AGENTS THAT CAUSE A HIGH FREQUENCY OF GENETIC CHANGE FROM [*psi*+] TO [*psi*⁻] IN *SACCHAROMYCES CEREVISIAE*

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Manuscript received November 21, 1980
Revised copy received June 12, 1981

ABSTRACT

The [*psi*] factor of yeast is cytoplasmically inherited. SINGH, HELMS and SHERMAN (1979) reported that high concentrations of KCl and of ethylene glycol induce the genetic change from [*psi*+] to [*psi*⁻]. In this study, the following agents have been shown to induce the same genetic change: guanidine hydrochloride at 1 mM, dimethyl sulfoxide at 2.5% v/v and ethanol or methanol at 10% v/v. It is likely that a number of other agents also cause the change, namely 2 M glycerol, M succinate, M glutamate and M $MgCl_2$. Most of these agents induce the change at very high frequencies; with some, the frequency is 100%. Although the observed phenotypic change can also occur as a result of chromosomal gene mutation, no changes of this type were identified. Some of the agents also cause mutation from [*rho*+] to [*rho*⁻] and from killer to sensitive.

THE [*psi*] factor is a non-Mendelian determinant that affects the efficiency of certain ochre and frameshift suppressors (COX 1965, 1972; CULBERTSON *et al.* 1977). In [*psi*+] strains, the serine-inserting ochre suppressor *SUQ5* has a read-through efficiency some ten to fifteen times that in [*psi*⁻] strains (LIEBMAN, STEWART and SHERMAN 1975). Mutation from [*psi*+] to [*psi*⁻] and back again can be caused by a variety of conventional mutagens, including 254 nm UV, ethyl methansulfonate and nitrosoguanidine (YOUNG 1969; TUITE 1978; TUITE and COX 1979). The UV-induced mutation from [*psi*+] to [*psi*⁻], like nuclear mutagenesis, depends partly on the formation of pyrimidine dimers and is under the same genetic control and subject to the same repair systems.

Many cytoplasmic genetic elements are subject to "curing" by agents that are not nuclear mutagens or that have relatively small mutagenetic effects. Mitochondrial genomes of yeast, unlike chromosomes, are extremely sensitive to acridine dyes and to ethidium bromide. (SLOMINSKI 1968; EPHRUSSI and HOTTINGUER 1951). Quite low concentrations of these compounds can cause 100% of the cells in a population to become ρ^-.

Other agents that cause cytoplasmic petite mutation are manganese (PUTRAMENT, BARANOWSKA and PRAZMO 1973), 5-fluorouracil (MOUSTACCHI and MARCOVICH 1963; OLIVER and WILLIAMSON 1976), thymidylic acid starvation

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(BARCLAY and LITTLE 1978), growth at high temperatures (*e.g.*, 40°) (SHERMAN 1959), heat shock (SHERMAN 1956, 1959), cycloheximide (FINK and STYLES 1972), guanidine hydrochloride (JULIANI, GAMBARINI and DA COSTA 1975) and dimethyl sulfoxide (DMSO) (YEE, TSUYUMA and ADAMS 1972). Some of these agents are effective in causing "killer" to "sensitive" mutation, namely 5-fluorouracil (MITCHELL, HERRING and BEVAN 1976), acriflavine (FINK and STYLES 1972), growth at a high temperature (WICKNER 1974), cycloheximide (FINK and STYLES 1972) and guanidine hydrochloride (TUITE 1978).

For the most part, these agents are ineffective in causing [*psi*$^+$] to [*psi*$^-$] mutations (YOUNG 1969; TUITE 1978). The exceptions are heat shock, which also causes nuclear mutations (EVANS and PARRY 1975), dimethyl sulfoxide and guanidine hydrochloride. SINGH, HELMS and SHERMAN (1979) reported that 2.5 M KCl and 1.75 M ethylene glycol cause [*psi*$^+$] to [*psi*$^-$] mutations.

We have discovered a number of agents that are extremely effective in causing [*psi*$^+$] to [*psi*$^-$] mutations. A study of these agents is reported in this paper. The nature of these agents has important implications for the nature and mode of inheritance of the [*psi*] factor.

When an apparently powerful mutagen is discovered, it is important to establish that it induces the mutations observed and does not merely select them from a background of spontaneously occurring mutations. Confusion is unlikely to arise when there is a large discrepancy between the spontaneous frequency and that observed after treatment or if there is an absolute increase in mutant frequency in the absence of cell multiplication. However, when the growth of cells is necessary for mutagenesis or when the agent has lethal effects, suitable experiments must be done to measure the effects of selection. Very often, a "reconstruction" experiment is carried out. We believe, on the basis of our experience with the agents we describe here, that this is not a sufficiently sensitive test and can be misleading.

We have adopted three criteria to indicate induction. First, if we can show clearly that the treatment selects *against* the mutant form that it induces, selection of spontaneously occurring mutants is obviously ruled out, and in increase in frequency with treatment must be due to induction. The second criterion is to demonstrate that mutant frequency increases when cells are treated in a medium that allows the proliferation only of nonmutant cells. The third criterion applies when the spectrum of mutant loci is different from the spontaneous spectrum giving rise to the identical phenotype. The application of this criterion to a case where there is powerful selection in favor of the mutant phenotype is described below.

An increase in the frequency of sectored colonies after treatment could also be an indicator of induction (SINGH, HELMS and SHERMAN 1979). However, there are two circumstances that might lead to this effect in the absence of induction. It might be possible for an agent to select cells with replicating instabilities. Such cells would give rise to sectored colonies when plated. Where a cytoplasmic determinant is involved, these could be "low copy-number" variants with a higher probability of segregation of 'zero copy-number' cells. There-

fore, sectored colonies must be replated to ensure that both sectors are stable. Second, an agent might easily cause an increase in "clumping' or of budded cells. This would cause an increase in the observed frequency of sectored colonies without any real increase in the frequency of mutations.

MATERIALS AND METHODS

Strains: The following strains were used for studies of the effects of agents on mutation of [*psi*+] to [*psi*⁻]:

507.4/2b: *α ade2-1 his5-2 can1-100 ura3-1 SUQ5* [*psi*+]
MT182/8d: *α ade2-1 his5-2 leu1 SUQ5* [*psi*+]
CM106/5a: **a** *ade2-1 SUQ5* [*psi*+]

These three strains have no antisuppressor mutation and are able to maintain the [*psi*] factor, *i.e.*, they are all *ASU*+, *asu*+, *PNM*+ and *pnm*+ (*vide infra* and Cox, Tuite and Mundy 1980).

Strains used for complementation tests to determine the genotype of nonsuppressed variants of these strains were

461/4b **a** *ade1 his2*
468/1a **a** *ade2-1 lys1-1 can1-100 ura3-1 SUQ5* [*psi*⁻]
483/1d **a** *ade2-1 lys1-1 can1-100 SUQ5* [*psi*⁻]
465/2a **a** *ade2-1 his5-2 can1-100 ura3-1* [*psi*+]
MT152/3c **a** *ade2-1 his5-2 can1-100 leu1 kar1* [*psi*+]

These strains were derived from strains described by Cox (1965), from a strain provided by R. K. Mortimer, X2314-8C: **a** *ade2-1 his5-2 lys1-1 trp5-48 can1-100 ura3-1 SUP2* and from one provided by G. Fink, JC8: **a** *kar1-1 leu1*.

The following strains were used to test by complementation that suppression in white strains was [*psi*]-dependent:

584/1d **a** *ade2-1 his5-2 lys1-1 SUQ5 sal3-5 PNM2-19* [*psi*⁻]
584/2b **a** *ade2-1 his5-2 lys1-1 SUQ5 sal3-5 PNM2-19* [*psi*⁻]

The following strains were used in drop-out tests to assay the effect of various compounds on suppression:

(a) [*psi*+]-dependent:
MT182/8d (q.v.)
446/4a **a** *ade2-1 his5-2 SUQ5* [*psi*+]
MT193/3a *α ade2-1 lys1-1 ura3-1 SUQ5* [*psi*+]
MT35 **a**/*α ade2-1/ade2-1 SUQ5/SUQ5* [*psi*+]
MT35 **a**/**a** *ade2-1/ade2-1 SUQ5/SUQ5* [*psi*+]
MT35 *α*/*α ade2-1/ade2-1 SUQ5/SUQ5* [*psi*+]

(b) [*psi*+]-independent:
562/2a **a** *ade2-1 lys1-1 ura3-1 SUP2-1* [*psi*⁻]
X2315-14C *α ade2-1 his5-2 lys1-1 trp5-48 can1-100 ura3-1 SUP3-1* [*psi*⁻].
Five strains of genotype:
ade2-1 SUQ5 [*psi*⁻] and either *sal1*, *sal2*, *sal3*, *sal4* or *sal5*; and
four diploids of genotype:
ade2-1/ade2-1 SUQ5/SUQ5 [*psi*⁻] and either *sal1/sal1*, *sal2/sal2*, *sal3/sal3* or *sal5/sal5*.

Other strains are described in the text.

Gene symbols: *ade*, *his*, *leu*, *lys*, *trp* and *ura* are requirements for adenine, histidine, leucine, lysine, tryptophan and uracil; *can* indicates resistance to canavanine and *kar* is a recessive mutation of a gene required for karyogamy. Zygotes found in *kar* matings commonly segregate haploid cells of both parental genotypes but few diploids are stably replicating heterokaryons (Conde and Fink 1976). *SUQ5* is an ochre suppressor dependent on the presence of [*psi*+] for its expression. It is the same as SUP16 (Ono, Stewart and Sherman 1979). *PNM* and *pnm* indicate, respectively, dominant and recessive mutations in genes required for the maintenance and, possibly, expression of the [*psi*+] factor (Young and Cox 1971; Cox, Tuite and Mundy 1980).

ASU and *asu* indicate dominant and recessive antisuppressor mutations (McCready and Cox 1973). The wild-type alleles of these mutations, allowing suppression or maintenance of [*psi*+], are indicated by a "+". *sal* symbolizes recessive "allosuppressor" mutations that allow the expression of *SUQ5* in a [*psi*⁻] background (Cox 1977). *ade2-1 his5-2 lys1-1 trp5-48* and *can1-100* are ochre mutations all suppressible by *SUQ5* in a [*psi*+] background or in the presence of *sal* mutations.

Media: Yeast complete (YC) is a complete growth medium described by Cox and Bevan (1962). Both *grande* and *petite ade1* and *ade2* mutants accumulate a red pigment when grown on YC. Alternatively, Y8 medium, composed of 1% extract, 1% peptone and 8% dextrose, was used. YEPD is the same with 2% dextrose.

YEPG agar consisted of 1% yeast extract, 1% peptone, 2% w/v glycerol and 1.5% oxoid agar. MB agar was YC adjusted to pH 4.8, with methylene blue added to 3 mM from a sterile stock solution just before the plates were poured. Minimal medium, YNB, for omission plates and other purposes, is Difco Yeast Nitrogen Base + 2% Dextrose, with growth factors added to 10 or 20 μg/ml. Canavanine sulfate, where needed, was added to 40 μg/ml.

Sporulation medium was that described by Hurst and Fogel (1964).

Additions: Methanol, ethanol or DMSO was added to sterilized liquid medium after cooling to 40°. The mixture was shaken and poured immediately. Guanidine hydrochloride was made up as a 0.5 M stock solution, sterilized by filtration and added in the same way. The various carbohydrates and fatty acids were made up double strength, autoclaved separately and mixed with double-strength medium.

The "well" test: This is a rapid preliminary test to determine any lethal, inhibitory or mutagenic effects of a compound on cells. Approximately 10^5 cells are spread on the surface of a plate, a disc of agar 1 cm. in diameter is cut from the center and 100 μl of a solution of the substance to be tested is put in this well. The plate is incubated, during which time the compound diffuses out from the well, establishing a concentration gradient. After two or three days, the plate may be examined. Zones of inhibition or morphological changes may be observed.

Genetic methods: Techniques of growth, mating, sporulation and tetrad analysis have been described by Cox (1965).

Nonsuppressed derivatives of 507.4/2b, MT182/8d or CM106/5a were identified as red colonies or red sectors on YC or Y8 agar medium. This red color indicated that the ochre *ade2-1* mutation in these strains was no longer suppressed, or that a further mutation had occurred in the *ade1* or the *ade2* locus. The genotypes of the red derivatives of 507.4/2b and MT182/8d were determined by complementation by the cross-streak method. Two *SUQ5* [*psi*⁻] testers were used in every test to improve its reliability. The patterns given by the major classes of nonsuppressed (red) variants are shown in Table 1.

Cross-streak tests were also carried out using the *PNM* testers described above to check the [*psi*+] status and [psi]-dependence of suppressed strains. Strains of genotype *ade2-1 SUQ5* [*psi*+] give red diploids when mated with these testers, due to elimination of the [*psi*+] factor by the *PNM* gene (Cox 1977).

Petites were scored by replica-plating colonies to YEPG agar; only *grandes* grow on this medium. The "killer" character was scored by replica-plating colonies to MB plates seeded with about 10^6 cells of a sensitive strain. Killer colonies generate a dark-blue clear halo in the pale blue background lawn of sensitive microcolonies after 3 days incubation at 25° (Somers and Bevan 1968).

RESULTS

Guanidine hydrochloride. Guanidine hydrochloride (GuHCl) is a denaturing agent (Levine *et al.* 1963) that is a potent inducer of cytoplasmic suppressive *petites*. It also eliminates the penicillinase plasmid of *Staphylococcus aureus* (Juliani, Gambarini and Da Costa 1975). The effect of this compound on cells of 507.4/2b and MT182/8d is illustrated in Figure 1 and Tables 2 and 3. Colonies

TABLE 1

The phenotypes of diploids formed by matings of various red, adenine-requiring, nonsuppressed mutants of strains of genotype ade2–1 SUQ5 [psi+] *with three tester strains*

Nature of genetic change	Testers (all *red, ade2–1*) A *SUQ5* [*psi*-]	B *suq*+ [*psi*+]	C *karl suq*+ [*psi*+]
SUQ5 → *suq5*+	W	R	R
[*psi*+] → [*psi*-]	R	W	W
pnm	R	W	R
PNM/ASU	R	R	R
asu	W	W	R

An *ade1* tester was included to identify nonmaters and *ade1* mutations. Second-site mutations at the *ade2* locus were distinguished by their ability to grow without histidine. The critical test for discriminating [*psi*-] derivatives from nuclear mutations affecting suppression by use of the "*kar*" mutation has been described in detail by COX, TUITE and MUNDY (1980). The rationale is that no nuclear mutation can be complemented unless nuclear fusion occurs (or stably replicating heterokaryons are formed); whereas, the cytoplasmic [*psi*-] mutation requires only plasmogamy for complementation.

in which the suppression system is functional are white; those in which it is inactive are red.

The following observations showed that the red variants were induced, rather than selected, by GuHCl). (1) There was 100% conversion to the red phenotype at concentrations that allowed survival of 85% of the original cells plated (Table 2). (2) The white suppressed sectors in the mosaic colonies clearly grew faster than the induced red sectors (Figure 1); selection obviously acted against the new phenotype. (3) Plating cells after growth in liquid media containing GuHCl

FIGURE 1—Colonies of MT182/8d (*ade2–1 his5–2 leu1 SUQ5* [*psi*+]) growing on (a) 1 mM guanidine hydrochloride and (b) 2.5 mM guanidine hydrochloride. Colonies growing on YC are wholly white.

TABLE 2

The phenotype of colonies of the suppressed strains 507.4/2b and MT182/8d, both α, ade2-1 SUQ5 [psi+], *grown in various media*

	507.4/2b				MT182/8d			
Concentration of GuHCl (mM)	% Survival	No. of colonies	Phenotype	% *rho*⁻	% Survival	No. of colonies	Phenotype	% *rho*⁻
0	100.	2051	white	0.5	100	856	white	2.1
0.5	94.0	545	white	0.3	—	—	—	—
1.0	95.4	1755	1751 mosaic 4 white	0.7	94.6	351	mosaic	2.6
2.5	97.2	681	red	2.1	85.2	527	526 red	6.8
5	78.2	548	red	38.9	85.4	528	red	7.2
7.5	—	—	—	—	79.9	494	red	13.6
10	0.7	2	red	100	6.5	3	red	100
50	0.001	0	—	—	—	—	—	—

Plates were scored after 3 days incubation.

yielded sectored colonies (Table 3). (4) Growth in counter-selection media containing GuHCl yielded nonsuppressed derivatives (Table 3). No induction of red colonies was observed when cells were treated in saline.

Colonies growing on lower concentrations of GuHCl ($\leqslant$ 1 mM) were almost all mosaic. Numbers of these were resuspended, sonicated and replated. Then, 96% of the colonies that grew were pure red or pure white, indicating that the mutant phenotype had arisen during growth of the original colony and was not a replicating instability.

Petites were also induced by the treatment, and on 5 mM *GuHCl* medium most colonies were mosaic for the petite phenotype.

Genetic analysis was carried out on 108 independent nonsuppressed isolates by complementation tests and on 16 randomly chosen colonies derived from a *suq*+ [*psi*+] strain after growth on GuHCl-containing medium. All of the nonsuppressed isolates were [*psi*⁻], as were 15 of the 16 colonies from the *suq*+ parent where visual selection was not involved (Table 4, line 4); 11 were also analyzed by tetrad analysis and found to be [*psi*⁻] (Table 5).

Tests were also carried out on various white clones recovered after treatment with GuHCl to determine whether they were refractory to GuHCl or otherwise distinguishable from the original cultures. The white clones chosen were the four wholly white colonies found growing on 1 mM GuHCl (Table 2, line 3), 15 sectors from different mosaic colonies and 10 white sectors from the colonies recovered after plating from liquid medium containing GuHCl. All these clones were found to be [*psi*+] (see MATERIALS AND METHODS) and to become [*psi*⁻] on treatment with GuHCl.

Guanidine hydrochloride does not affect the expression of ochre suppressors. Suspensions of cells of suppressed strains of various genotypes were "dropped out" on YC + 2.5 mM or YC + 10 mM GuHCl plates at approximately 10^4 cells

TABLE 3

The number of nonsuppressed colonies and colonies with nonsuppressed sectors observed on YC plates after cells grown in various liquid media were plated

Medium	Viable titer/ml	No. of colonies White	No. of colonies Red	Sectored	% Nonsuppressed	% *rho*⁻	% killer⁻
(a) *507.4/2b*							
YC	2.29×10^8	2747	0	1	0.04	0.1	—
YC + 5 mM GuHCl	1.27×10^8	2481	47	9	2.21	2.7	—
YC + 50 mM GuHCl	6×10^3	1108	66	92	12.48	6.5	—
(b) *MT182/8d*							
YC	1.8×10^8	3799	0	0	0	0.3	0
YC + 1 mM GuHCl	5.06×10^7	522	1230	3	70.3	22.7	0
YNB + ade + his + leu + 1 mM GuHCl	1.07×10^6	592	1	0	0.2	0.5	0
YNB + ade + his + leu + 2.5 mM GuHCl	1.36×10^6	560	104	17	17.7	2.4	0
Counter-selection media							
YNB + leu	3.08×10^6	1963	0	0	0	0.4	0.3
YNB + leu + 1 mM GuHCl	5.64×10^6	3327	3	0	0.09	0.6	0
YNB + leu + 2.5 mM GuHCl	1.99×10^6	1931	43	18	3.1	28.2	3.3
Non-growth conditions							
Saline + 2.5 mM GuHCl	2.27×10^3	530	0	0	0	4.9	—

Ten ml volumes in conical flasks were inoculated with 10^5 cells and shaken at 28° for 3 days before sampling. The frequency of petites and, where tested, the frequency of clones sensitive to "killer" are also recorded.

TABLE 4

The genotype of nonsuppressed derivatives obtained in various strains by treatment with guanidine hydrochloride

Treatment	Numbers of clones of strains						
	507.4/2b (*SUQ5* [*psi*$^+$])		MT182/8d *SUQ5* [*psi*$^+$]		465/2a *suq*$^+$ [*psi*$^+$]		
	Tested	[*psi*$^-$]	Tested	[*psi*$^-$]	Tested	[*psi*$^-$]	[*psi*$^+$]
"Well test"	11	11	—	—	—	—	—
2.5 mM GuHCl in YC agar	12	12	12	12	—	—	—
1 mM GuHCl in YC agar: red sectors from mosaic colonies	15	15	—	—	—	—	—
1 mM GuHCl in YC agar	—	—	—	—	16	15	1
YC agar (untreated control)	—	—	—	—	10	0	10
YC + 5 mM GuHCl (liquid)	25	25	—	—	—	—	—
YNB + leu + 2.5 mM GuHCl (liquid)	—	—	10	10	—	—	—
YC + 1 mM GuHCl (liquid) (whole red)	—	—	15	15	—	—	—
(liquid) (red sectors)	—	—	8	8	—	—	—

Genotypes were determined by complementation tests.

per drop. Six of these strains were dependent on [*psi*$^+$] for the suppression phenotype, and 11 were suppressed by various chromosomal mutations (MATERIALS AND METHODS). All six psi-dependent suppressed strains grew as red cultures, and all 11 strains in which *ade2–1* was suppressed exclusively by chromosomal genes remained white.

We conclude that GuHCl induces the change from [*psi*$^+$] to [*psi*$^-$]. At relatively low concentration, it does so with remarkable efficiency. Induction depends on cell division. GuHCl does not interfere with the activity of suppression. The

TABLE 5

Genotypes of eleven red variants of MT182/8d

Revertant no.	Number of tetrads with ratios of:	
	2 suppressed: 2 nonsuppressed	3 suppressed: 1 nonsuppressed
1	9	1
2	7	2
3	7	0
4	9	0
5	5	1
6	6	0
7	7	0
8	8	1
9	6	0
10	7	0
11	8	0

Genotypes were determined by tetrad analysis after crossing with 465/2a (*ade2–1 his5–2* [*psi*$^+$]. [*psi*$^-$] variants are expected to yield 2 suppressed: 2 nonsuppressed clones in each tetrad.

frequency with which *petites* and sensitives (killer negative) were recovered in these experiments is recorded for comparison in Tables 2 and 3. The induction of inactivation or loss of the three cytoplasmic determinants was essentially independent of the others.

Dimethyl sulfoxide: DMSO is commonly used in the laboratory as a cryoprotective agent and as an organic solvent. DMSO has proved negative in the AMES mutagenesis tests (AMES *et al.* 1973) but YEE, TSUYUMI and ADAMS (1972) report that it induces [*rho*$^-$] mutations. It is known to protect *S. cerevisiae* from UV-induced death and mutation (SINGH, MAHATAN and KRISHNAN 1976).

It became clear that DMSO inhibits the growth of [*psi*$^+$] more than that of [*psi*$^-$] strains. The effect is illustrated in Tables 6 and 7. When cells of 507.4/2b (*ade2–1 SUQ5* [*psi*$^+$], etc.) were plated on media containing 5.5% DMSO or less, red papillae appeared on a background lawn of white growth. At a concentration of 6% DMSO or higher, there was no background growth, but many red colonies grew; 75 red papillae or colonies were purified and found to be [*psi*$^-$] by complementation tests.

A test was carried out to determine the genetic basis of the inhibition. A set of adenine-requiring derivatives of 507.4/2b (*ade2–1 ADE1*$^+$ *SUQ5* [*psi*$^+$] was obtained after UV treatment and classified by complementation tests (COX, TUITE and MUNDY 1980). The mutations fell into three categories: (1) A second, not suppressible, mutation had occurred in either the *ADE1* or the *ade2* locus, but ochre suppression was still actively expressed; (2) a mutation had occurred in the suppressor locus (*suq5*$^+$) or an antisuppressor locus (*ASU*$^-$ or *asu*$^-$) abolishing suppression, but leaving the strain [*psi*$^+$]; (3) a mutation had occurred rendering the strain [*psi*$^-$] *i.e.*, the strain was either *PNM*, *pnm* or had mutated from [*psi*$^+$] → [*psi*$^-$]. The original strain and each mutant was plated on YC-DMSO agar medium. The results are shown in Table 7. It is clear from the growth pattern on the plates that it was the [*psi*$^+$] genotype alone that determined inhibition of growth by DMSO in this strain.

The data presented in Table 6 also suggest that DMSO induces the [*psi*$^-$] mutation since there was an absolute increase in numbers of [*psi*$^-$] colonies on plates that permitted no residual division of [*psi*$^+$] cells. A counter-selection experiment similar to that recorded in Table 2 was performed with MT182/8d. There was an increased frequency of red colonies recovered after growth in counter-selection media containing 5% or 10% DMSO, compared with controls containing none (Table 8, lines 2, 4 and 7); 15 of the wholly red and 4 of the sectored colonies were tested by complementation tests. All reds were found to be [*psi*$^-$] and the white sectors [*psi*$^+$].

Because of the heavy selection for [*psi*$^-$] cells by DMSO, it is difficult to establish that the compound also induces the mutation. The following observations indicate that DMSO induces [*psi*$^-$] mutations: (1) There was an increased *number* of [*psi*$^-$] mutants, compared to controls, on plates containing concentrations of DMSO inhibitory to division of [*psi*$^+$] cells. (2) There was a higher frequency of mutations in counter-selection media containing DMSO, compared to controls. (3) There was an increased frequency of sectored colonies when cells grown in

TABLE 6

The growth of 507.4/2b cells and the numbers of red colonies observed on YC plates containing various concentrations of DMSO

No. of experiments	No. of plates	No. of cells plated × 10^{-4}	% DMSO	No. of red colonies or papillae	Frequency × 10^4	Growth of background
5	50	1.0148	0	1*	0.99	discrete colonies
1	10	3.85	5.0	5	1.3	confluent
3	30	109.8	5.5	1365	12.4	confluent but weaker
5	50	103.0	6.0	3,378	32.8	a few residual divisions
2	20	26.4	6.5	1,180	44.7	no residual divisions
4	24	12.5	7.0	366	29.3	no residual divisions

507.4/2b cells were suspended in saline and plated on the media at various densities. The figures are the summary of five separate experiments.

* This was revertant at the suppressor locus, that is *suq5*+.

liquid media containing DMSO were plated out (Table 8, lines 3, 4, 5 and 7). (4) All the nonsuppressed variants recovered and tested were [*psi*⁻] (104/104). The significance of this observation is that there are two kinds of mutations that can give rise to a [*psi*⁻] state, namely, mutations of the [*psi*] factor itself and recessive or dominant mutations in nuclear genes (*PNM* or *pnm*) required for the maintenance or expression of [*psi*⁺] (YOUNG and Cox 1971; Cox, TUITE and MUNDY 1980). All three types are equally resistant to DMSO (Table 7). If plating or growth in DMSO merely selected [*psi*⁻] cells from a background of spontaneous mutations, then the spectrum of mutations recovered from DMSO plates

TABLE 7

The growth of 507.4/2b and an isogenic series of adenine-requiring mutants derived from it after 7 days on YC agar + DMSO

Genotype of the 507.4/2b derivative	% DMSO			
	0	5.0%	5.5%	6.0%
Original: *ade2-1 SUQ5 ADE1*+ [*psi*+] *ASU*+ *asu*+ *PNM*+ *pnm*+	++	++	+	±
Derivatives: *ade1* [*psi*+]	++	+	±	—
ade2-x [*psi*+]	++	+	±	—
asu [*psi*+]	++	+	±	—
suq5+ [*psi*+]	++	—	—	—
[*psi*⁻]	++	++	++	++
PNM [*psi*⁻]	++	++	++	++
pnm [*psi*⁻]	++	++	++	++

The derivatives were induced by UV and classified by complementation tests (TUITE and Cox 1979); 500 cells of each strain were plated on each kind of medium.

Symbols: ++ = very good; + = poor; ± = very poor; — no growth (estimated by colony number and colony size).

TABLE 8

The number of nonsuppressed mutants of MT182/8d found after growth in complete and defined media containing DMSO

Medium + DMSO (v/v)	Colonies scored	Viable cell titer/ml	Whole red	Sectored red/white	Mosaic	% Mutant colonies	Total colonies scored	% *petite*	Total colonies scored	No. of NK
1. YC	3799	1.80×10^8	0	0	0	0.03	1494	0.3	705	0
2. YNB + leu	1963	3.08×10^6	0	0	0	0.05	735	0.4	309	1
3. YC + 5%	2110	1.68×10^7	26	10	0	1.7	634	9.5	343	34
4. YNB + leu + 5%	1807	6.82×10^5	5	2	0	0.4	536	17.7	398	1
5. YNB + ade + his + leu + 5%	1068	6.11×10^5	31	2	0	3.1	404	1.5	NT	
6. YC + 10%	4	13.33	1	0	0	25.0	NT		NT	
7. YNB + leu + 10%	555	1.08×10^4	128	11	0	25.0	333	5.4	253	2
8. YNB + ade + his + leu + 10%	0	0	0	0	0	0	0		0	

Flasks containing 10 ml of medium were inoculated with 2.5×10^5 cells and shaken at 28° for 5 days. The cultures were then diluted and plated on YC agar. [*psi*$^-$] cells of this strain do not grow on YNB + leu, with or without DMSO.

would reflect that occurring spontaneously. MUNDY (1979) and COX, TUITE and MUNDY (1980) found that, over a number of separate experiments, spontaneously occurring [*psi*$^-$] mutants were distributed as follows: *PNM*, 20.1%; *pnm*, 27.6%; and cytoplasmic [*psi*$^-$],43.4%. This contrasts with the recovery of only cytoplasmic [*psi*$^-$] revertants from media containing DMSO. Clearly, if DMSO merely selected the revertants recovered in these experiments, it was from populations of cells grossly abnormal in their distribution of spontaneous mutations.

The effect of DMSO on suppression was tested, as before, by dropping saline suspensions at 5×10^6 cells/ml on YC + 6% DMSO. The five *sal* strains remained white on both media. The two class I suppressor strains formed a pink growth on DMSO, but reverted to white when recloned on YC. All the other strains grew numerous red papillae on YC + 6% DMSO. Six papillae from the diploid MT35 **a**/α were sporulated and six to 10 tetrads analyzed from each. All six cultures yielded only nonsuppressed spore clones, that is, tetrad ratios of 0 white : 4 red. This demonstrates that DMSO also generates [*psi*$^+$] to [*psi*$^-$] mutations in diploids. However, 5% v/v DMSO in saline did not induce [*psi*$^-$] mutation in 507.4/2b.

Methanol and ethanol: We observed that many colonies of the strain CM106/5a (*ade2–1 SUQ5* [*psi*$^+$] had large red sectors when grown on YEPD plates containing methyl or ethyl alcohol. Red sectors did not appear to grow faster than the white portions of the colonies, and the size of the sectors suggested that the mutation had often arisen during one of the first three or four divisions of the clone.

The frequency of sectored colonies on plates containing various concentrations of methanol is illustrated in Figure 2. At a plating efficiency of 30%, on plates containing 10% methanol, more than 90% of the colonies had large sectors. Red nonsuppressed clones were also produced during growth in liquid media containing methanol (Table 9).

Altogether, 140 red sectors and 20 red colonies derived from liquid YEPD + 7.5% methanol cultures independently inoculated with 20 clones of 507.4/2b were tested by complementation tests; 155 red cultures were found to be [*psi*$^-$] and five were *pnm*$^-$.

Figure 3 shows that there is a delay in the appearance of [*psi*$^-$] clones in cultures that are inoculated with stationary-phase cells. This would account for the appearance of only sectored colonies on plates containing methanol or ethanol, rather than wholly red colonies. Overall, there does not seem to be any difference in the growth of [*psi*$^+$] and [*psi*$^-$] strains in methanol medium sufficient to account for the accumulation of [*psi*$^-$] clones by selection. Table 9 shows that [*psi*$^-$] clones accumulated in high frequency in counter-selection conditions. Although their numbers are not recorded separately, colonies carrying large red sectors were also found after cells in this media were plated on YC. However, it was also observed that ethanol and methanol caused severe 'clumping' of cells in liquid media, clumps that could not be readily dispersed by ultrasonication. The increased clumping could account for the increased frequency of sectored colonies after plating from such media. Methanol was also found to induce [*psi*$^-$] muta-

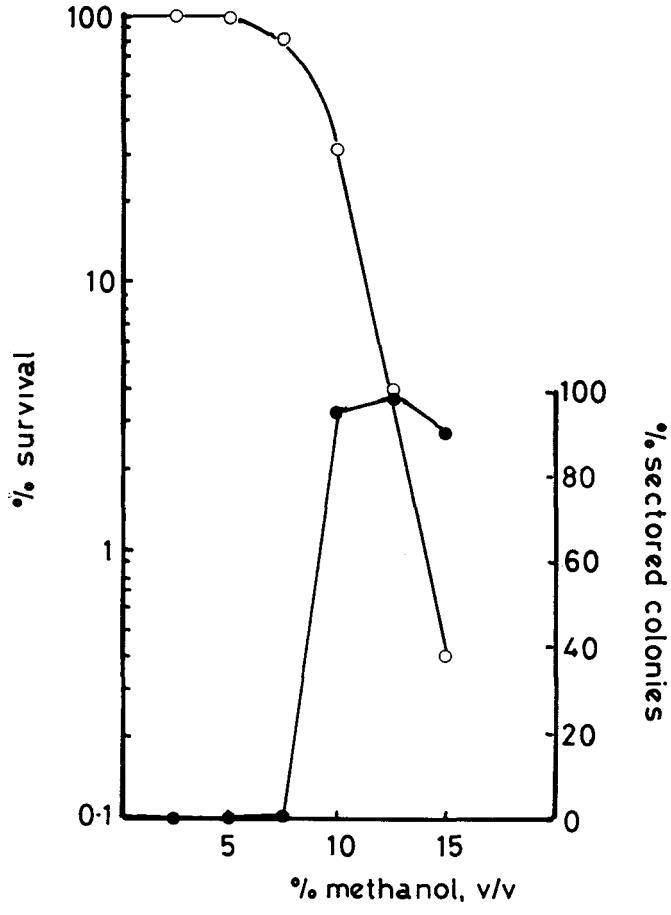

FIGURE 2—Survival and the frequency of nonsuppressed revertants of CM106/5a plated on YC containing various concentrations of methanol. ○ = survival; ● = frequency of sectored colonies. No wholly red colonies were observed.

tions in the diploid MT35 and in unsuppressed [*psi*$^+$] strains. It has no effect on suppression. We conclude that growth in media containing methanol or ethanol induces [*psi*$^-$] mutations.

Other agents that induce [*psi*$^-$] *mutation*: It has been reported that KCl and ethylene glycol induce [*psi*$^-$] mutation (SINGH, HELMS and SHERMAN 1979). We have repeated the experiments and found similar effects, except that we were unable to demonstrate the appearance of [*psi*$^-$] clones during growth in counter-selection media containing KCl (Table 10). Concentrations of KCl that were effective in generating [*psi*$^-$] mutations were extremely inhibitory to growth, depressing yields by three orders of magnitude. Furthermore a large proportion of the colonies showing nonsuppressed sectors after growth in 2M KCl were "mosaic" with a "bicycle-wheel" effect. Two of these colonies were resuspended and replated and found to maintain replicating instabilities (Table 11). In these clones

TABLE 9

The number of nonsuppressed variants of CM106/5a (ade2-1 SUQ5 [psi+]) *grown in various media*

Medium	Culture no.	Viable cell titer ml	Colony counts		
			White	Red	% Non-suppressed
YNB + adenine	1	3.3×10^7	65	0	0
	2	7.1×10^7	142	0	0
	3	7.0×10^7	140	0	0
YNB + adenine + 7.5% methanol	1	6.0×10^7	555	44	7.3
	2	4.0×10^7	382	18	4.5
	3	3.9×10^7	380	12	3.1
YNB	1	2.9×10^7	288	1	0.3
	2	2.1×10^7	307	0	0
	3	2.2×10^7	220	0	0
YNB + 7.5% methanol	1	5.2×10^7	478	47	9.0
	2	2.0×10^7	191	10	5.0
	3	2.0×10^7	177	26	12.8

10^5 log-phase cells were inoculated into flasks containing 10 ml of medium and incubated at 28° for 7 days. [*psi*⁻] cells of this strain do not grow in YNB, with or without methanol.

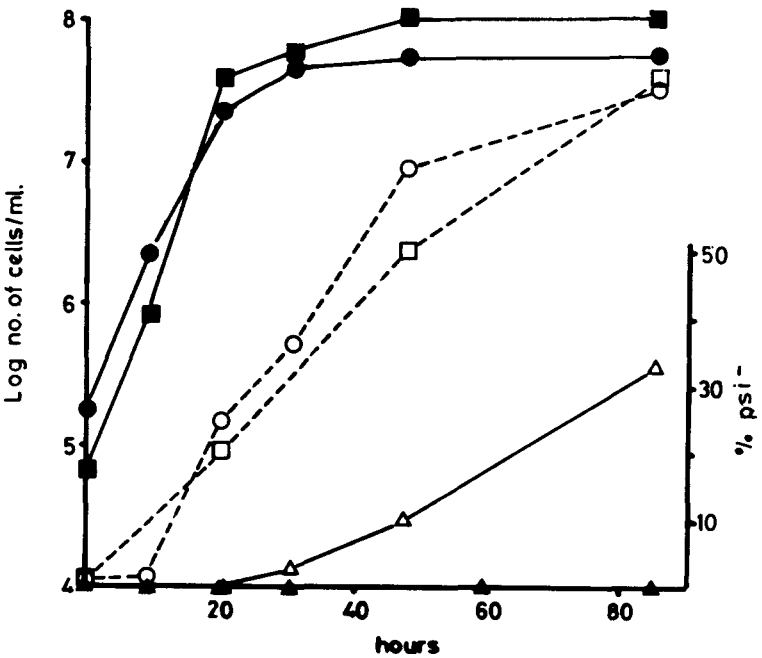

FIGURE 3—Numbers of cells in cultures of [*psi*+] and [*psi*] variants of CM106/5a and the frequency of variant clones recovered after plating the cultures on YC.

Growth of: ■ [*psi*+] cells in YEPD; ● [*psi*⁻] in YEPD; □ [*psi*+] cells in YEPD + 10% methanol; ○ [*psi*⁻] cells in YEPD + 10% methanol.

▲ % of [*psi*⁻] cells in YEPD; △ %of [*psi*⁻] cells in YEPD + 10% methanol.

TABLE 10

Induction by KCl: the numbers of nonsuppressed variants found in cultures of 507.4/2b and MT182/8d after 10 days growth in various liquid media

Strain	Medium	Viable cell titer	Colonies scored	Nonsuppressed colonies			% Revertant	% *petite*
				Whole	Sectored	Mosaic		
507.4/2b	YC	1.2×10^8	2415	0	0	0	0.04	0.15
	YC + 2 M KCl	1.8×10^5	3061	79	8	55	4.64	0.26
MT182/8d	YNB + leu + ade + his + 2 M KCl	8.3×10^4	29	0	0	0	—	—
	YNB + leu + 2 M KCl	2.53×10^5	1598	0	0	0	0	—

Inoculations were made at 10^4 cells/ml. Samples were plated on YC to estimate viable titer and numbers of mutants.

the pattern of segregation shows that the red phenotype is unstable: stable white clones are produced, but no stable reds. It is possible that the unstable clones are low copy-number variants that revert to [*psi*$^+$] cells with a normal copy number.

KCl is the one compound of those we have tested where the observations more strongly indicate selection than induction. One observation, however, suggests induction; all of the nonsuppressed clones recovered and tested (73) proved to be [*psi*$^-$].

Agents related to methanol and ethanol: We conducted a brief survey of substances that might have metabolic roles related to those of methanol or ethanol to determine whether they induce [*psi*$^-$] reversion. First, a series of media were made, based on YC but containing these substances made up to 1 M or 2 M (approximately equivalent to the molarity of 10% v/v ethanol). Twelve separate cultures of each medium were inoculated with different clones of CM106/5a. After five days of incubation, with aeration, loopsful of each culture were streaked on YC. We found that, in many of the cultures, large numbers of cells giving rise to red clones had accumulated. The organic acids pyruvate (1 M), glutamate (1 M) and succinate (1 M) were, apparently, very potent; glycerol (2 M) ethanol (2 M) sorbitol (2 M) and mannose (2 M) less so, and glucose, galactose and fructose (all (2 M) were virtually ineffective. The experiment was repeated using defined counter-selection media supplemented with 1 M succinate. It was clear that succinate induced the nonsuppressed variants at very high frequency in counter-selection conditions, with more than 50% of clones from each of ten cultures being red.

Second, suspensions of cells of MT182/8d were plated onto solid YC media containing the compound. On many of the media, the colonies that grew were small and of unusual appearance. They were replicated onto standard YC plates and, after further incubation, scored for the appearance of nonsuppressed red clones. The results are shown in Table 12. Up to 10 independent nonsuppressed clones or sectors were isolated from plates following each treatment and tested by complementation; all were [*psi*$^-$]. It is clear that [*psi*$^-$] mutations are induced on many of these media. On some media, there were very few colonies with red sectors, and it cannot be assumed that these were induced by the treatment.

Agents that do not induce [psi$^-$] *mutation*: A number of agents that have been more-or-less exhaustively tested to determine whether they can induce [*psi*$^-$] mutations from [*psi*$^+$] have given negative results (TUITE 1978). Some of these

TABLE 11

Numbers of red, white and mosiac colonies obtained after sonicating and recloning two mosaic and one sectored colony of 507.4/2b, found after growth in YC + 2 M KCl

Nature of mutant	Total colonies	Whole red	Whole white	Mosaic	Sectored red/white	Sectored mosaic/white
mosaic-1	260	0	73	172	0	15
mosaic-2	349	0	77	265	0	7
½ red/½ white sectored	484	320	161	0	3	0

TABLE 12

The effectiveness of various agents in producing [psi⁻] *derivatives of MT182/8d* (SUQ5 [psi+] *when present in plates in high concentrations*

Agent and concentration	No. of colonies examined	No. of sectored colonies	% Sectored	No. tested and genotype
(a) *Ionic*				
1.8 M KCl	16	14 L*	88	10 × [*psi*⁻]
1.0 M MgCl2	9	8 L	90	10 × [*psi*⁻]
1.0 M glutamate	84	82 L	98	10 × [*psi*⁻]
(b) *Alcohols*				
1.8 M methanol	68	0	0	
1.8 M ethanol	86	0	0	
1.8 M ethylene glycol	93	All colonies wholly red	—	10 × [*psi*⁻]
1.8 M glycerol	94	39 L	41	7 × [*psi*⁻]
1.8 M sorbitol	58	1 H*	2	
(c) *Sugars*				
1.8 M glucose	51	0	0	
1.8 M galactose	49	0	0	
1.8 M fructose	56	6 H	11	1 × [*psi*⁻]
1.8 M sucrose	60	4 L	7	4 × [*psi*⁻]
1.8 M mannose	91	0	0	

Equal numbers of cells were plated on all media. Nonsuppressed clones appear as red sectors or wholly red colonies.

* "H" indicates the sectors were hairline, "L" that they were large (up to half the colony).

are listed in (Table 13. Others were: methylene blue (20 μg/ml); nystatin (5–10 μg/ml); streptomycin (200 μg/ml); neomycin (100 μg/ml), urea (by a "well" test) and protamine sulfate (100 μg/ml).

DISCUSSION

Table 13 summarizes and compares the affects of various agents on cytoplasmic determinants in yeast. It is apparent from the table that agents fall into five classes. These are: (1) Agents that induce genetic change in all three categories of genes: nuclear, mitochondrial and [*psi*]. These are all "conventional" radiomimetic chemicals or physical mutagens. (2) Agents that selectively affect the mitochondrial genome. Examples are ethidium bromide, acriflavine and thymidylate starvation. (3) Agents that affect both the [*psi*] factor and the mitochondrial genome. Examples are guanidine hydrochloride and dimethyl sulfoxide. (4) Agents that exclusively affect the [*psi*] factor. These are the compounds described here and in SINGH, HELMS and SHERMAN (1979) that are effective when present in growth media at very high concentrations. (5) An agent common to mutagenesis of [*psi*] and nuclear genes, but that does not, apparently, effect *petite* mutagenesis. This is error-prone repair of UV damage, controlled by the *rad6–rad18* pathway.

We have argued elsewhere that the susceptibility of [*psi*] to the standard mutagens, all of which interact with nucleic acids, indicates that the [*psi*] factor is a nucleic acid, probably DNA. Furthermore, the control of [*psi*] mutagenesis

TABLE 13

A summary of the effects of various agents on the induction of different cytoplasmic mutations in yeast

Treatment	[*psi*$^+$] [*psi*$^-$]	Mitochondrial drug resistance	[*rho*$^+$] [*rho*$^-$]	Killer sensitive
(EMS) Nuclear mutagens (NTG) (UV)	+[1,5]		+[20]	+[21]
Error-prone repair	+[5]		0[19]	
Ethidium bromide 10 μg/ml	0[1]		+[6]	0[8]
Acriflavine 10 μg/ml	0[1]		+[7]	(+)[8]
5FU 50 μg/ml	0[2]		+[9]	+[11]
$MnCl_2$ 8.8 mM	0[2]	+[12]	+[17]	
Thymidine starvation	(0)[2]	+[13]	+[13]	(0)[2]
High temperature growth (37°)	0[2]		+[14]	+[15]
52° heat shock	+		+[14]	
Cycloheximide 1 μg/ml	0[2]		+[8]	+[8]
Erythromycin 250 μg/ml	(+)[2]		+[10]	
Guanidine hydrochloride 2.5 mM	+		+[16*]	+
DMSO 2.5% v/v	+		+[23]	(+)
MeOH/EtOH 10% v/v	+		0[2]	
2 M KCl/M $MgCl_2$	+[4,*]			
1.8 M ethylene glycol	+[4,*]		(0)	
2 M glycerol	+[3]		(0)	
2 M mannose	(+)			
2 M glucose	(0)			
2 M fructose	(0)			
2 M galactose	(0)			
2 M sorbitol	(0)			
M pyruvate	(+)			
M glutamate	+			
M succinate	+			
cdc4, restrictive temperature	0[2]		0[2]	
cdc7, restrictive temperature	0_2		+[2]	
cdc8, restrictive temperature	+[2]		+[2,22]	
cdc9, restrictive temperature	0[2]		0[2]	
cdc21, restrictive temperature	(0)[2]		+[18,22]	0[2]

Parentheses indicate that the evidence is based on preliminary observations only.
+ = active; 0 = inactive.

1. YOUNG 1969; 2. TUITE 1978; 3. MUNDY 1979; 4. SINGH, HELMS and SHERMAN 1979; 5. TUITE and COX 1979; 6. SLONIMSKI 1968; 7. EPHRUSSI and HOTTINGUER 1951; 8. FINK and STYLES 1972; 9. MOUSTACCHI and MARKOVITCH 1963; 10. WILLIAMSON, MAROUDAS and WILKIE 1971; 11. MITCHELL, HERRING and BEVAN 1976; 12. PUTRAMENT, BARANOWSKA and PRAZMO 1973; 13. BARCLAY and LITTLE 1978; 14. SHERMAN 1956; 15. WICKNER 1974; 16. JULIANI, DA COSTA and BACILA 1973; 17. NAGAI, YANAGASHA and NAGAI 1961; 18. J. C. GAME, personal communication; 19. MOUSTACCHI 1968; 20. Various sources; 21. VODKIN 1977; 22. NEWLON, LUDESCHER and WALTER 1979; 23. YEE, TSUYUMU and ADAMS 1972; *This study.

by *rad1* excision and by the *rad6–rad18* error-prone repair pathways indicates that the metabolism of [*psi*]-DNA and nuclear DNA have much in common.

The heterokaryon test demonstrates that the [*psi*] factor is cytoplasmically located (JINKS 1963; Cox, TUITE and MUNDY 1980); yet, it is insensitive to acriflavine and ethidium bromide. This suggests that the enzymes of mitochondrial

metabolism, which are probably responsible for its sensitivity to these dyes (MAHLER and BASTOS 1974), are confined to that organelle. This conclusion is supported by the observations of the effects of thymidylate starvation, which is highly mutagenic for mitochondrial genomes, but seems to have no effect on [*psi*], (TUITE 1978).

There were two chemicals in Class 3 that induce both [*psi*$^-$] mutations and, at lower frequencies, *petites*, but do not appear to cause nuclear mutations. There is no evidence that either of these compounds reacts with DNA to cause mutation. DMSO, which is used as a solvent for many carcinogens and potential carcinogens, has proved negative at about 2.5% v/v in controls of mutagenesis studies with the AMES test (AMES *et al.* 1973). It appears, however, to have a plethora of other biological effects, including the activation of promoters, both *in vivo* and *in vitro* (NAKANASHI *et al.* 1974), and increasing membrane permeability (see SCOTNIKI and ROLFE 1977; FRIEND and FREEDMAN 1978). JULIANI, DA COSTA and BACILA (1973), who were the first to show that guanidine hydrochloride induced *petites* in *Saccharomyces cerevisiae*, also demonstrated that the DNA modification that occurred as a result of treatment behaved as if a "sensitive site" for the action of GuHCl had been removed, since there was no progressive degradation of mitochondrial DNA in successive treatments.

There is nothing to suggest that the mode of action of DMSO and GuHCl has anything in common. There are two striking differences in their effects. First, the concentration ranges over which each is active in mutageneiss differ by three orders of magnitude. Second, whereas DMSO selects heavily for [*psi*$^-$] cells in YEPD medium, GuHCl selects against them.

What is the mode of action of the various compounds? There are four kinds of events that could reasonably be expected to affect phenotypes controlled by plasmid-borne genes: (1) Curing, meaning the loss of the plasmid; (2) mutation, which we take to include any changes in the structure of the genetic material, including those due to recombination-like events; (3) low copy-number variants; and (4) a "switch-off" in a self-sustaining regulation system that governs the activity of a structural gene or the replication of the plasmid.

None of the compounds described here is normally regarded as a mutagen. It will be important, therefore, to establish the nature of the [*psi*] "mutation" that they produce because this may throw light on the nature of the [*psi*] factor and on the control of its replication and expression. Also, it would yield important information about the interaction of these compounds with biological systems.

One of us (M.F.T.) is indebted to the Science Research Council of Great Britain for a research studentship. The work was supported by a Science Research Council Support grant.

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