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Osmotic-Sensitive Mutants of *Saccharomyces cerevisiae* as Screening Organisms for Promutagens and Procarcinogens

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An attempt to improve the response of yeast cells to promutagens and procarcinogens in mutagenicity assay was made by using osmotic-sensitive mutants of a yeast, *Saccharomyces cerevisiae*. Four osmotic-sensitive mutants of yeast which showed increased sensitivity to 1.5 M KCl were induced by ethylmethanesulfonate treatment. One of the mutants, strain C658-K42, was highly sensitive to antibiotics such as mitomycin C, novobiocin, nalidixic acid, chloroquine and rifampicin at concentrations showing no growth-inhibitory effect on the original strain, *S. cerevisiae* C658. Strain C658-K42 was considered to have a defect in the cell membrane.

These osmotic-sensitive mutants were tested for suitability for screening by using well-known procarcinogens (promutagens), dimethylnitrosamine, 3,4-benzpyrene and 2-acetylaminofluorene. The response of these mutants in a mutagenicity assay (Trp^+ reversion) was apparently increased compared with that of the original strain. The yeast cells which were harvested from a late logarithmic phase culture could activate procarcinogens to genetically active forms without any exogeneously added metabolic activation system.

Keywords——*Saccharomyces cerevisiae*; osmotic-sensitive mutant; procarcinogen; promutagen; metabolic activation; mutagenicity assay

A majority of potential carcinogens and mutagens have been detected by a combination of several short-term assay methods using bacteria, fungi, phages, cultured cells and so on. Yeast, *Saccharomyces cerevisiae*, is a eukaryotic organism and provides a model of higher organism cells. Yeast cells are available for detecting not only mutational events (including mitochondrial mutations) but also other genetic events such as mitotic crossing-over, mitotic gene conversion, chromosome non-disjunction and chromosome aneuploidy simultaneously on the selection media.[1)]

Yeast cells contain a microsomal cytochrome P-450[2)] which is in many respects similar to cytochromes found in mammalian microsomes.[3)] Unlike almost all bacteria, yeast cells can activate some procarcinogens and promutagens to genetically active products.[4)] Most *in vitro* mutagenicity assay systems contain mammalian hepatic microsomal fractions such as S-9 fraction. In such systems, procarcinogens and promutagens are activated to the proximate or ultimate products outside of the indicator organisms. If the yeast activation system is available, the production of active metabolites would be carried out in the yeast cells, much closer to chromosomal deoxyribonucleic acid (DNA) than in the case of *in vitro* activation systems.

On the other hand, several factors are known to affect the response of yeast cells to genotoxic agents[1d,e)] such as cell permeability, DNA repair ability and a high ribonucleic acid (RNA)-to-DNA ratio. The cell permeability may be a problem especially for large aromatic substances. Major permeability barriers of yeast cells are the cell membrane (plasma membrane) and the rigid cell wall. Many chemical, physical and enzymatic methods have been used for permeabilizing yeast cells.[5)] Permeabilization methods using organic solvents, detergents, antibiotics and lytic enzymes are less appropriate when the decrease of metabolic activation ability and/or viability of yeast cells is considered. Thus, mutants with increased

permeability such as osmotic-sensitive mutants[6] and fragile mutants[7] are favorable since they are viable and need no special treatment. Most permeability mutants have been selected for the ability to take up antibiotics.[5]

In the present paper we report the induction and the isolation of osmotic-sensitive mutants of yeast which have increased sensitivities to potassium chloride and some antibiotics. Metabolic activation and mutagenicity assay of some procarcinogens (promutagens) was also studied using the isolated osmotic-sensitive mutants. Dimethylnitrosamine was used as a test procarcinogen since its metabolic pathway has been well studied. Large aromatic procarcinogens, 3,4-benzpyrene and 2-acetylaminofluorene, were also used since they have been reported to have no genotoxic activity on a normal yeast.[4c]

Experimental

Yeast Strains——*Saccharomyces cerevisiae* C658 (*a*, *ade*2, *trp*1, *rho*$^+$) was used as the original strain. Ethylmethanesulfonate-induced mutants sensitive to KCl and antibiotics (C658-K7, -K18, -K35 and -K42) were also used.

Chemicals——Dimethylnitrosamine (DMN, *N*-nitrosodimethylamine), 3,4-benzpyrene (BP, benzo[*a*]pyrene) and 2-acetylaminofluorene (AAF, *N*-2-fluorenylacetamide) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). For treatment, DMN was dissolved in distilled water, and BP and AAF in dimethylsulfoxide (DMSO, spectrophotometric grade, Wako Pure Chemical). The final concentration of DMSO in the mutagenicity assay medium was adjusted to 10%. Mitomycin C (Wako Pure Chemical), nystatin (Serva), chloroquine diphosphate (Sigma) and novobiocin sodium (Boehringer Mannheim) were dissolved in distilled water, nalidixic acid (Aldrich) in ethanol, and rifampicin (Boehringer Mannheim) and amphotericin B (Sigma) in DMSO. All antibiotics stock solutions were prepared just before the experiment under subdued lighting.

Isolation of Mutants Sensitive to Osmotic Tension and Antibiotics——*S. cerevisiae* C658 was grown in 5 ml of YPD medium (1% Difco yeast extract, 2% Difco polypeptone and 2% glucose, pH 5.3) at 30 °C to the late logarithmic phase. The yeast cells were harvested, washed twice and resuspended in 0.1 M sodium phosphate buffer (pH 7.0) at 5×10^7 cells/ml. Ethylmethanesulfonate (EMS) was added to a final concentration of 3% (v/v) and incubated at 30 °C for 2 h. The EMS-treated cells were washed with 5% sodium thiosulfate to quench the reaction and plated on YPD agar medium to form about 200 colonies. After a 30 h incubation at 30 °C, small colonies on the plates were replicated onto YPD plates containing 1.5 M KCl and incubated for 3 d. Colonies which failed to grow on YPD with 1.5 M KCl were transferred from the original YPD plates to each screening medium using a multipoint inoculator.

Screening Media——YPD medium with 1.0 or 1.5 M KCl was used for checking osmotic sensitivity.[6] YPG medium which contained 2% glycerol as a carbon source instead of glucose in YPD medium was used for detecting respiratory-deficient (RD) mutation. Growth on YPD medium containing antibiotics was used as an index of cell permeability. All agar media were incubated at 30 °C for 3 d in the dark and the growth of each strain was compared with that on control YPD medium.

Mutagenicity Assay——Each strain was cultured at 30 °C with shaking in 5 ml of YPD liquid medium to the late logarithmic phase of growth ($OD_{660} = 0.8$—0.9). The yeast cells were harvested, washed twice with 0.1 M sodium phosphate buffer (pH 7.4) and suspended in the same buffer at 6—7×10^8 cells/ml. Treatment was performed with shaking at 30 °C for 4 h with 0.3 ml of yeast cell suspension, 1.5 ml of S-9 mix or 0.1 M sodium phosphate buffer (pH 7.4) and 0.2 ml of a solution of a procarcinogen (total 2.0 ml). The treated cells were washed twice and suspended in 2.0 ml of sodium phosphate buffer. For detection of Trp$^+$ revertants, 0.1 ml portions of the cell suspension (final about 1×10^7 cells) were spread onto minimal medium (0.67% Difco yeast nitrogen base w/o amino acids and 2% glucose) supplemented with adenine sulfate (10 mg/l) and incubated at 30 °C for 6 d. The results were expressed as Trp$^+$ revertants/10^7 survivors. Colony-forming ability on YPD medium was used as the criterion for survivors.

Preparation of S-9 Mix——S-9 fraction was purchased from Oriental Yeast Co., Ltd. (Tokyo). The fraction was prepared from male Sprague–Dawley rats treated with phenobarbital and 5,6-benzoflavone as described by Ames *et al.*[8] S-9 mix was prepared as described by Yahagi *et al.*[9] and contained per ml: 0.3 ml of S-9 fraction, 8 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of glucose-6-phosphate (G6P), 4 μmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 0.5 unit of G6P-dehydrogenase with 100 μmol of sodium phosphate as a buffer (pH 7.4). Cofactors in the S-9 mix were purchased from Oriental Yeast Co., Ltd.

Results

Isolation of KCl-Sensitive Mutants of Yeast

The survival ratio of *S. cerevisiae* C658 treated with 3% EMS for 1 h was about 40%

TABLE I. Effects of Potassium Chloride and Various Antibiotics on the Cell Growth of the Original and Osmotic-Sensitive Strains of *Saccharomyces cerevisiae*

Media	Strains				
	C658	K7	K18	K35	K42
KCl (M)					
None	+++	+++	+++	+++	+++
1.0	+++	+	++	+	++
1.5	+++	−	−	−	−
Antibiotics (μg/ml)					
Mitomycin C					
200	+++	+++	+++	+++	++
400	+++	+++	+++	+++	+
Nalidixic acid					
200	+++	+++	+++	+++	+
400	+++	++	++	++	−
Novobiocin					
800	+++	+++	+++	++	+
1500	+++	+++	++	+	−
Chloroquine					
800	+++	+++	+++	+++	++
1500	+++	+++	++	+++	−
Rifampicin					
800	+++	+++	+++	+++	++
1500	+++	+++	++	+++	−
Nystatin					
2	+++	+++	++	++	+++
4	++	−	−	+	+++
8	−	−	−	−	+++
Amphotericin B					
2	+++	+++	++	++	+++
4	++	+	+	−	+++
8	−	−	−	−	+++

Fresh yeast cells were inoculated onto each agar medium and incubated at 30 °C for 3 d. The growth is expressed relative to that of the original strain on YPD medium.

TABLE II. Effects of DMSO on Viability and Trp^+ Reversion of the Original and Osmotic-Sensitive Strains of *Saccharomyces cerevisiae*

Strains	Survival ratio in medium with 10% DMSO (%)	Trp^+ revertants per 10^7 survivors	
		−DMSO	+DMSO
C658	90±4	2.5±1.0	2.7±0.7
K7	87±6	3.2±0.1	3.5±0.5
K18	83±9	5.4±0.1	5.6±0.5
K35	77±4	3.5±1.0	3.4±1.0
K42	31±7	2.0±0.6	3.1±0.4

Each strain was suspended in the assay medium with or without 10% DMSO at 1×10^8 cells/ml and incubated at 30 °C for 4 h. Colony-forming cells on YPD and minimal agar media were scored after a 3 d incubation. Survival ratio is given as a percentage of the DMSO-free control. Results are the averages of 3 to 6 independent experiments with 7 plates ± S.D.

throughout the experiment. Among 52000 colonies tested, 850 did not initially grow on YPD medium with 1.5 M KCl. These first lots were not stable and the majority of the KCl-sensitive strains had reverted after one transfer on YPD medium. After 5 subsequent transfers on YPD medium, 4 strains in Table I (C658-K7, -K18, -K35 and -K42) were isolated as osmotic-sensitive mutants. Since these strains showed normal growth on YPG medium, they do not have a respiratory-deficient mutation (data were not shown).

Effect of Antibiotics on the Growth of Yeast Cells

The original strain C658 showed normal growth on YPD plates containing antibiotics at the concentrations shown in Table I. Novobiocin, nalidixic acid and rifampicin were used at the solubility limit. Only strain K42 showed high sensitivity to the antibiotics tested, except nystatin and amphotericin B. This strain was less sensitive to these polyene antibiotics than

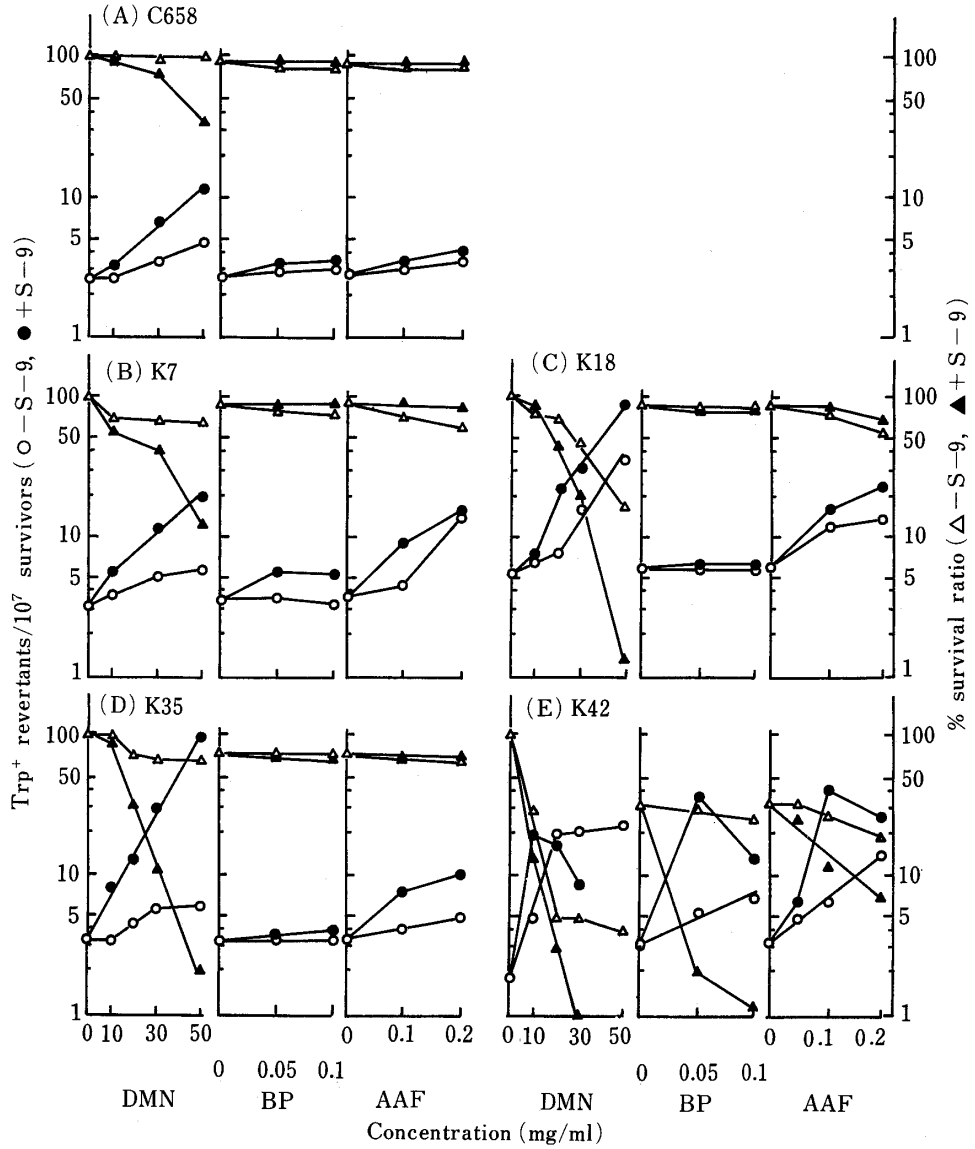

Fig. 1. Cell Survival and Induction of Trp$^+$ Revertants in the Original and Osmotic-Sensitive Strains of *Saccharomyces cerevisiae* Treated with Procarcinogens

Late logarithmic phase cells of each strain were exposed to DMN (dimethylnitrosamine), BP (3,4-benzpyrene) and AAF (2-acetylaminofluorene) with or without S-9 mix at 30 °C for 4 h. Treated cells were washed, spread onto YPD and minimal media and incubated at 30 °C. Survivors and Trp$^+$ revertants were scored after incubation for 3 and 6 d, respectively. The results are the averages of 3 to 6 independent experiments with 7 plates.

the original strain was. The other 3 osmotic-sensitive strains, K7, K18 and K35, showed slightly increased sensitivity to some of the antibiotics including nystatin and amphotericin B.

Effects of DMSO on Yeast Cells

As shown in Table II, the final concentration of 10% DMSO in a reaction mixture showed rather small effects on the survival ratio. Viable cell count of the original strain C658 showed a 10% decrease after a 4 h incubation with 10% DMSO. Osmotic-sensitive mutant K42 was most sensitive to DMSO and showed only one-third survival as compared with the original strain. Spontaneous reverse mutation rate at the *trp*1 gene was 2.5×10^{-7} in the original strain C658 and this ratio was not affected by the addition of 10% DMSO. Osmotic-sensitive mutants, except for strain K42, also showed almost the same reversion frequency regardless of the presence of DMSO. The Trp^+ reversion in the osmotic-sensitive strain K42 increased from 2.0 to 3.1 per 10^7 survivors in the presence of 10% DMSO.

Mutagenicity Assay of Procarcinogens by Using Osmotic-Sensitive Mutants of Yeast

Though S-9 mix was present in the assay medium, 0.1 mg/ml of BP and 0.2 mg/ml of AAF could induce few Trp^+ revertants in the original strain C658 (Fig. 1A). When the original strain was treated with increasing concentrations of DMN, moderately increased frequency of Trp^+ reversion was observed even in the absence of S-9 activation system.

As in the case of the original strain, the Trp^+ reversion by BP in the osmotic-sensitive mutants, K7, K18 and K35, showed little increase, while those by DMN and AAF showed some increases with or without S-9 mix (Figs. 1B—1D). As shown in Fig. 1E, strain K42 was most sensitive to the procarcinogens tested and was the only strain that could detect the mutagenicity of BP. The frequency of Trp^+ reversion of strain K42 by optimal concentrations of procarcinogens with S-9 activation was about 10 times higher than that of the control (no carcinogen). The Trp^+ reversion by DMN showed the same level in strain K42 regardless of the presence or the absence of S-9 mix. There exists a clear inverse relationship between the Trp^+ reversion ratio and the survival ratio in all strains. A decrease in Trp^+ reversion was observed in strain K42 at higher concentrations of procarcinogens. When cells of strain K42 were treated with increasing doses of each procarcinogen with S-9 activation for 4 h, the survival ratio decreased linearly.

Discussion

When yeast cells are used as the indicator organism for mutagenicity assay, the rigid cell wall and cell membrane provide a barrier against the uptake of test substances. Therefore, permeability mutants such as an osmotic-sensitive mutant of yeast with a defective cell wall and/or cell membrane are favorable. Venkov *et al.*[7] reported an osmotic-sensitive fragile mutant of yeast which had a defective cell wall and could grow only in a medium containing 10% sorbitol as an osmotic stabilizer. They used this mutant for the uptake of antibiotics[10] and DNA.[11] Gause and Laiko[12] used a mutant with a distorted cell membrane as a prescreen for anticancer agents. All of these mutants were more susceptible to antibiotics than their parent strains.[7,12] Since the osmotic-sensitive mutants isolated in this experiment showed normal growth in YPD medium without an osmotic stabilizer and did not show any morphological change in water or in 5% saline solution (data were not shown), it is considered that they had little or no defect in the structure of the cell wall. It is interesting to note that strain K42 had a lesser sensitivity to polyene antibiotics, nystatin and amphotericin B. Strain K42 could grow on the medium with 8 μg/ml of nystatin or amphotericin B, which did not allow the growth of any other strain (Table I). It was reported that amphotericin B binds with sterol and produces holes in the cell membrane.[13] It is considered, therefore, that conformational changes in the cell membrane of strain K42 inhibit the formation of the

amphotericin B-ergosterol complex reported by Kruijff and Demel.[13b]

A final concentration of 10% DMSO in the assay medium reduced the survival ratio of strain K42 to one-third of that of the DMSO-free control. It was reported that loss of sterol and phospholipids after DMSO treatment makes the cell membrane less rigid.[14] The cell membrane of strain K42 may be affected much more by DMSO than that of other strains. This, in turn, increased the susceptibility of strain K42 to BP and AAF. However, DMSO does not assist DMN, since the mutagenicity of N-nitroso compounds was repressed by the addition of DMSO.[15]

The response of yeast cells to procarcinogens, though only 3 compounds were tested, was increased by osmotic-sensitive mutation. The original strain, C658, showed little response to BP and AAF even in the presence of S-9 activation system. Both BP and AAF have been reported not to be recombinogenic on a normal yeast. In *Salmonella typhimurium* TA100 with S-9 mix, 10 μg/ml BP and 50 μg/ml AAF induced about 100 and 340 His^+ revertants/10^7 survivors, respectively.[15b] It is considered, therefore, that little of these aromatic compounds or their active metabolites was incorporated into the original strain. Since AAF could induce Trp^+ reversion in the osmotic-sensitive mutants without S-9 activation, it is clear that AAF was incorporated into yeast cells and metabolized by them. Another procarcinogen, BP, was less able to permeate into yeast cells, and only strain K42 showed a response to BP regardless of the presence of S-9 mix. The water-soluble procarcinogen, DMN, was mutagenic to both the original and osmotic-sensitive strains. It was also metabolized into a genetically active form in the yeast cells.

The survival curves showed a clear inverse relationship to the Trp^+ reversion curves in all cases. When strain K42 was treated with increasing doses of DMN, the survival curve declined linearly without any shoulder, while those of the other three osmotic-sensitive mutants showed a shoulder followed by a linear decline in survival ratio. These observations suggest the presence of DNA repair-deficient mutation[16] in addition to the osmotic-sensitive mutation in strain K42. Detailed studies on the possibility of the double mutation in strain K42 are in progress.

Though the response of yeast cells as an indicator organism is lower than that of the *Salmonella typhimurium* assay system,[8] they still have practical advantages, such as being able to detect several genetic events simultaneously[1] and to assay antibacterial agents. We have enhanced the frequency of induced reverse mutation markedly by using osmotic-sensitive mutants, and detailed biochemical and genetic studies on the mutants are under way. Further work remains to be done to show that other genotoxic agents can be detected by these osmotic-sensitive mutants. Attempts are also in progress to isolate stable osmotic-sensitive mutants of *S. cerevisiae* D7. This strain was constructed for mutagenicity assay by Zimmermann *et al.*[1c] and can detect reverse mutation, mitotic gene conversion and mitotic crossing-over simultaneously.

Utilization of a metabolic activation system in yeast cells is another important problem. Though it was clear that the three procarcinogens tested were metabolized into genetically active forms, the metabolic activation by the yeast cells was less effective than S-9 activation. Recently we have reported that in growing yeast cells ethanol can increase the content of cytochrome P-450[17] which is in many respects similar to cytochromes in mammalian microsomes.[3] Further improvement of the activation system in yeast cells should facilitate their application as a screening organism.

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References

1) *a*) R. K. Mortimer and T. R. Manney, "Chemical Mutagens," Vol. 1, ed. by A. Hollaender, Plenum Press, New

York, 1971, pp. 289—310; *b*) F. K. Zimmermann, *ibid.*, Vol. 3, ed. by A. Hollaender, Plenum Press, New York, 1973, pp. 209—239; *c*) F. K. Zimmermann, R. Kern and H. Rasenberger, *Mutat. Res.*, **28**, 381 (1975); *d*) M. Hollstein and J. McCann, *ibid.*, **65**, 133 (1979); *e*) E. Moustacchi, *Arch. Toxicol.*, **46**, 99 (1980).
2) A. Lindenmayer and L. Smith, *Biochim. Biophys. Acta*, **93**, 445 (1964).
3) *a*) K. Ishidate, K. Kawaguchi, K. Tagawa and B. Hagihara, *J. Biochem.* (Tokyo), **65**, 375 (1969); *b*) Y. Yoshida and H. Kumaoka, *ibid.*, **71**, 915 (1972); *c*) A. Wiseman and T.-K. Lim, *Biochem. Soc. Trans.*, **3**, 974 (1975).
4) *a*) D. F. Callen and R. M. Philpot, *Mutat. Res.*, **45**, 309 (1977); *b*) D. Siebert, U. Bayer and H. Marquardt, *ibid.*, **67**, 145 (1979); *c*) V. F. Simmon, *J. Natl. Cancer Inst.*, **62**, 901 (1979); *d*) D. F. Callen, C. R. Wolf and R. M. Philpot, *Mutat. Res.*, **77**, 55 (1980); *e*) V. W. Mayer and C. J. Goin, *ibid.*, **78**, 243 (1980); *f*) P. Wilcox and J. M. Parry, *Carcinogenesis*, **2**, 1201 (1981); *g*) G. Bronzetti, C. Bauer, C. Corsi, C. Leporini, R. Nieri and R. del Carratore, *Mutat. Res.*, **89**, 179 (1981); *h*) E. R. Nestmann, E. R. Stephen, D. J. Kowbel and A. Nasim, *Can. J. Genet. Cytol.*, **24**, 771 (1982); *i*) R. D. Mehta and R. C. von Borstel, *Mutat. Res.*, **92**, 49 (1982); *j*) E. C. McCoy, M. Anders, M. McCartney, P. C. Howard, F. A. Beland and H. S. Rosenkranz, *ibid.*, **139**, 115 (1984).
5) H. Felix, *Anal. Biochem.*, **120**, 211 (1982).
6) *a*) C. D. Hawthorn and J. Friis, *Genetics*, **50**, 829 (1964); *b*) A. Singh, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 305 (1977); *c*) J. F. T. Spencer and D. M. Spencer, *Z. Naturforsch.*, **34c**, 131 (1979).
7) P. V. Venkov, A. A. Hadjiolov, E. Battaner and D. Schlessinger, *Biochem. Biophys. Res. Commun.*, **56**, 599 (1974).
8) B. N. Ames, J. McCann and E. Yamasaki, *Mutat. Res.*, **31**, 347 (1975).
9) T. Yahagi, M. Degawa, Y. Seino, T. Matsushima, M. Nagao, T. Sugimura and Y. Hashimoto, *Cancer Lett.*, **1**, 91 (1975).
10) P. V. Venkov, L. I. Stateva and A. A. Hadjiolov, *Biochim. Biophys. Acta*, **474**, 245 (1977).
11) P. V. Venkov and V. P. Ivanov, *Current Genet.*, **5**, 153 (1982).
12) G. F. Gause and A. V. Laiko, *Antibiot. Chemother.*, **23**, 21 (1978).
13) *a*) S. C. Kinsky, *Ann. Rev. Pharmacol.*, **10**, 119 (1970); *b*) B. de Kruijff and R. A. Demel, *Biochim. Biophys. Acta*, **339**, 57 (1974).
14) B. G. Adams, *Anal. Biochem.*, **45**, 137 (1972).
15) *a*) T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada, *Mutat. Res.*, **48**, 121 (1977); *b*) T. Morita, M. Sawaki and I. Mifuchi, *Chem. Pharm. Bull.*, **29**, 1758 (1981).
16) R. H. Haynes and B. A. Kunz, "The Molecular Biology of the Yeast *Saccharomyces*-Life Cycle and Inheritance," ed. by J. N. Strathern, E. W. Jones and J. R. Broach, Cold Spring Harbor Laboratory, New York, 1981, pp. 371—414.
17) T. Morita and I. Mifuchi, *Chem. Pharm. Bull.*, **32**, 1624 (1984).