ADVANCES IN THE STUDY OF RESPIRATION-DEFICIENT (RD) MUTATION IN YEAST AND OTHER MICROORGANISMS[1]

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I. INTRODUCTION

Investigation of respiratory deficiency in yeast has opened a unique field of microbial genetics in respect to the so-called biochemical mutations. Various types of yeasts, especially species of *Saccharomyces*, which possess both respiratory and fermentative mechanisms to provide the energy needed for living, sometimes lose the former permanently but still maintain life by means of the latter. This loss has been found to be heritable through both vegetative and sexual reproductions when the genetic lineage is carefully traced. The respiration-deficient yeast also shows a clear-cut difference from the respirationally normal yeast with respect to morphological, physiological, and enzymatic characters, and hence constitutes a class of mutant.

The respiration-deficient mutant of yeast is less efficient in the utilization of sugars than the normal yeast and is, therefore, slower in growth. Colonies of the mutant are much smaller than the normal colonies when the sugar concentration in the nutrient medium is low. This outstanding character first drew the attention of French investigators. They gave, therefore, the designation "petite colonie" to the mutant. By deliberately performed cross breeding, they found that the mutation is effected by a certain factor which resides in the cytoplasm as well as by a nuclear gene mutation. It was inferred that the "cytoplasmic" factor which controls the formation of a series of respiratory enzymes is autonomous in its replication but is dependent on a dominant nuclear gene in its function. The inspiring, imaginative monograph by Ephrussi (18) is an outstanding landmark which has led many interested newcomers into this frontier of genetics, biochem-

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istry, and cellular physiology. However, it still leaves various problems yet to be solved by further investigations. It is also noteworthy that several Americans before Ephrussi independently noticed the occurrence of respiratory deficiency in yeast. Such defective cultures were produced by exposure to cyanide (128) and ethylene oxide (137). The genetic pedigrees of defective cultures were traced but got lost in a few generations (59: Chapter 15; Depletion mutation).

The present review deals with the recent advances in the studies of respiration-deficient mutation in yeast and other microorganisms, to present a broad survey of various findings in relation to fundamental biological problems such as growth, differentiation, inheritance, selective toxicity, and adaptation. It covers mainly the information accumulated since 1953, during which period these investigations have proceeded into a second era of progress.

II. Types of the RD Mutants in Yeast

Respiration-deficient mutants of yeast besides the "petite colonie" mutant have been encountered in various independent investigations. The colony size has not always been "petite." They appear in various designations, although identical to each other at least with respect to the phenotypic respiration deficiency. They have been called *W* variant (145), *aer* (60, 94), secondary colony variant (138), M_k mutant (113) and R^- (144). Therefore, a simple abbreviation, RD, after a recent work (72) will be used as the general designation in this review to comprise those respiration-deficient mutants. The RD mutants have been found or produced not only in *Saccharomyces cerevisiae* (baker's and brewer's yeast) but also in *S. chevalieri*, *S. carlsbergensis*, *S. saké*, *S. ellipsoideus* (wine yeast), and the Carbondale hybrid yeast. A pathogenic yeast, *Candida albicans*, also gave rise to a similar mutant (3).

With respect to genetic behavior, two types of the RD (petite) mutant, namely, vegetative (cytoplasmic) and segregational (nuclear), have been described. Vegetative RD mutants are frequently produced by exposure to various chemical agents which will be mentioned later. Ultraviolet (UV) radiation produces vegetative, segregational, and also double mutants which have mutated in both cytoplasmic factor and nuclear gene (18, 104).

An important finding on the vegetative RD mutants is a new factor, suppressiveness (25). Some haploid, vegetative RD cultures gave rise to RD zygotes when mated with normal haploid cultures of the opposite mating type, and were designated as "suppressive" petites. Other RD cultures, hitherto known to produce only normal zygotes by mating with normal cultures, were accordingly designated as "neutral" petites. The RD mutants produced by heat also showed various degrees of suppressiveness as well as neutrality (117, 118).

Ordinary yeast cultures contain about 1% or less of RD mutants in the populations grown under normal conditions. But some strains, especially haploids, are unstable and contain the RD mutants in very high frequencies even when cultured under normal conditions. It is likely that the cellular metabolism of such unstable strains is much different from that of stable strains (*see* Section IV-D).

III. Methods of Diagnosis

The RD (petite) mutant drew attention first by virtue of its small colony size. This is a fairly good criterion when the sugar concentration is appropriately limited. More precise methods of diagnosis are based on differences in enzymatic characters. Spectroscopic examination shows the absence of the absorption bands of cytochromes *a* and *b* in the RD mutant. Lack of cytochrome oxidase in the mutant makes the Nadi test negative. However, the colony size is often unreliable. Small colonies are not necessarily RD mutants. It is extremely time consuming to perform the Nadi or spectroscopic test on all the colonies. The diagnosis up to 1954, especially the populational scoring of the mutants, inevitably involved errors due to such difficulties.

Later came another line of diagnostic methods based on differential growth. Carbon sources which can be utilized only by oxidative degradation do not support the growth of the RD mutant. Nutrient media, in which sugar is omitted (104) or replaced by acetate (93, 145), lactate (94, 145), glycerol, and succinate (145), are effective in differentiating the RD mutant from normal yeast. Lactose and rhamnose are also useful for nonfermenters of these sugars (149). Combinations of the replica plating technique with such differential media were found to be useful to identify small numbers of normal colonies mixed

with the RD mutants in the majority (95, 144).

The third and the most useful method recently developed employs 2,3,5-triphenyltetrazolium chloride (TTC) as the color indicator (78, 95, 145). Melted soft agar (1 to 1.5%) containing 1 mg per ml of TTC is gently poured onto the colonies grown on a normal nutrient agar. Plate cultures thus covered are incubated at 30 C. Very clear-cut color differentiation between normal (red) and RD (remaining white) colonies appears in 1 to 3 hr as shown in Fig. 1. This method facilitates the most precise and efficient diagnosis of numerous colonies (up to 500 per dish) at once without regard to the colony size and other unreliable criteria. Furthermore, the color differentiation reveals sectoring and variegation as combinations of red and white regions in dubious colonies, which have been designated as "scalloped" (*festonné*) in Ephrussi's description (18, 22). Recently improved modification of this method employs TTC cut down to 0.5 mg per ml and 5 mg per ml of glucose to economize and speed up the procedure (79, 150). Validity of this method has been repeatedly confirmed by other work (72, 117, 118). Exact nature of the enzymatic locus effecting the reduction of TTC into red pigment (formazan) is not yet identified, although succinic dehydrogenase, which catalyzes the succinate-to-fumarate conversion at a redox potential near that of TTC, and which is absent in the RD mutant, is assumed to be responsible. Differential staining of colonies was also tried in some earlier work by adding TTC to the nutrient agar medium (103, 138). Normal colonies stained red, but the RD colonies also appeared red tinted. Color differentiation between the two types was therefore vague. As it was later demonstrated that TTC by itself produces the RD mutant (57, 145), to diagnose the mutant by means of TTC added to culture medium was found to be impos-

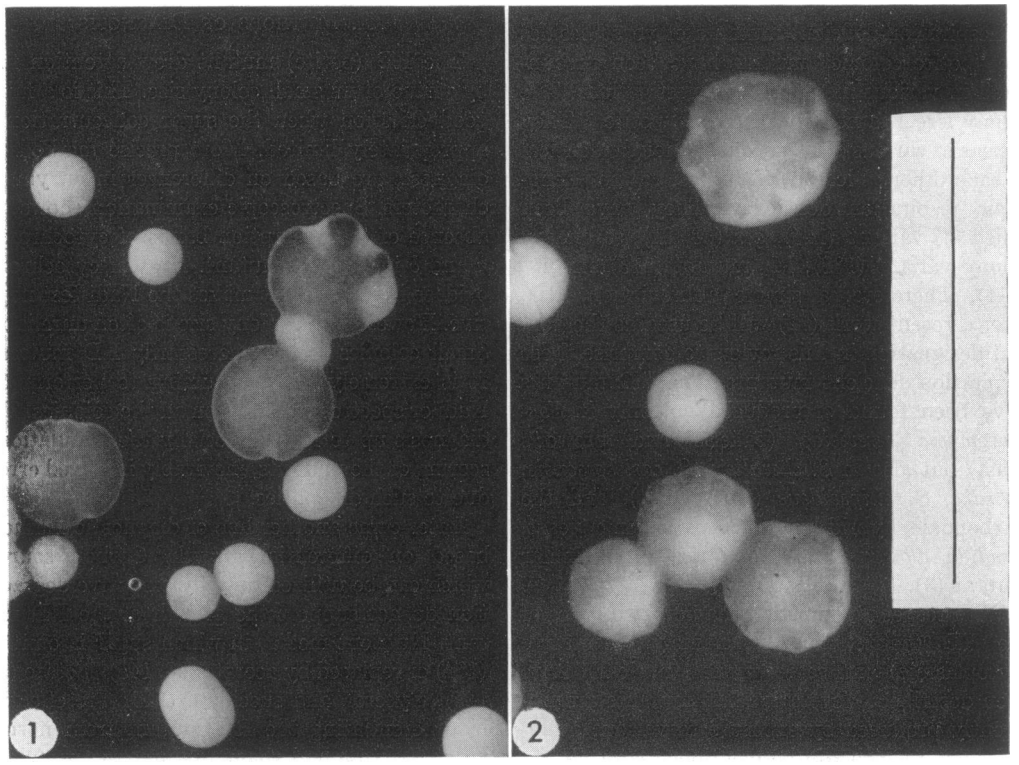

FIG. 1. *Normal, RD, and sectored colonies of baker's yeast diagnosed by the triphenyltetrazolium chloride overlay after Nagai (79). Normal (dark) colonies and regions are red in the actual color.*

FIG. 2. *Normal looking (large) and RD colonies of an unstable strain (R6U2) under the triphenyltetrazolium chloride overlay. Note the red (dark) top and the white fringe. Scale indicates 1 cm.*

sible, and this procedure was replaced by the TTC-agar-overlay method.

Another diagnostic method by means of color differentiation was worked out by Russians (31). It employs the leuco base of methylene blue added to the nutrient agar. Normal colonies grown thereupon stain blue, whereas the RD colonies remain white.

Color differentiation accompanying the RD mutation also appears in the adenineless mutant of yeast which develops a light red color in the respirationally normal state. The RD mutants derived from such red cultures turn brown, orange, or white, and are visually distinguishable from the normal strain. Several investigations of the RD mutation used such red cultures for experimentation (44, 61, 109).

IV. Induction and Prevention of the RD Mutation by Various Agents

The frequency of the RD mutants spontaneously appearing in ordinary yeast cultures is in the neighborhood of 1%. Such a spontaneous level is independent of the ploidy and is appreciably high as compared with that of other gene-controlled biochemical mutants. Furthermore, application of various physical and chemical agents increases the mutant frequency up to 100% under appropriate conditions. Generally speaking, these agents are more or less injurious to the yeast cells and kill them when applied in excess. Some of them have been known to be mutagenic to species of *Drosophila*, *Neurospora*, and some bacteria.

A. General Survey of the Inducers

1. Chemicals. The chemical agent which has been most extensively studied is acriflavine, an antiseptic acridine dye. It was used throughout the early works of Ephrussi and his collaborators. It was later found that the active component in pharmaceutical preparations (pharmacopoeia specifications in many countries except Japan, where the JP specifies euflavine as the sole component) of acriflavine is euflavine (3,6-diamino-*N*-methyl-acridine chloride, numbering after *Chemical Abstracts*), whereas proflavine (3,6-diamino-acridine sulfate), constituting about one-third, is only slightly effective. Further investigation on the relationships between the chemical structure and the RD-inducing effect was made by Marcovich (66) using some acridine derivatives and basic triphenylmethane dyes. This author seems, however, to have had some difficulty in diagnosing the RD mutant simply by the colony size because of the considerable variability of the colony size when some dyes other than euflavine were tested. Although involving some errors unavoidably, appreciable production of the RD mutant close to 100% was observed with 3-amino-*N*-methyl-acridine and rosanilin in addition to euflavine. Pararosanilin, pentamethyl-, and hexamethyl-rosanilins were also tested but were vaguely stated to have somewhat increased the frequency of the RD mutant. A serious ambiguity appeared with 9-amino-*N*-methyl-acridine. Further information on the acridine derivatives was presented by Slonimski (120). The effects of synthetic dyes were later tested by Japanese investigators more extensively and precisely employing the TTC-overlay technique for diagnosis. Purity of the dyes was also examined by means of paper chromatography (79, 149, 150; *see* Table 1).

Another agent of particular interest is TTC, which was found to be similarly effective in producing the RD mutants (57, 145). The relationship between the RD-producing effect and the usefulness in the diagnostic method (color differentiation) has not yet been fully explained.

The next group of agents includes heavy metals such as copper, nickel, cobalt, and manganese (62, 81, 83, 146, 147). Manganese (chloride and sulfate) was found to be most effective in producing the RD mutants while being weak in toxicity.

Some enzyme inhibitors were also found to be effective. Sodium azide apparently produced the RD mutants, although they were designated under a vague name, "secondary colonies," by the authors (138). Crystal violet and brilliant green were also tested and found to be effective in the same studies. Separate and more systematic investigations demonstrated that sodium azide and some nitrophenols produce the RD mutants. Among them, 2,4-dinitrophenol and *p*-nitrophenol are fairly strong, whereas the *ortho* and *meta* isomers are far less effective (148).

The last group of miscellaneous chemicals includes allylglycine (111), camphor (31), caffeine (82), and some antifungal antibiotics (72). Various antifungal antibiotics were tested for dif-

TABLE 1. *Classified list of chemical agents found to produce the RD mutants out of normal yeast inoculum*

Chemical agent	Effective concn		Reference
	mg/liter	*M*	
Basic dyes:			
Acriflavine	0.4–1.5	$1–5 \times 10^{-6}$	79, 124
3-Amino-*N*-methyl-acridine	0.2–2.0		66
Crystal violet	0.2–0.6, 6–18		79, 138
Ethyl violet	0.2–0.4		79
Methyl violet	2.0–4.0		79
Malachite green	1.0–3.0		79
Brilliant green	10.0–12.5		138
Rosanilin	2.0–10.0, 3.0–8.0		66, 79
Pararosanilin	2.0–6.0		79
Victoria blue 4R*	6.0–10.0		79
Victoria blue B*	6.0–20.0		79
Pyronin B	2.0–6.0	$0.5–1.0 \times 10^{-5}$	79, 149, 150
Pyronin Y	2.0–6.0		79
Acridine red 3B	12.0–40.0		79
Acridine orange*	100		113
Phenosafranin	6.0–80.0		S. Nagai, *unpublished*†
Pinacryptol green	50.0–200.0		
Safranin T	4.0–100.0		
Safranin O	8.0–100.0		
Mauveine	80.0–240.0		
Amethyst violet	4.0–50.0		
Janus green B	0.6–3.0		
Night blue*	1.0–5.0		
Heavy metal salts:			
$CuSO_4$	130–250	$0.5–1.0 \times 10^{-3}$	62, 146, 147
$CoCl_2$	130–250		62
$NiCl_2$	380–500		62
$MnCl_2$, $MnSO_4$	1,700–3,300		62, 81
$CdCl_2$		$0.6–1.0 \times 10^{-3}$	H. Nakamura, *unpublished*‡

TABLE 1—*Continued*

Chemical agent	Effective concn		Reference
	mg/liter	*M*	
Enzyme inhibitors:			
Sodium azide*	2.6–3.1	$0.05–0.1 \times 10^{-3}$	138, 145, 146
p-Nitrophenol*		$0.5–1.0 \times 10^{-3}$	145, 146
2,4-Dinitrophenol*		$0.1–0.2 \times 10^{-3}$	N. Yanagishima, *unpublished*‡
Miscellaneous:			
TTC	0.01–0.1, 80–100		57, 145
Camphor	2,500		31
Caffeine	1,500–2,500		79, 82
Allylglycine		2×10^{-3}–2×10^{-2}	109

* Selective against RD mutant.

† Test organism and procedures identical with those of Nagai (79).

‡ Test organism and procedures identical with those of Yanagishima (145).

ferential toxicity upon prototrophic (wild type) and auxotrophic (mutant) yeasts in an attempt to isolate the mutants out of the mixed cultures in a manner similar to the penicillin method for isolating auxotrophic mutants of bacteria. Some of them such as Endomycin and Amphotericin B strongly inhibited the prototroph while allowing the auxotroph (adenineless) to grow. However, these antibiotics produced RD mutants from the prototroph but not from the auxotroph. As prototrophic RD mutants thus produced were, unfortunately, more resistant to the antibiotics than the respirationally normal prototroph and grew as well as the auxotroph the trial was only partially successful.

The efficacy of these agents apparently depends on the test organism and culture conditions, particularly temperature, and composition of the basal medium to which those agents are added. It is therefore difficult to compare the efficacy simply by the mutant production versus the doses (concentrations) in various researches performed separately. But, an approximate comparison shows that the effective doses of most of

the agents range from 10^{-6} to 10^{-5} M. Outstanding exceptions are manganese, camphor, and caffeine, which are effective at 10^{-2} M (0.2 to 0.3%).

Another problem of interest is the relation of the RD-inducing effect to the lethality (120). Most of the chemical inducers of the RD mutation tested in series of concentrations bring about the RD mutant production in the ranges where the total populational growth is only slightly inhibited. Further increase in the concentration leads to decreased mutant production and finally to the entire suppression of growth (79). Marcovich's observation with euflavine, 3-amino-*N*-methyl-acridine, and rosanilin shows the same trend as well (66). In addition, the effect of euflavine depends on the pH in both the RD induction and lethality. Higher concentration is required when the pH is lowered from 7 to 3. An almost concentric zonation appears between the two effects with respect to the concentrations required at each of the pH values tested (67). The RD-inducing action in this respect appears, to a certain extent, to be parallel with lethal action so far as the effective RD-inducers are concerned, although many other agents are toxic but are ineffective to induce the RD mutation.

2. Ultraviolet (UV) radiation. It was found by Raut that UV radiation produces the RD mutants from the survivors in very high frequencies (103, 104). The effect was actually an induction and not a selection in favor of the mutants due to a differential killing of normal and mutant cells. Furthermore, some of the RD mutants thus produced were nuclear gene mutants (segregational RD). Some of the nuclear mutants showed frequent reversion to the respirationally normal state. Double mutants (RD for both the cytoplasmic factor and nuclear gene) were also produced. X-ray only killed the cells but did not produce any RD mutant (105). Similar experiments repeated later (98) also confirmed the effect of UV radiation. A recent finding of interest is the photoreactivation phenomenon (109), in which the production of the RD mutants by UV radiation was reduced by subsequent exposure to visible light. The photoreactivation was observed only with the aerobically adapted (normally respiring) yeast, and not with aerobically unadapted (immediately after harvesting from anaerobic culture). Survival of the irradiated yeast (both aerobically adapted and unadapted) showed remarkable differences depending on the medium on which the irradiated cells were plated. Survival before the photoreactivation was better on lactate medium than on glucose medium with both aerobically adapted and unadapted cells, whereas the survival after photoreactivation was the same on lactate and glucose media with both the adapted and unadapted cells. Later investigations (100, 101) showed that the photoreactivation occurs similarly to the killing by UV radiation, and that the production of the RD mutants by UV radiation was independent of the ploidy when haploid, diploid, and tetraploid yeasts were collaterally tested.

3. Heat. Production of the RD mutants by elevated temperature was first reported by Yčas (151). A parallel but more detailed investigation was carried out by Sherman (116–118). Diploid and haploid yeasts cultured at 38 to 40 C gave rise to the RD mutants which finally constituted 10 to 100% of the population, the frequency depending on the respective strains tested.

The actual induction of the RD mutation was proved through both populational estimation and single cell (bud) isolation by a micromanipulator (*see* Section IV-B). It was noticed that only very rich nutrient media containing 2 to 6% yeast extract could support the unarrested growth of the yeast at such high temperatures. Heat shock (54 C, up to 80 min) killed about 99% of the yeast cells but produced the RD mutants constituting up to 40% of the survivors. Furthermore, the RD mutants were always more sensitive to heat than the original (normal) cells.

4. Anaerobiosis. Much attention has been paid to the effect of anaerobic cultivation on the production of the RD mutants. While it was claimed that haploid and tetraploid strains of Carbondale hybrid yeast cultured anaerobically produced the RD mutants at a rate above the spontaneous frequency (44, 61), another study with a diploid baker's yeast showed no increase in the RD mutants (37). The effect, if any, seems almost insignificant as compared with that of other agents, namely chemicals, UV radiation, and heat. On the other hand, the yeasts precultured anaerobically (aerobically unadapted cells) were more susceptible to the RD-inducing effect of acridine dyes, manganous ion, and UV radiation, respectively (111). Furthermore, the production of the RD mutant by *p*-nitrophenol was more ef-

fective under anaerobic conditions than under aerobic conditions (145). The photoreactivation phenomenon in relation to the effect of UV radiation was not observed with the cells precultured under anaerobic condition (*see* Section IV-A-2).

B. Proof of the Induction

Laborious experiments were made in earlier work to demonstrate that the increased production of the RD mutants in the presence of a given agent was due to actual induction and not to selection in favor of the mutants. The critical examinations have been achieved by: (i) removing the new buds formed in the presence of euflavine by means of a micromanipulator and finding the great majority of them to be RD while the mother cell still remained normal (21); (ii) allowing a normal inoculum to grow in the presence of acriflavine (euflavine) and finding the accumulation of the RD mutants in the population at a rate higher than that of the previously isolated RD culture growing alone under the same conditions (23, 118); and (iii) isolating many single cells (by means of a micromanipulator) from a homogeneous normal culture, placing them separately in drops of nutrient media with and without TTC, allowing them to grow into small clones (about 200 cells), respectively, and finding the mutation rate calculated by the Luria-Delbrück test to be higher in the lot of clones exposed to TTC than in the control (57). It was demonstrated later that the use of a selective culture medium, which does not permit the proliferation of the RD mutants (*see* Section III), considerably simplifies the procedure to prove the induction. With respect to acriflavine (80), manganese ion (81), caffeine (82), copper (149), and pyronin B (150), a significant increase in the RD mutants in both number and proportion was found when the normal yeast was allowed to proliferate in glycerol medium containing those agents, respectively. It may be said that the above-mentioned procedure (ii) is simplified by eliminating one fundamental factor, namely, the self-multiplication of the RD cells which have been contained in the inoculum or produced during the subsequent proliferation. There remained, however, a possibility that under these conditions some metabolic products of the growing normal cells might be fed to the RD cells and enable them to proliferate. To test this possibility, RD cells were embedded in agar discs and immersed in liquid glycerol medium containing copper or pyronin B inoculated with normal cells. The RD cells in agar showed no proliferation, whereas the normal cells grew well in the surrounding medium, and hence such a possibility was ruled out (149).

It is obvious that the increase in the exact mutation rate in terms of the mutant production per cell per generation as determined by (iii) should be the convincing evidence for the induction by a given agent. The procedure to determine the mutation rate was much simplified by Ogur et al. (96) by means of a selective medium which contained lactate (*see* Section III). It was demonstrated through both mathematical derivation and experimental estimation that the relative frequency of the RD mutant equals the mutation rate when population equilibrium has been reached after growth in exponential phase in a medium which is totally selective against the RD mutant. The exponential growth was maintained by successive transfer every 24 hr and incubation at 30 C with shaking. Mutation rates in various stable and unstable strains (*see* Section IV-D) were found to range around 1 per generation per 10^3 cells in stable cultures and up to 20 in unstable cultures, respectively. Although these authors did not test the cultures supplemented with inducers under the corresponding conditions, it is almost doubtless that increased mutation rates over the control will be found in such cultures and hence fortify quantitatively the validity of the above-described procedure using a selective medium to prove the induction by the given agent (inducer).

Apart from using selective media, Slonimski and de Robichon-Szulmajster (124; *see* Section IV-C) employed a highly buffered, glucose-rich synthetic medium and restricted the populational scoring to an early period of the growth (within 8 hr after inoculation). Competition and selection were excluded under such conditions and hence the changes in the RD mutant frequency could be ascribed to the actual induction by euflavine and its modification by additional cofactors.

Although the actual induction of the RD mutation has been proved by various procedures, either complicated or simplified, for most of the agents hitherto tested, some agents are by themselves selective against the RD mutant. Sodium azide, *p*-nitrophenol, 2,4-dinitrophenol (145), acridine orange (113), cyanide (117), Victoria blue

B, Victoria blue 4R, and night blue (S. Nagai, *unpublished data*) were found to suppress entirely the growth of previously isolated RD mutants in the concentration ranges at which those agents produce considerable RD mutants from normal inoculum. It is, therefore, automatically clear without any particular operation that those agents actually induce the RD mutation. In the case of elevated temperatures also, the RD mutants were found to be always more sensitive to heat than the normal cells (117, 118).

C. Interactions between Some Inducers and Other Agents

Various types of antagonistic interactions have been known in metabolic and mutagenic processes. For instance, competitive enzyme inhibitors hinder the action of enzymes on their normal substrates, the inhibition being reversed by addition of the normal substrate in excess. Plant auxins are antagonized by antiauxins. Effects of mutagens are alleviated by antimutagens. On the other hand, promotive or synergistic interactions are also encountered in various cases. Partly similar but partly dissimilar situations have been found with respect to the induction of the RD mutation as well. Some extra agents applied in addition to the inducer often modify the effect of the latter and lead to decreased or increased production of the RD mutants. Analysis of such situations will give an important clue to the mechanism of induction of RD mutation.

Addition of some nucleic acid components (adenine and guanine, either separately or in combination) and "cofactor E" (derived from erythrose, a C_4-sugar) counteracted the effect of acriflavine, whereas "cofactor T" (derived from threose, another C_4-sugar) stimulated it (124). Addition of adenosine and guanosine in combination remarkably counteracted the effect of caffeine induction of the RD mutation (82). Production of the RD mutants by allylglycine was prevented by addition of sulfur-containing amino acids such as cysteine, homocysteine, and methionine (111). The extra agents in the cases mentioned above are not inducers when applied alone, respectively, to the yeast. They appear in some respects to shield or repair the process of the production of respirationally normal progeny from normal mother cells against the attacks by the inducers.

Quite interesting findings came from tests of the combinations of some agents which are known to induce the RD mutation when applied singly in sufficient doses (S. Nagai, *unpublished data*). Combinations of an inducer in sufficient dose and another inducer in insufficient dose (too small to induce the RD mutation when applied singly) were made between acriflavine, pararosanilin, pyronin B, pyronin Y, acridine red 3B, and caffeine. As seen in Table 2, the effects of the respective inducers were either counteracted or promoted, depending on the partners of the combinations. Total populational growth was not significantly affected throughout the tests. Such interactions are quite similar to an earlier finding called "therapeutic interference" in the chemotherapy. Indeed, the trypanocidal effect of acriflavine was antagonized by small (subtherapeutic) doses of pararosanilin when those two dyes were applied together to the trypanosome-infected mice, the therapy being annulled (6). Some interpretations presented by Albert (2: Chapter 3) comprising a number of later findings appear to apply to the interferences between the inducers of the RD mutation as well, although pararosanilin in this case affects promotively when supplemented to acriflavine. The possibilities are: (i) the two agents compete with each other for a common receptor (the "Chemorezeptor" in the Ehrlichian view) and one of them, which has a stronger affinity for the receptor, excludes the other; (ii) the two agents bind with two different but closely neighboring receptors, respectively, and one of them, which occupies the receptor of lesser importance, spatially blocks the access of the other agent to the other vitally important

TABLE 2. *Interferences between some selected inducers of the RD mutation in yeast*

Effect of sufficient doses of	Counteracted by insufficient doses of	Promoted by insufficient doses of
Acriflavine (AF)	PB, CA	PR, PY
Pararosanilin (PR)		AF, PB, PY, AR, CA
Pyronin B (PB)	CA	PR, PY, AR
Pyronin Y (PY)	CA	PR, PB, AR
Acridine red 3B (AR)	CA	PR, PB, PY
Caffeine (CA)	PR, PB, PY, AR	

Based on unpublished data of S. Nagai. Test organism and procedures identical with those of Nagai (79).

receptor; and (iii) the two agents react with each other to form micelles, namely temporary polymeric association [*see also* (69) for further details] and the actual concentrations of the therapeutically active monomers become reduced. Further investigations are needed of the interferences between the RD inducers with attention to these possibilities. In the experiment with *p*-nitrophenol also, addition of 2,4-dinitrophenol and sodium azide increased the production of the RD mutants above the level accomplished by a given dose of *p*-nitrophenol alone, whereas *p*-chloromercuribenzoate, sodium fluoride, and monoiodoacetate decreased it, respectively (148).

Another type of counteractive effect is the photoreactivation by visible light reducing the effect of UV radiation (*see* Section IV-A-2).

D. Unstable Strains

Some yeast strains, especially haploids, are inherently unstable and produce the RD mutants in unusually high frequencies. Their populations in equilibrium in ordinary sugar media often contain more than 50% RD mutants. Such an instability was found to be a single-gene-controlled recessive character, segregating regularly into two-to-two out of the crosses between some stable and unstable strains (22). Not much progress has been made with respect to the nature of the instability as compared with the induced production of the RD mutants, although the estimation of the mutation rate in those unstable strains was much simplified recently (*see* Section IV-B), and an unstable strain of *Saccharomyces carlsbergensis* was derived by a serial selection out of an originally stable culture (34). A phenomenon of the phenotypic lag in such unstable strain was demonstrated by means of a selective culture medium (97).

An unstable strain investigated by James and Spencer (54) showed an extreme complexity because of the appearance of "abortive cells" in addition to normal and RD cells. Furthermore, an appreciable fraction of the cells in a population went extinct when plated out. They were alleged to be lethal variants, death of which could be, however, averted by the RD mutation. It was also inferred that the production of the RD mutant in such an unstable culture occurred as a chance phenomenon rather than as a result of sequential processes in cell metabolism.

A preliminary attempt to impose an experimental regulation upon such an instability was informally communicated by Slonimski and de Robichon-Szulmajster (124) in connection with the cofactors affecting the production of the RD mutants (*see* Section IV-C). Addition of adenine and cofactor E increased the production of the RD mutants by an unstable strain, whereas the production was decreased by cofactor T. Effects of those cofactors appeared in an inverse relationship as compared with the cases of a stable strain exposed to euflavine.

A recent finding on the effect of caffeine upon an unstable haploid strain is of particular interest in this respect (S. Nagai, *unpublished data*). Small doses of caffeine (not more than 1,200 mg per liter), which are ineffective to produce the RD mutants from normal baker's yeast, added to an ordinary glucose medium considerably reduced the spontaneous mutant frequency in this strain from 50% in the control down to about 5%. Normal looking (large) colonies of this strain growing on a caffeine-free agar medium appeared scalloped in outline and, when overlaid with TTC-agar, revealed a white fringe surrounding a red, serrated top in the center, as shown in Fig. 2. On the agar medium containing caffeine, however, the large colonies appeared smoothly round and solidly red under the TTC overlay, just like the normal colonies of a stable strain shown in Fig. 1. Doses of caffeine from 600 to 1,200 mg per liter thus reduced the heterogeneity of normal looking colonies (clones), while not influencing the growth of RD colonies at all. Caffeine in this case appears to exert a stabilizing effect upon such an unstable strain.

V. Enzymatic Changes Involved in the RD Mutation

The RD (petite) mutants have been known to be lacking in cytochromes *a* and *b*, but they show the absorption band of cytochrome *c*, which appears even stronger than that of the normal yeast (122). On the other hand, one of the haploid RD mutants produced by exposure to UV radiation was lacking in cytochromes *a* and *b*, and showed only a faint band for cytochrome *c* (103). This mutant strain (W1) appeared quite similar to the segregational RD mutant when crossed with other haploid strains either normal or RD. Later investigation showed that it was deficient in catalase and hematin and had a metabolic block, perhaps another single-gene mutation different from

the segregational RD, in the pathway of glycine synthesis. This block makes the synthesis of cytochrome *c* inoperative, for glycine is needed as a precursor. In fact, an enriched supply of glycine and of protoporphyrin restored the formation of cytochrome *c* and catalase (153, 154).

Although the RD mutants are unable to respire at all, it was noticed that even normal yeast is unable to respire immediately after being harvested from culture under anaerobic conditions. The anaerobically grown yeast lacks some respiratory enzymes at first but later forms them adaptively when exposed to oxygen. This development of respiratory competence, designated as "*adaptation respiratoire*" by Slonimski, takes place independently of cell proliferation (119–121, 123). It appears that oxygen induces the synthesis of respiratory enzymes in general. Magnitude of the oxygen-induced increase in the activity of the respective enzymes is greater the closer the enzyme is to oxygen in the chain of the redox system. The respiratory adaptation is inhibited by cyanide, amino acid analogues, and some other toxic agents. Acriflavine is the most powerful inhibitor of this process at about the same concentrations as those employed for production of the RD mutant. Other closely related compounds such as acridine derivatives are more or less inhibitory in good correspondence to their efficacy in producing the RD mutants. Those agents, however, do not inhibit the activity of the enzymes already formed before exposure to the inhibitor. It is, therefore, inferred that the RD mutation is induced by the specific block of the formation of respiratory enzymes. It is also mentioned that 2,4-dinitrophenol inhibits the respiratory adaptation while being unable to induce the RD mutation (120). However, it is shown by later work that 2,4-dinitrophenol, as well as *p*-nitrophenol, induces the RD mutants in *Saccharomyces ellipsoideus* (N. Yanagishima, *unpublished data*). Anaerobically grown yeast is generally unable to show photoreactivation after exposure to UV radiation, whereas the photoreactivation is remarkable in the normally respiring yeast (100, 101, 109). A kinetic investigation of the respiratory adaptation was performed with respect to cytochrome oxidase and the succinic dehydrogenase complex (42). It was again confirmed that these enzymes are associated with the mitochondrial fraction of mechanically disrupted cells. These particles contained much lipid and showed a marked increase in the enzyme activities during the exposure to oxygen. Addition of ergosterol to the medium of preculture under anaerobic conditions increased the speed of subsequent formation of the enzymes. A recent reappraisal of the succinic oxidase system in the anaerobically grown yeast by an improved method shows that the succinic dehydrogenase level is about the same as that of the normally respiring yeast, whereas the succinic-cytochrome *c* reductase activity is absent (43). The situation is even more complicated by the alleged appearance of hemoglobin in the RD mutants and anaerobically grown yeast. A faint band at 580 mμ appearing in those yeast cultures is ascribed to hemoglobin. It disappears at the end of the respiratory adaptation of anaerobically grown yeast (56, 152). Disagreement on the interpretation of the variation in cytochrome bands during the respiratory adaptation still remains in spite of the improvement in the spectrophotometric measurements (64).

Although the RD mutant is unable to respire, it undergoes remarkable changes in respiratory enzyme composition as shown by Slonimski (121) and a team of Belgians led by Chantrenne (8). It lacks cytochrome *c*, cytochrome *c* peroxidase, and catalase when grown anaerobically. These enzymes are adaptively formed when the anaerobically grown culture of the RD mutant is subsequently aerated. Cytochrome *c* and cytochrome *c* peroxidase appear immediately after the beginning of aeration, but catalase appears after a certain delay. It is thus likely that there is a chain of induced enzyme formation. Various hydrogen donors such as ethanol and formate as well as exogenously supplied cytochrome *c* affect the speed of the enzyme formation (114). Moreover, tracer experiments show that the induced enzyme formation is accompanied by supplementary incorporation of adenine and uracil into cellular ribonucleic acid (RNA) (7, 8). UV radiation inhibits these processes to a certain extent (4), whereas X-ray affects them rather promotively (9). Slonimski and Tysarowski (125) also found that the anaerobically grown yeast has a particular type of lactic dehydrogenase (enzyme N), which is later converted into another type (enzyme O) during the respiratory adaptation.

Ephrussi (19) has presented a comprehensive survey of these enzymatic changes. Regarding the RD mutants and the respiratory adaptation, he considers the failure of respiratory enzyme

formation as an event which happens either to the apoenzyme or to the prosthetic group. The former may result from disturbance in the amino acid metabolism, and the latter from some defect in the pathway of heme synthesis, as seen in Raut's mutant (103). This view in the light of more recent knowledge may be extended to the macromolecular replication mechanism which controls the fabrication of those enzymes or their essential components (12).

On the other hand, a pathogenic yeast, *Candida albicans*, shows enzymatic characters quite different from those of *Saccharomyces* species. Its respiratory function is independent of the cytochrome system and is carried out by flavoproteins. The flow of hydrogen transfer, which is firmly coupled to the cell division mechanism in normal candidae, "spills over" in the filamentous (divisionless) mutant at a level of redox potential in the neighborhood of that of TTC (88, 90). This is an outstanding example which indicates that some different kinds of shunt mechanism are possible even within a group of organisms designated as yeast in a broad sense.

VI. Physiological Background of the RD Mutation

A. Possible Mode of Action of the Inducers

The work hitherto mentioned has been aimed, more or less, at the mechanism and process of the production of the RD mutants. Although the exact turning point from the normal to mutated state is not yet known, the possibilities conceived may be summarized as the following: (i) permanent inactivation or elimination of one or many of the respiratory enzymes; and (ii) inhibition of the formation of the enzyme(s) by a specific block or disturbance in the cell metabolism. The latter may involve: (iia) inhibition of the fabrication of the enzyme molecules from amino acids and perhaps hemes through a temporary inactivation of the assembly line, namely the template; and (iib) inhibition or alteration of the replication of the template through an intervention into the metabolism of the template material, perhaps RNA.

So much information on the inducers of the RD mutation and inhibitors of the respiratory adaptation has been accumulated recently that a presumptive survey of the mode of action of these agents with attention to the above-mentioned possibilities may be put forward. Concerning the chemical inducers of the RD mutation, at least three major classes, namely basic dyes, heavy metal salts, and respiratory enzyme inhibitors are distinguishable as seen in Section IV-A. Of particular interest is the chemical structure of the basic dyes and their interaction with some cellular constituents. Most of these dyes have been employed for staining of the nucleus and nucleic acids in cytological and cytochemical investigations. The relationships between efficacy as inducer and chemical structure also give some suggestions about the mode of action of these agents. Throughout the observations using acridine dyes, triphenylmethane dyes, xanthene dyes, and some azine dyes, the general trend in efficacy appears, within a certain limit, to depend on the basic strength which results from various substitutions in the skeletal structures of the respective dyes. However, the efficacy also appears to depend on the spatial configuration of the molecule, which affects the attachment or fit of the dye to some essential receptor of the cell.

In emphasizing the important relations of these properties to the antibacterial efficacy of various drugs, Albert (2: Chapter 8) suggests that the receptor of the antibacterial drugs of this class perhaps involves nucleic acids. Some earlier work (69, 73) on the interaction of basic dyes with nucleic acids indicates that the addition of RNA and deoxyribonucleic acid (DNA) to dye solutions shifts the spectral absorption maximum to a longer wavelength region and that the polymeric association of the dye molecules is concomitantly abolished. Recent work (13) with living ascites tumor cells also indicates that the nucleic acid forms in vivo a firmly bound complex with the dye and creates some changes in the spectral absorption as compared with that of free dye molecules. A general inference is that the dye molecules (cations), attracted by the acidic groups of the nucleotide, fit into the spaces between the planes of the purine and pyrimidine rings, owing to a certain characteristic structure which conforms to the configurational requirements imposed by the nucleotide structure. This view again suggests that these basic dyes, when acting as the RD inducer, bind with nucleic acid, perhaps RNA rather than DNA, and create a disturbance in the subsequent process of replication.

Such a disturbance in the metabolism of RNA, which plays an important part in the protein synthesis, may lead to failure in the formation of enzyme proteins. The problem of how such a dis-

turbance operates with a highly differentiated specificity, restricted to a few respiratory enzymes, is yet unsolved. Another task of future investigation will be to integrate these metabolic and physiological aspects with the "dilution model" derived from various observations in earlier work [*see* (117) for detailed discussions].

The inducers of other classes such as heavy metals and enzyme inhibitors also appear to act through some disturbance in the metabolic processes which are essential for enzyme synthesis. For instance, exposure to *p*-nitrophenol, an effective inducer, considerably reduced the affinity of normal cells for pyronin B (N. Yanagishima, *unpublished data*).

Another line of observations, namely the effect of cofactors on the euflavine-induced RD mutation and that of guanosine and adenosine counteracting caffeine, also suggests the significant role of nucleic acid as the receptor which is primarily affected by the inducers. The findings that UV radiation is most effective at 260 mμ approximately corresponding to the absorption maximum of RNA (105), and that manganous ion is effective when the ionic ratio Mn^{++}/Mg^{++} is greater than 4 (110), also suggest the significance of nucleic acid.

Those points of the physiologic view hitherto discussed may be applicable to other types of induced mutations (in narrow sense) which have rather been regarded as an abrupt, discontinuous change caused by an unspecific blow at a mysterious spot, namely the gene molecule. Indeed, a recent observation (127) of the interferences, either promotive or counteractive, between some mutagens in the induced mutation of *Escherichia coli* suggests the presence of certain receptor(s) corresponding to the mutagens of different types. Another fact of interest is that a certain metabolic conditioning related to protein or nucleic acid synthesis is needed for stabilization of the incipient mutations caused by various mutagenic treatments (16, 87, 129, 142).

B. Aftermath of the RD Mutation

A considerable variability often appears in the susceptibility of yeast cells to various toxic agents. When a toxic agent is potentially an inducer of the RD mutation, the variation in the susceptibility also affects the occurrence of the RD mutation. An example is the development of resistance to copper in various strains of yeast. The tolerable concentration range of copper depends on the inherent resistance of the yeast, but is variable when the yeast adaptively develops an increased resistance. Production of the RD mutant occurs during the process of adaptation which gives rise to more resistant progeny out of the survivors. Substrains which have thus acquired an increased resistance to copper produce far fewer RD mutants when again exposed to the same concentration of copper to which they are already resistant. In other words, the increased resistance to the toxic effect of copper is accompanied by the increased resistance to the RD-inducing effect (146, 147). Copper-sensitive cultures growing under the intoxication by copper are white and also remain white under the TTC overlay even when they are respirationally normal, whereas the copper-resistant, normal cultures growing in the presence of tolerable concentrations of copper are brown and also turn red under the TTC overlay (83). Copper-resistant cultures which have developed through adaptation are also brown in the presence of copper so far as they are respirationally normal. The brown color is developed by deposition of copper sulfide produced by reaction with the hydrogen sulfide which comes out of the reduction of sulfate by respirationally normal cells (85). The RD mutant culture shows far less ability to produce hydrogen sulfide and therefore fails to develop brown color (147). A fact of similar interest is that a concentration of azaserine which is strong enough to exert the mutagenic effect on a strain of *E. coli* is no longer effective on a mutant substrain which has developed an increased resistance to azaserine (53). On the other hand, the resistance of yeast to crystal violet was increased by successive transfers to higher concentrations. However, the cells were 100% converted into the RD mutants accompanying the increasing resistance to the dye. In other words, the increased resistance to the dye cost the yeast its respiratory competence (138).

Another feature of interest appearing in the RD mutant is the dimorphism phenomenon in wine yeast. Some RD mutant cultures show a noticeable alternation of unicellular (yeast form) and pseudomycelial phases depending on the cultural conditions, whereas the normal cultures always appear in the yeast form under the corresponding conditions. The dimorphic changes take place only when the cultures are almost deprived of fermentable substrates and are furnished

with sufficient oxygen (N. Yanagishima, *unpublished data*). From a recent finding that under sufficient oxygen supply an RD culture of brewer's yeast showed increased endogenous fermentation (degradation of carbohydrate and production of ethanol and of carbon dioxide) above the anaerobic rate (10), it appears clear that the RD mutants can respond to external oxygen (*see also* Section V). As the mycelial form seems to be ancestral compared to the yeast form in the evolutionary descent of the yeast organism, such a dimorphic reversion to the mycelial form may be in some relation to the loss of the respiratory competence and obligate dependence on the fermentative mechanism, which is ancestral in the biochemical evolution.

A pathogenic yeast, *Candida albicans*, shows dimorphic changes back and forth between the mycelial and yeast phases, but sometimes mutates to a permanently divisionless (filamentous) form. The changes appear to depend on the nutritional conditions and suggest an important relation of the respiratory mechansim to the processes governing the cell division which is necessary for the maintenance of the yeast form (88, 89).

VII. Cytological and Cytochemical Observations

In spite of extensive studies, controversies continue about yeast cytology, especially about the nucleus and mitochondria (140). Observations on the mitochondria with respect to the RD mutants were presented by some American and French authors, both having associates from Japan (24, 139). These discussions remain unreconcilable with respect to the shape and size of mitochondria, whether they are spherical, elongated, or threadlike, because only the extreme examples were shown in either of the differing views. Earlier work by another group of Americans (38, 74) showed that the mitochondria are present in the RD mutant cells as well as in the normal cells. They employed Janus green, Sudan black, Nadi reagent, and TTC to find the difference between the normal and mutant cells with respect to the respiratory status of the mitochondria. Apparent differences were seen between the mitochondria from the two types of yeast cells. However, the shape did not differ appreciably throughout the materials examined. On the other hand, a recent observation on the mitochondria of normal and RD yeast cells collaterally with normal and "slow growing" Neurospora mutants, which have some respiratory defects, shows that the mitochondria of the RD yeast and Neurospora mutants are long and threadlike, whereas those in normal cells are round. In addition, the normal Neurospora hyphae grown in the presence of acriflavine (0.005 mg per ml) produced long, threadlike mitochondria (26). The mitochondria of normal yeast in recent electron microscopy appear round or oval, and are clearly differentiated from the surrounding cytoplasm. They also show the presence of cristae mitochondriales in the interior (1, 36).

A fact of interest is the Gram stain reaction. Yeast cells stain intensely gram-positive immediately after appropriate fixation. The staining is rendered weaker by treating the fixed cells with hydrochloric acid, perchloric acid, sodium hydroxide, or particularly ribonuclease prior to the staining, and is eventually converted into the negative state by prolonged exposure. Comparing normal and RD cells, the latter showed better persistence of the stainability. However, the granular remains in the cytoplasm which appear during the partially gram-negative state in the normal cells are rapidly lost in the RD cells (77, 145).

The shape and size of whole cells of the RD mutants hitherto mentioned are essentially the same as those of the normal cells. A remarkable exception is an RD mutant produced through fluorochrome staining of baker's yeast with acridine orange. The RD cells are small and round, and contain far fewer cytoplasmic granules than the normal cells (113). The RD strains of haploid yeast often show better separation into single cells than the normal cultures, which appear in heavy clusters.

Another cytochemical approach is the differential fractionation of disintegrated material. Cytoplasmic granules obtained by an improved method of mechanical disruption are satisfactorily characterized as intact mitochondria by morphological, cytochemical, and enzymatic criteria (65). A recent investigation of a similar preparation from anaerobically grown normal yeast shows structural changes which gradually proceed during the respiratory adaptation. The enzyme activity represented by cytochrome oxidase appears to be firmly associated with flattened, spherical vesicles ranging from 0.2 to over 1 μ in size (41). An observation on *Candida albicans* showed that the mitochondria are easily released from the cells grown in the presence of a metal-chelating agent,

ethylenediaminetetraacetate (EDTA), and remain awhile intact outside the cells (68).

Summarizing the observations mentioned above, it may be said that mitochondria are present in the RD cells, but differ from those of the normal cells not only in the associated enzymes but also in structural stability. Even in normal cells, the mitochondria show appreciable differences depending on cultural conditions and respiratory status. The recently found possibility that subcellular materials (naked protoplast or its fragments) of yeast, obtained by mechanical disintegration (86) or hypertonic treatment with strontium chloride (B. T. Yamamoto, *unpublished data*), when kept under appropriate conditions, are viable and are even able to regenerate into entire cells suggests that these subcellular elements are sturdier than hitherto believed. Contrasted views also appear yet unsettled on the origin of the mitochondria in newborn buds. One says that they are supplied ready-made from the mother cell (76), while the other assumes a formation de novo from submicroscopic primordia (75). Further work is needed to extend knowledge of the mitochondria as structural and functional entities.

VIII. Nuclear Gene Control as Affected by the RD Mutation

It has been said generally that the RD mutation does not affect other gene-controlled characters such as nutritional requirements, fermentative ability, and resistance to toxic agents. However, the RD mutation is found to affect the manifestation of some gene-controlled characters, at least in their phenotypic expressions. Long-term adaptation to galactose fermentation in *Saccharomyces chevalieri* occurred much more slowly or in far less frequency in the RD strain than in the normal strain (99). A UV-induced RD strain of Carbondale hybrid yeast which was phenotypically a melezitose nonfermenter was found to be a fermenter in the genotype by a cross with a respirationally normal nonfermenter. It was inferred that the phenotypic expression of the fermenter character was concealed by the respiratory defect (63). Another example of complication by the RD mutation appears in the color variation of the adenineless mutant which is red. The color varies from dark red to pink depending on the sugar content of the culture medium and aeration. The colony growing on agar medium is red only at the surface but white in the interior. In addition, the RD substrains also show color variation from reddish brown to white. Consequently. the phenotypic expression of the gene-controlled adenine deficiency appears very complicated when various crosses and the segregants from them are diagnosed under different conditions. For instance, two separate analyses on an identical strain resulted in the single-gene interpretation by one (59, 60) and the three complementary-gene interpretation by the other (108). Further analyses of the adenine deficiency led Roman (106) to the assumption that more than seven genes are involved and that only two are related to color production. Secondary mutants, white and pale pink, appear out of the red, adenineless progenitor. Those secondary mutants outgrow the progenitor, after repeated transfers, due to a selection in favor of the secondary mutants. The RD mutation in such cultures retarded the populational shift as compared with respirationally normal cultures (106).

Inheritance of the ability to ferment galactose has presented considerable inconsistencies since the very beginning of yeast genetics. Previous investigations indicate that dominant alleles of three complementary genes are required to be present in a rapid fermenter of galactose. One of them, g_3, is assumed to control the ability to form de novo a cytoplasmic factor concerned with the adaptive synthesis of enzymes for fermentation of galactose. It is probable that the RD mutation disturbs the process controlled by that gene (17, 40).

An interesting experiment on the heterokaryon state of yeast, which transiently occurs after mating, was performed with some nuclear gene markers (arginine-, thiazole-, Actidione-resistance) and the extranuclear (vegetative) RD character. Production of parental type buds from heterokaryons was confirmed. Although the RD character was exchanged by cytoplasmic fusion, no recombination of nuclear gene markers through extranuclear transmission was detected (144).

It may be said, therefore, that one should exclude RD mutation first when he begins cross breeding for genetic analysis. But it is interesting and important to include the RD mutant in the pedigree and see how it intervenes in the nuclear control, because the RD mutation occurs ubiquitously so often and may lead to unneces-

sary complications unless it is carefully taken into account.

IX. RD and Related Mutations in Other Microorganisms

Various types of mutations resembling the RD mutation occur in some other microorganisms as well. They fall into two classes. One includes respiratory defects in a famous mold, *Neurospora crassa*, and bacteria. The other includes miscellaneous heritable changes, but not respiratory defect, as affected by several agents which induce the RD mutation of yeast.

Mutant microorganisms which are closely similar to the RD mutant of yeast are the "slow growing" Neurospora mutants. A mutant strain, "poky" was found to be far less vigorous in growth than the wild type (normal) strain and to possess somewhat defective respiration. Inheritance of the poky character indicates that it is governed by a cytoplasmic factor which is transmitted maternally through the characteristic pattern of sexual reproduction. Other phenotypically similar mutants (C115, C117) were found to be defective in respiration but to be nuclear-gene mutants by inheritance (70, 71).

Although the respiratory defect in the mold mutants is not so overwhelming as in the RD yeast, a similar defect appears in the composition of respiratory enzymes such as cytochromes *a* and *b* and succinic dehydrogenase (39, 71). Mitochondria of the mold mutants are much elongated or threadlike, whereas those of normal mold are rod-shaped or round. Acriflavine caused similar changes of mitochondrial forms in the original normal mold (26).

Most of the RD inducers mentioned in Section IV-A do not work similarly upon bacteria, but copper shows a noticeable effect on *E. coli*. Copper sulfate added to a synthetic medium in a low concentration (5×10^{-6} M) produced small-colony mutant cells from *E. coli* strain B. The mutant (copper coli) constituted almost 100% of the population in 30 hr of growth. Besides the reduced colony size (about one-fourth that of the normal colonies), the rate of oxygen consumption of the copper coli was reduced to about one-third of that of the normal strain and they were unable to utilize lactose as the sole carbon source (135). Similar small-colony mutants were also produced from *E. coli* strain K12 by the action of copper but not from *E. coli* strain 15 (11, 135). This type of mutant is indeed the bacterial counterpart of the RD yeast. Respirationally impaired mutants were found in later investigations as well. They were produced from *E. coli*, *Staphylococcus aureus*, *Bacillus mycoides*, and *Bacterium paracoli* by exposure to UV radiation and urethane (28, 29). The respiration is reduced to one-half to one-third of that of normal strains. They apparently show remarkable differences from the normal strain in cytochrome composition. A strain of BCG mycobacterium adapted to isonicotinic acid hydrazide also shows remarkably reduced cytochrome oxidase activity as compared with the unadapted strain (126). Respirationally impaired mutants of bacteria have not been produced so far by the inducers of RD yeast, except for UV radiation and copper.

Some species of bacteria such as *E. coli*, *Salmonella bovismorbificans*, *Salmonella schottmülleri*, *Salmonella enteritidis*, and *Paracolobactrum arizona* showed remarkable changes in respiratory competence similar to the respiratory adaptation of anaerobically grown yeast. The endogenous respiration rate of these bacteria, especially the last-named one, precultured anaerobically, increased during subsequent exposure to oxygen. The increase occurred without cell proliferation and was accompanied by changes in the absorption bands of cytochromes (47).

The chemical agents which have been known to induce the RD mutation of yeast also cause miscellaneous heritable changes in some other microorganisms. A remarkable case is the effect of acriflavine (euflavine) and of pyronin Y on *E. coli*. These dyes successfully induced the mutation to T1 phage resistance, although the procedure of treatments (nonproliferating condition, short exposure, nearly 99% killing by very high concentrations up to 0.05% acriflavine and 2% pyronin Y) was different from that for the production of the RD yeast (141). Acriflavine was found to eliminate the F factor, which controls the mating of *E. coli* strain K12. It is inferred that F factor is an extranuclear element which is, however, dependent on a nuclear gene (46). The effect of acriflavine and of proflavine eliminating the F factor was counteracted by addition of other dyes such as thionin and methylene blue (45). It seems important also that the mutagenic effect on *E. coli* of manganous chloride and of azaserine showed a synergistic or additive increase when the two agents were jointly applied (127). Acriflavine also

caused a remarkable filamentation of *E. coli* when added to the culture medium (102). Acriflavine applied to a culture of *Trypanosoma gambiense* produced mutant cells, AK, which lacked the kinetoplast and appeared to be retrogressive as compared with the normal cells (51).

Manganous ion, although a powerful mutagen on *E. coli* and an RD inducer on yeast, was found to prevent the somatic segregation in a diploid *Penicillium chrysogenum* which had been rendered unstable by exposure to nitrogen mustard and UV radiation (115).

The miscellaneous findings mentioned here indicate that respirationally defective mutants appear in a wide range of microorganisms. The effect of the so-called RD inducers is not necessarily monolithic but is rather diverse with respect to the heritable changes they cause.

X. Cancer and the RD Mutation

It is beyond the scope of this article to discuss the whole problem of cancer. However, amazingly much attention has been coming from the field of cancer research to the RD mutation, or mutations in general, on the presumption that the carcinogenetic process may share some principle in common with the mutations. A number of comprehensive articles emphasizing this possibility have appeared (49, 50, 107).

The decline of respiration which ostensibly characterizes the malignant tumors led Warburg to a hypothesis that cancer is initiated by a permanent inhibition and eventual destruction of the respiratory function accompanied by increased glycolytic activity, the energy provision being carried by the latter (132). Although this hypothesis is not yet fully substantiated, is faced with criticisms (91, 136), and is ignored in a recent symposium on carcinogenesis (143), it still appears to harbor some fertile seeds which may grow out in the future.

Some possibility of respiratory impairment by carcinogenic agents was indicated in early work on the cells of plant (orchid) ovules and salivary glands of *Chironomus* larva. Cytoplasmic granules, which in the intact state develop color by reoxidizing leuco bases of cresyl volet, Janus green B, methylene blue, toluidine blue, thionin, and phenosafranin, remain uncolored under intoxication by 20-methylcholanthrene, 3,4-benzpyrene, and styryl 430 (55). Respiratory inhibitors are not necessarily carcinogenic. A good example is Janus green B, which inhibits respiration at high concentrations and produces RD mutants in baker's yeast (Table 1), but does not produce tumors in rats (91). Warburg in this respect elaborates further his view that for a respiratory inhibition to be carcinogenic, it has to persist even after the cells have been freed from the agent and allowed to proliferate to become a tissue (133). The question may arise whether an agent could be potentially carcinogenic if it does not inhibit respiration of the original cells but does produce a permanent respiratory incompetence in the progeny. Such is the case with the production of the RD mutants by acriflavine, pyronin B, and perhaps many other inducers.

At present, however, it may not be very sound to regard the RD mutant as an unequivocal counterpart of cancer in the yeast oragnism. In fact, *p*-dimethylaminoazobenzene, 3,4-benzpyrene, and 20-methylcholanthrene (and cigarette smoke, too) failed to produce the RD mutants of baker's yeast (S. Nagai, *unpublished data*). Conversely, the known RD-inducers may not necessarily be cancer hazards. Recent development of quantum chemistry has introduced another approach to the carcinogenic activity of various chemicals based on their electronic structures. Improved methods of molecular orbital computations (27, 84) show the presence of specific centers of activity in various carcinogens. Future advance may lead to a more generalized molecular interpretation comprising the specific effects of mutagens and RD inducers as well as carcinogens. On the other hand, it was claimed that the filamentous cell growth, which occurs divorced and uncontrolled from the cell division mechanism in a mutant of *Candida albicans*, is more closely similar to "malignancy" than the RD mutant state, in which cell division and cell growth are supported by anaerobic energy supply, but are nevertheless regularly linked (134).

Passing over these fundamental uncertainties, however, some practical trials based on the Warburgian view have been underway in America, Europe, and Russia in the search for anticancer drugs. A selective toxicity for some inherently anaerobic microorganisms or respirationally defective mutants, concomitant with harmlessness to the respirationally normal counterparts, has been sought as a criterion of the anticancer potentiality. If it is germane and successful, microbial cultures, perhaps with gradient or ditch

plate technique, will replace laborious tests with living animals or tumor cultures in vitro to make a considerable saving in the screening of prospective or suspected drugs. Ten species of anaerobic bacteria were employed by Americans to test many drugs. The inhibitory effect on *Clostridium feseri* appeared to correlate well with the anticancer effect (5). Russians of a team led by Gause claim that they have very efficiently selected some promising anticancer antibiotics out of numerous preparations. They collaterally used respirationally normal and defective strains of *Staphylococcus aureus*, *Bacterium paracoli*, *Bacillus mycoides*, *Saccharomyces cerevisiae*, and *Candida albicans*. *Saccharomyces* and *Staphylococcus* strains were found to be particularly useful. Some preparations, which selectively inhibited the growth of respirationally defective mutants but allowed the normal cultures to grow uninhibited, showed a carcinostatic effect in animal tests (28–30). German work on the production of partially cytochrome-deficient mutants of *E. coli*, *S. aureus*, and mycobacteria appeared along a similar line (131). Importance of the RD mutation in yeast and other microorganisms has been emphasized by all those authors to reinforce the validity of the approach.

Carcinogenic effects of various agents have often been compared with their mutagenic effects (14, 15, 32, 112). There is indeed a parallel relationship which is presumably due to a common mode of action, perhaps a destructive attack on the nuclear material. It is striking that some prospective anticancer drugs such as nitrogen mustards, triethylene melamine, and azaserine are mutagenic when tested in terms of the induced mutation frequency in *E. coli* (15, 52). A pessimistic view regards these drugs as rather hazardous (15), but they may be preferentially destructive to cancer cells (35, 130). However, there is still a possibility that effective and versatile anticancer drugs may be finally discovered as examples of the mutagen-antimutagen relationship. In fact, some mutagens are counteracted by structurally analogous agents as seen in caffeine and theophylline versus adenosine and guanosine (92), and 4-nitro-6-hydroxybenzimidazole versus 4-hydroxy-6-nitrobenzotriazole (33). Furthermore, the "counteractive interference" between some mutagens for bacteria as well as some RD inducers for yeast (*see* Section IV-C) makes the prospect somewhat brighter. A mutagen (in the broad sense) may annul the effect of another mutagen when the combination is appropriate. A certain drug, although it may be carcinogenic when applied alone, could still fight cancer through intervention into the carcinogenetic process caused by another agent (carcinogen), no matter whether it is endogenous or foreign. The observation that an inherently unstable strain of yeast could be "stabilized" by small doses of caffeine, an RD inducer (*see* Section IV-D), also appears to favor such a speculation.

It is tempting to imagine those four potencies, carcinogenic, mutagenic, RD-inducing, and respiration-inhibitory, as four floodlamps of different colors placed at the four corners of the ceiling and throwing light aslant onto the floor, respectively. Various objects spotted on the floor would accordingly appear in subtly blended shades, being rendered to varied extents tumorous, mutated, respirationally defective, or respirationally inhibited. The effect might be utterly lethal in a small region at the center where the four beams overlap together. Further advance along such a line should make a significant contribution to the strategic approach of the future.

XI. Concluding Remarks

Survey of the numerous reports on the RD mutation suggests three major lines of further investigation. They are: (i) to determine the nature of the receptor(s), which might be different depending on the types of the inducers; (ii) to trace the process from the initial attack by the inducer up to the final failure of respiratory enzyme formation; and (iii) to find out the mode of action of the nuclear control over the cytoplasmic factor involved in the formation of respiratory enzymes. These lines, when converged, should not only unravel the mechanism of the RD mutation but also give a useful clue to show how the code-information-decoding system operates in hereditary transmission.

One should not, however, hasten to amend the general principle of inheritance, because the RD mutation still appears to conform with the Mendelian law so far as the cytoplasmic factor depends on nuclear gene control in the formation of respiratory enzymes.

It may be helpful also to solve the problems involved in the differentiation and dedifferentiation of cells and tissues in higher organisms. Ephrussi (20) in discussing the problems of somatic cell

variation also points out the significance of the interrelations between genetic, epigenetic, and cytoplasmic mechanisms participating in the constancy and variability of hereditary determinants in both unicellular and multicellular organisms. This should be, indeed, a geneticist's broad approach to one of the general biological problems. Lederberg's discussion (58) on symbiotic evolution with the concept of "plasmid" also suggests the importance of extranuclear constituents, although it is not yet clear whether they have come out of some inseparably accommodated symbiont or have been secondarily established in subordination to the nucleus. Such investigation will certainly help to extend knowledge of the origin and evolution of life, with respect to the nucleocytoplasmic relations in various forms of cellular organization (48), no matter whether Lederberg's view be substantiated or some other pathways be conceived.

Looking back to the very beginning of these investigations, the problem of the RD mutation was, so to speak, a tiny grove on a trail in the wilderness. It is nowadays a key junction of several routes which connect major fields of contemporary biology.

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