U (200 μg/ml), TD (80 μg/ml), or neither one was supplemented, were plated after 12 hours of incubation. Values obtained by plating on the agar medium containing 50 μg/ml of U are parenthesized.

Discussion

It is considered as mentioned above that FU is incorporated into RNA and leads to misformation of protein, which can be reversed by simultaneous addition of U, while FU inhibits DNA synthesis through its conversion to fluorodeoxyuridylate, this being reversed not by U but by T or TD (12). The present investigation eliminated the possibility that FU causes death of yeast cells by inhibiting cell well synthesis, as is known in bacteria (15, 16).

Yeast growth as determined by an increase in the amount of cellular substances as well as in the cell numbers was not inhibited by bromodeoxyuridine or bromouracil, but it was markedly inhibited by FU. The inhibition by FU was partially recovered by supplementing U, but further supplementation of T did not improve the recovery. It was tried to get T or TD requiring auxotrophic mutants from a haploid yeast strain by using various agents and methods, but

all efforts were unsuccessful. This may suggest that this yeast can not utilize extraneous T, TD and thymine analogs, owing, for example, to impermeability to them or absence of thymidine kinase. If so, it is understandable that FU inhibition was not completely recovered by either U or U and T, as was the case with *E. coli* infected and uninfected with a bacteriophage (12). The assumption is supported by the findings (Fig. 4) that a later addition of U to FU-containing cultures did not improve the recovery from the FU-effect.

It was shown that FU and FDU considerably induced RD mutants. As to the effect of FDU on the growth and RD induction U showed a slight reversing effect, and that only during an early period of the treatment.

Unpublished experiments have shown that an amino acid analog, ethionine, is also a powerful inducer of RD mutation. About half of colonies which produced RD mutants appeared as a sector or sectors even on the peptone-containing plate after ethionine treatment (19). It has been suggested that synthesis of protein and/or RNA is required for the initiation of DNA replication (20). Proteins modified by incorporation of an amino acid analog or proteins directed by FU-containing messenger RNA may interfere with the replication of the nochromosomal gene and result in producing RD mutants. The fact that cell division was increased and RD mutation induction was alleviated by U (Fig. 8) in an early period of FDU-treatment may be explained by assuming that FU, formed by splitting of FDU, inhibited the replication of the nonchromosomal gene in that way and so the disturbance was recovered by U. This consideration is based on the observation that the nonchromosomal gene seems to be much more labile than chromosomal genes and its mutation can be induced by various agents with high frequencies.

Thymine deprivation of a thymine requiring mutant of *E. coli* was first reported to cause "thymineless death" (21) and mutation (22). A mutagenic activity of FDU was reported in another fungus, *Neurospora* (23). As shown in Table 3, FU induced nuclear gene mutation, namely, to copper resistance, but the frequency was much less than that of RD mutation. Assuming that the site of lethal effect of UV irradiation is on chromosomal genes and that of RD mutation of it is on the nonchromosomal gene, Uchida (24) concluded that the activity of dark repair of UV damage is very weak in the "cytoplasm". Pauling & Hanawalt (25) pointed out that dark repairing enzyme(s) may also contribute to the recovery from "thymineless death". The weak activity of dark repairing mechanism in yeast "cytoplasm" may be a factor for the unstable nature of the nonchromosomal gene. Therefore the assumption that RD mutants induced by FU and FDU are for the most part nonchromosomal may be verified.

From these and other observations it is postulated that FU and FDU, intracellularly inhibiting synthesis of thymidylate, may cause thymineless state in yeast cells and induce RD mutation. If this is so, one can suppose that the nonchromosomal gene is of DNA nature rather than of RNA nature.

General occurrence of DNA in mitochondria of yeast and other organisms seems to be established (4, 26) and mitochondrial DNA is going to be accepted as

a molecular basis of the nonchromosomal gene. However, Uchida (24) is rather sceptical about it according to his experimental results in which RD mutation by UV was studied in various culture stages.

Further investigations are necessary for the elucidation of the unstable nature and localization of the nonchromosomal gene of yeast.

Acknowledgements

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