OSMOTIC-REMEDIAL MUTANTS. A NEW CLASSIFICATION FOR NUTRITIONAL MUTANTS IN YEAST[1]

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INDEPENDENT isolates of a given nutritional mutant in a microorganism can frequently be distinguished from one another when examined under a variety of conditions or treatments which can effect a restoration of prototrophic growth. Some of the properties in which they may differ are their response to external suppressors (YANOFSKY and BONNER 1955), to changes in temperature (MITCHELL and HOULAHAN 1946), or pH of the medium (STOKES, FOSTER and WOODWARD 1943) and their susceptibility to phenotypic reversal by 5-fluorouracil (CHAMPE and BENZER, 1962), or streptomycin (GORINI and KATJA, 1964). Other useful characterizations are the patterns of complementation in diploids or heterokaryons (WOODWARD, PARTRIDGE and GILES 1958), and the back mutation rates following treatments with specific mutagens (MARGOLIN and MUKAI 1961), or the intervention of a meiotic cycle in a diploid organism (MAGNI 1963), and "self transduction" in bacteria (DEMEREC 1963).

To these characterizations there can be added the ability to grow in the minimal medium when it has been modified by the addition of a relatively innocuous solute to raise the osmotic pressure. A variety of mutants with this property have been found in yeast, *Saccharomyces cerevisiae*. The description of several representative "osmotic-remedial" mutants is given below.

MATERIALS AND METHODS

From the University of Washington collection of Saccharomyces breeding stocks, haploid auxotrophs amenable to multiple testing on agar plates, *i.e.* those showing a minimal amount of cross feeding, were selected to be screened for osmotic-remedial mutants. Aerobically grown, stationary-phase cells were harvested from YEP broth (1 percent yeast extract, 2 percent peptone, and 2 percent glucose), washed and spotted (about 10^4 cells per drop) on agar plates of the diagnostic media (WICKERHAM 1946) with 1.0 M KCl, 0.5 M KCl or no extra salt. The plates were incubated at 33°, 25°, and 18°C to screen for temperature mutants as well as to see if the osmotic-remedial response was temperature dependent. Confluent growth was the criterion for a positive score.

In subsequent tests, liquid media with a variety of solutes—KCl, glycerol, glucose, sorbitol, and diethylene glycol—were used to obtain growth curves for mutants which had shown a positive response to the extra KCl in the above screening. The liquid media (5 ml in 11 × 100 mm test tubes) were inoculated with 10^5 cells and left standing except for the momentary shaking needed to suspend the cells for turbidity measurements. The measurements were taken with a Klett colorimeter with filter No. 59. The Klett readings recorded are within the range where

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there is a 1:1 correspondence between turbidity and dry cell weight. At the end of the experiments, samples were taken from the liquid cultures and plated on the normal diagnostic medium to see if the presence of back mutants could account for the observed growth, and, occassionally, a point on the yield *vs.* molarity plots was lost because of the appearance of a revertant population.

RESULTS

The occurrence of osmotic-remedial mutants: Thirty-six osmotic-remedial mutants were found in the collection of 231 mutants examined (Table 1). Twenty-three of 116 adenine mutants, 3 of 30 isoleucine mutants, 5 of 17 histidine mutants, 1 of 17 tyrosine or phenylalanine mutants, and 3 of 13 threonine mutants possessed this property. No osmotic-remedial mutants were found in the sample of 16 tryptophan, 9 lysine, 7 uracil, and 6 leucine mutants.

It was possible to confirm the initial classification of most of the osmotic-remedial mutants by testing additional strains carrying the osmotic-sensitive allele. In each case, the response pattern was similar to the original scoring. Thus, it is assumed that the ability to grow under increased osmotic pressures without the normally essential supplement is a characteristic of the mutant allele and is not brought about by modifiers segregating independently of the locus in question. Support for this interpretation will be given later with the analysis of crosses involving osmotic-remedial mutants.

The efficacy of various solutes: Following the initial observation that 0.5 M or 1 M KCl in the diagnostic medium would permit the growth of certain nutritional mutants, other agents were tested to see if this phenomenon was a response to potassium or chloride ions or to the increased osmotic pressure of the medium. Some of the solutes, NaCl, sorbitol, and diethylene glycol, were gen-

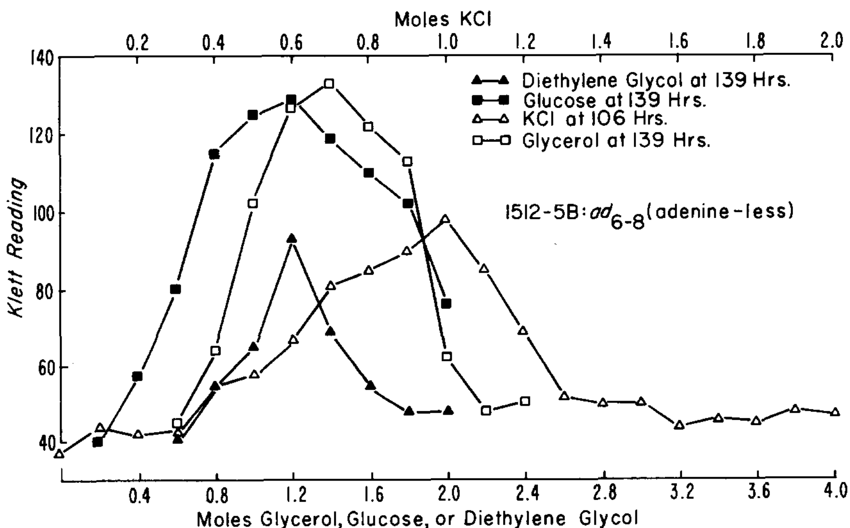

FIGURE 1.—Yields of an osmotic-remedial adenineless mutant. ad_{6-8}, when grown (30°C) on adenineless media supplemented with diethylene glycol, glucose, glycerol, or KCl.

TABLE 1

Days required for a positive score by mutants which respond to increased osmotic pressures in the test media

	Culture conditions: Temperature and KCl concentration										
	33°C			25°C			18°C				
Mutant*	0M	.5M	1M	0M	.5M	1M	0M	.5M	1M	Strain	Source†
ad_{1-1}	..	7	5	..	10	7	..	..	..	1694–8A	ELT
ad_{2-8}	..	..	10	..	7	7	..	..	..	5135D	CR
ad_{2-13}	..	..	10	7	5	5	5	3	3	4718B	CR
ad_{2-14}	..	..	5	5	3	3	7	5	7	4722A	CR
ad_{2-19}	..	..	15	..	5	5	..	7	10	6219D	CR
ad_{3-2}	..	..	..	5	5	..	10	7	7	1427–4A	HR
ad_{3-7}	..	..	..	..	10	5	22	7	5	4025B	HR
ad_{3-9}	7	7	7	..	..	7	..	7	5	4043C	HR
ad_{4-13}	7	5	3	7	5	5	..	..	10	4093A	HR
ad_{5-19}	..	..	7	..	..	10	..	..	..	3515B	HR
ad_{6-7}	..	..	..	..	..	15	..	..	15	3684A	HR
ad_{6-8}	..	10	7	10	5	5	..	10	10	3689B	HR
ad_{6-10}	..	15	10	10	5	3	7	5	5	3697D	HR
ad_{6-41}	..	15	10	..	15	7	..	15	7	BW1F–14C	EWJ
ad_{6-44}	..	..	22	..	15	10	..	15	22	BW5F–4C	EWJ
ad_{7-1}	..	7	5	10	7	5	15	10	10	4214A	HR
ad_{7-3}	..	..	..	..	10	5	22	7	5	3773A	HR
ad_{7-11}	..	7	15	7	5	5	10	7	15	1630–2A	DCH
ad_{7-15}	..	5	3	5	3	3	5	5	7	1614–3C	DCH
ad_{7-19}	..	..	..	..	7	7	15	10	15	1617–1A	DCH
ad_{7-23}	..	..	..	..	15	15	22	15	15	1608–4A	DCH
ad_{7-24}	..	15	5	7	3	3	10	5	7	1640–1C	DCH
ad_{7-25}	..	..	..	..	..	7	..	10	10	1609–5D	DCH
ad_{7-32}	..	..	..	..	7	3	15	5	7	1627–4D	DCH
hi_{1-5}	..	..	..	..	3	3	3	3	7	JB–13	RKM
hi_{1-7}	..	..	..	..	..	..	..	..	15	JC–6	RKM
hi_{2-1}	5	5	7	10	7	10	..	..	..	1446–168B	DCH
hi_{6-1}	..	5	3	..	..	7	..	..	..	1323–26A	RKM
hi_{8-1}	..	5	5	..	5	5	..	10	10	1512–5B	RKM
is_{2-4}	..	..	5	..	3	5	..	5	7	AT281D	SNK
is_{2-8}	..	..	7	..	7	5	..	..	..	Nr211–1aM	WL
is_x	..	..	7	..	7	3	..	5	3	M–19	SNK
thr_{2-3}	..	..	..	..	7	7	10	7	22	JB–46	RKM
thr_{2-5}	..	..	..	..	15	10	..	10	10	1680–4C	DCH
thr_{2-6}	..	..	..	..	..	..	..	..	22	7D31–1	DCH
$ty_{2,4-2}$	..	5	..	3	5	..	5	7	..	JD–7	RKM

* The symbols stand for genes controlling requirements for adenine, histidine, isoleucine + valine, threonine + methionine, and tyrosine + phenylalanine.

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erally less satisfactory than KCl in alleviating the nutritional requirement, since at the higher concentrations they were inhibitory to both wild-type and mutant strains. Glucose and glycerol proved effective (Figure 1), but since they could be metabolized by the cells, the osmotic pressure of the medium was subject

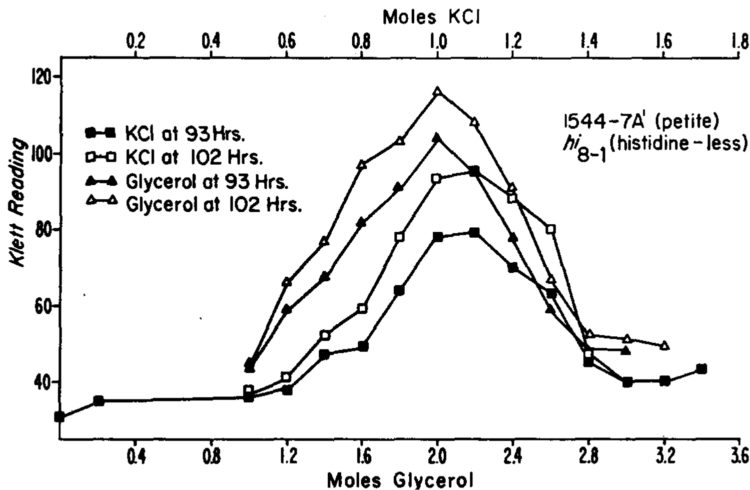

FIGURE 2.—A comparison of yields obtained on KCl supplemented and glycerol supplemented histidineless media for a petite isolate of the osmotic-remedial histidineless mutant, hi_{8-1}, grown at 30°C.

to change as the molarity of the substrate and end products varied during the course of the experiment.

A direct comparison of yield *vs.* molarity plots for KCl and a nontoxic organic solute was sought. For this purpose, petite (respiratory deficient) clones were isolated and glycerol, which no longer could serve as a substrate, was used as the solute. Under these conditions, KCl and glycerol give comparable optima; the results of a representative experiment are summarized in Figure 2.

The yield *vs.* molarity plots, Figures 1 and 2, show the optimal concentrations of the various solutes for the fastest growth rate. Each graph is a representation of the cross-section of 10 to 20 growth curves of the mutants in adenineless media (Figure 1) or histidineless media (Figure 2) with different concentrations of solute. At the time chosen for a plot, none of the cultures in the series had reached the stationary phase. This procedure was followed since the final yields reflected the toxicity of the solutes. For example, the ad_{6-8} mutant, 1512-5B, when grown in the adenineless medium with 1 M KCl, the KCl concentration which gave the shortest generation time (Figure 1), gave a final Klett reading of 137; however, with 0.3 M KCl, the minimal concentration which permits growth at 30°C without an adenine supplement, a Klett reading of 182 was attained. The same mutant strain, 1512-5B, grown in the synthetic complete medium (20 mg adenine sulfate/ml) gave a Klett reading of 210 and a cell count of 9×10^7 cells/ml. In complete media with 1 M KCl and 2 M KCl, the readings and cell counts were 140, 5×10^7 cells/ml, and 90, 2.5×10^7 cells/ml, respectively.

Only a restoration of enzyme activity can account for the growth in the diagnostic media with KCl, since the inoculum of 10^5 cells was too small to provide the reserves of adenine or histidine necessary for the several-hundred fold increase in cell numbers, and no revertants to wild type were found in the sample

of 10^4 cells taken from the fully grown cultures at the end of the experiment. A fuller description of the physiological aspects of the osmotic response will be presented elsewhere (FRIIS and HAWTHORNE, in preparation).

The interdependence of temperature and osmotic pressure: Many of the osmotic-remedial mutants can also be classified as temperature mutants. For example, ad_{2-13}, ad_{6-10}, ad_{7-11}, ad_{7-24} and hi_{1-5} strains will grow on the diagnostic medium with no extra salt when the incubation temperature is lowered from 30°C, the temperature used in the routine tests for nutritional requirements, to 25° or 18° (Table 1). If the incubation temperature is raised to 33° or 35°, a greater salt concentration is needed for the growth of these mutants. This relationship is illustrated in the rise of the osmotic optima for the growth of the ad_{2-13} mutant at 18°, 25°, 30°, and 35° (Figure 3).

The phenomenon of an increase in the osmotic optima with the higher incubation temperatures was observed for several mutants which ordinarily would not be recognized as temperature mutants. Strains with ad_{7-3} or ad_{7-32} were unable to grow at 18°C without 0.5 M or 0.2 M KCl, respectively, in the adenineless medium and required a higher molarity for growth at the higher temperatures (Table 2).

An inverse correlation of temperature and osmotic pressure was noted for the mutants ad_{1-1}, ad_{4-13}, ad_{5-19}, hi_{2-1}, and hi_{6-1}. With these mutants, the higher osmotic pressures were required for growth at the lower temperatures (Tables 1 and 2).

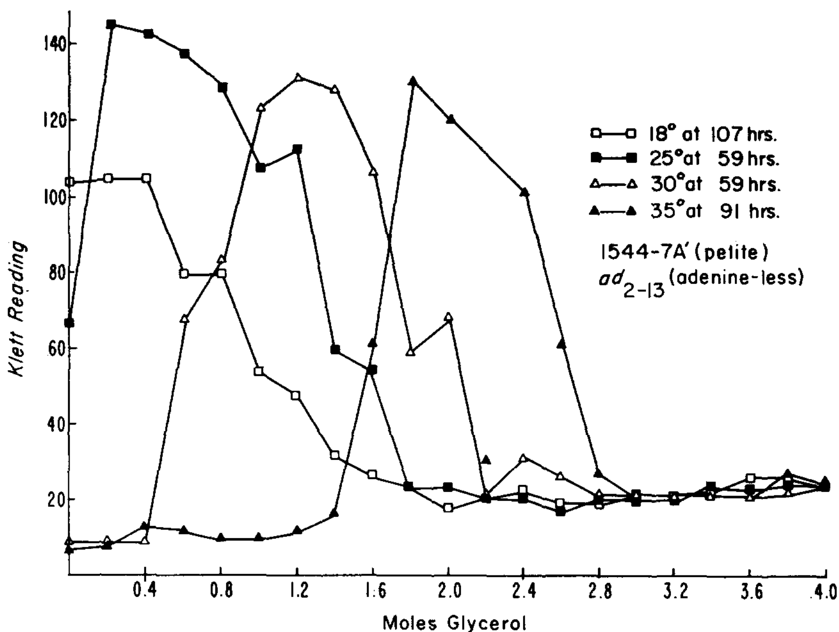

FIGURE 3.—The interaction of temperature and osmotic pressure on the growth of a temperature-sensitive, osmotic-remedial adenineless mutant, ad_{2-13}, on adenineless media supplemented with glycerol.

TABLE 2

Interdependence of temperature and osmotic pressure

Mutant	Osmotic optima: Molarity of KCl			Growth without extra salt
	33°C	25°C	18°C	
ad_{2-13}	1.2M*	0.3M	0	25°C
ad_{3-9}	0, 1.1M	1.0M	0.6M	33°C
ad_{7-3}	2.0M	1.4M	1.0M	. .
ad_{7-32}	. .	1.1M	0.4M	. .
hi_{2-1}	0.4M	0.8M	. .	33°C
hi_{6-1}	1.3M	2.0M	. .	. .

* Osmotic optimum determined at 35°C.

Still other mutants showed unique response patterns to the interaction of temperature and osmotic pressure. The most complicated pattern was displayed by an adenineless mutant, ad_{3-9}, which grew well in the normal adenineless medium at 33°C, was inhibited by the addition of KCl up to 0.6 M, and then grew better with an increasing salt concentration and showed a second optimum at 1.1 M KCl. At lower incubation temperatures, there was no growth in the normal medium and the optimal concentration of the added KCl dropped to 0.6 M at 18°.

As indicated above, the osmotic-remedial mutants were likely to be temperature mutants as well. The converse of this observation also holds. In fact, all but three of the 21 temperature mutants detected during the screening of the stock collection were also classified as osmotic-remedial mutants and therefore included in Table 1.

To see if there might be a similar coincidence of the osmotic response with a sensitivity to pH, five osmotic-remedial histidineless strains, hi_{1-5}, hi_{2-1}, hi_{6-1}, hi_{8-1}, and ad_{3-2} (ad_3 imposes a requirement for both histidine and adenine) which displayed various temperature-osmotic pressure interactions, were selected for the following investigation. In this experiment, the osmotic pressure was kept constant (0.1 M buffer was incorporated in the histidineless medium KCl/HCl for pH 3.5, K-acetate/acetic acid for pH 4.8 and pH 5.7, and K_2HPO_4/KH_2PO_4 for pH 6.5 and pH 7.4) while three temperatures, 18°C, 25°C, and 33°C, were used in assaying the effect of varying the pH on the expression of the mutant phenotype. For one of the temperature mutants, hi_{1-5}, there was an extension of the permissive temperature range to 25° at pH 6.5. The other two temperature mutants, hi_{2-1} and ad_{3-1}, also showed a pH optimum, pH 3.5, but they grew only at the permissive temperatures, as noted in Table 1. The remaining mutants—hi_{6-1}, characterized in Table 2, and hi_{8-1}, which shows no interaction of temperature with the osmotic response—failed to grow under any of the above conditions.

Osmotic-remediability, suppressibility, and complementation: There is a negative correlation for the osmotic response and the susceptibility to phenotypic suppression by "super-suppressors" (HAWTHORNE and MORTIMER 1963). The osmotic-remedial mutants and the suppressible mutants appear to be in mutually exclusive subsets. Twenty-three suppressible mutants were included in the

screening and none responded to the increased osmotic pressure. Fifteen osmotic-remedial mutants have been tested and found to be unaffected by the presence of a super-suppressor. Both types of mutants can occur at the same locus.

Amongst 27 ad_7 alleles tested for both characteristics, there were five with a suppressible phenotype and nine osmotic-remedial mutants. An examination of the ad_7 alleles for intragenic complementation at 30°C divided this sample into a set of 16 complementing mutants and a set of 11 noncomplementing mutants. None of the suppressible mutants was in the complementing class, while seven of the nine osmotic-remedial mutants were classified as complementing mutants. The remaining two osmotic-remedial mutants were found to complement when the tests were conducted at 21°. The osmotic-remedial mutants were not restricted to any particular section of the complementation map (Figure 4).

It should be noted that all "ad_7" mutants can be thought of either as being complementing mutants in that they complement all "ad_5" mutants or as being defective in the second cistron of a bipartite locus, ad_5-ad_7, since the *trans* diploids grow like wild type on adenineless medium. The argument for a single complex locus is based on the occurrence of a third general class of mutants for this locus, designated ad_{5-7} which fail to complement either ad_5 or ad_7 (ROMAN 1956).

Crosses with osmotic-remedial mutants: In addition to noncomplementing combinations of ad_7 alleles from the above study, diploids heteroallelic for an osmotic-remedial and a nonresponding mutant have been constructed and tested on high salt media. In most cases, the osmotic response proved dominant, although generally the diploid grew more slowly than the osmotic-remedial parent.

For several combinations, a tetrad analysis of four-spored asci has demonstrated the expected 2:2 segregation for the response to the extra KCl in the diagnostic medium: hi_{1-5}/hi_{1-1}, five asci; ad_{2-13}/ad_{2-20}, seven asci; ad_{6-8}/ad_{6-3}, five asci; and ad_{6-10}/ad_{6-43}, eight asci. The tetrads from the first two crosses were also scored for growth on the normal diagnostic medium at 18°C and again two of the segregants in each ascus grew, the same two which grew at 30° when 1 M KCl was added to the histidineless or adenineless media.

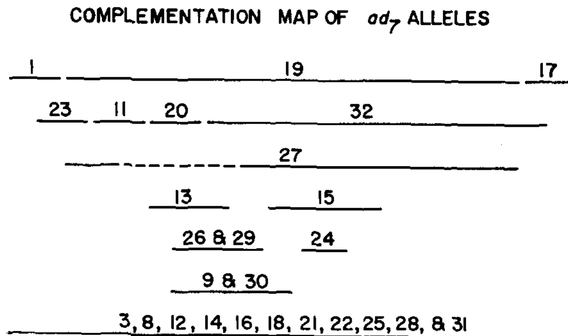

FIGURE 4.—The complementation map of the ad_7 alleles based on the 30°C assay. (At 30°, one of the temperature-sensitive alleles, ad_{7-10}, grew so well as to preclude scoring for complementation; however, at 21°, the complementation pattern for ad_{7-10} could be discerned so it was classified as a complementing allele.)

DISCUSSION

An objective in the characterization of the mutant alleles of a gene is to relate the mutant classes to particular categories of defects in the DNA code. This is done at a molecular level in the studies concerned with the effectiveness of base analogs and specific mutagens such as nitrous acid, ethylmethanesulfonate, proflavine, etc., in producing back mutants. The defect is interpreted in terms of a transitional or transversional base substitution, hence a missense or nonsense coding unit, when the base analogs, or the alkylating agents are effective (FREESE 1959; BRENNER, BARNETT, CRICK, and ORGEL 1961; KRIEG 1963). If proflavine proves to be the only efficient agent in giving revertants, a loss or an addition of a base pair to give a reading-frame shift is indicated (CRICK, BARNETT, BRENNER, and WATTS-TOBIN 1961).

A physiological description of the defect is possible sometimes without resorting to the above treatments. Mutants which have a higher spontaneous back mutation rate during a meiotic division than during mitosis are also postulated to have a reading-frame shift which can be corrected by unequal crossing over (MAGNI 1963). Temperature-sensitivity is a characteristic attributed to missense defects (EDGAR, DENHARDT, and EPSTEIN 1964). A means of recognizing mutants with a common nonsense triplet by their response to external suppressors would follow from the most plausible explanation of how a suppressor gene can act upon a variety of different mutants and yet be specific in suppressing only a fraction of the alleles at a given locus (BENZER and CHAMPE 1962). It has been proposed that the suppressor mutation modifies an s-RNA so as to make "sense" or "missense" of the mutant codon which originally was "nonsense", *i.e.* it did not specify any amino acid. This could be accomplished, for example, by a base change at the triplet recognition site of a redundant s-RNA.

The investigation of another explanation (see below) of how such super-suppressors in yeast might discriminate between alleles while suppressing many different phenotypes had a fortuitous consequence, the discovery of osmotic-remedial mutants. A group of suppressible mutants were subjected to extreme variations in the culture conditions (salt concentrations, pH of the medium, and temperature) to see if the action of the super-suppressor in restoring the wild-type phenotype could be duplicated. None of the suppressible mutants was affected by the changes in the media or temperature; however, one of the non-suppressible mutants included as a control was the his_{8-1} strain which grew on the histidineless high salt medium.

Originally, the testing of the suppressible mutants had been undertaken to investigate the possibility that they represented a group of missense mutants where the tertiary folding of the mutant protein had gone awry because of the failure to form a critical hydrogen, hydrophobic, or salt bond involving the substituted amino acid. It was reasoned that if the substituted amino acid were similar to the original, then it might be possible to alter the normal physiological environment of the protein either by introducing a suppressor or by changing the culture

conditions to permit the formation of a more sensitive bond. This now seems unlikely for the suppressible mutants, but it still may serve as an explanation for the behavior of the osmotic-remedial mutants.

The assumption that the osmotic-remedial mutants produce a defective protein under normal culture conditions is supported by the observation that seven of the nine osmotic-remedial ad_7 alleles are complementing mutants. It is presumed that intragenic complementation in yeast has the same mechanism as that demonstrated for Neurospora where the two parental species of defective polypeptides have been combined *in vitro* to give a hybrid polymer with enzymatic activity (WOODWARD 1959; FINCHAM and CODDINGTON 1963).

The observation that many of the osmotic-remedial mutants can also be "cured" by changing the incubation temperature is consistent with the concept of a change in the mutant protein making it more sensitive to physical parameters which can be varied without detriment to the other enzymes necessary for the growth of the cell. With a few exceptions, the interaction of temperature and osmotic pressure in overcoming the postulated bonding difficulties of the mutant protein takes either of two general patterns. For the predominant group of mutants, lowering the incubation temperature has an effect similar to raising the osmotic pressure. It is proposed that here the common difficulty is the failure, under normal conditions, to form an essential bond involving the substituted amino acid. For the second group of mutants, increasing the osmotic pressure has the same effect as raising the incubation temperature. In this case an undesirable bond may be formed early in the folding of the protein and thus lead to the misalignment of the polypeptide for the formation of subsequent bonds. Temperature would have a consistent effect in the above interpretations: the bonds in question would be formed only at the lower incubation temperatures. However, it must be postulated that an increased osmotic pressure can prevent as well as promote the formation of the same bond. This can be adduced from a consideration of the growth-yield plots (Figures 2 and 3) if we assume that the osmotic response profile, with a breadth that frequently ranges less than $\pm$ 0.3 M KCl from the optimum molarity, reflects the formation and disruption of a single sensitive bond.

A variation of the above interpretations would have the defect impair the formation of dimers or polymers needed for enzymatic expression. It seems likely that aggregated enzymes would generally be more sensitive to missense changes than monomers since such changes could affect polymerization as well as the conformation of the active site. Thus among the mutants of a gene for a complex enzyme one would expect a greater proportion of missense mutants which in turn might be subject to cure by temperature or osmotic pressure variation. In fact, the several genes in this study for which it has been possible to demonstrate intragenic complementation, thus implying the formation of a polymer, have given a relatively high frequency of osmotic-remedial alleles: ad_2 4/14, ad_3 3/4, ad_7 9/31, and thr_2 3/4. Unfortunately, the tests for complementation are not extensive enough for us to say whether or not there are any genes with osmotic-remedial alleles where complementation appears unlikely. And, since we have

no direct evidence on the complexity of the enzymes affected in the various osmotic-remedial mutants, the validity of the above restriction on the class of proteins capable of showing this phenomenon remains undetermined.

Osmotic-remedial mutants should occur in other microorganisms used for genetic studies; the only apparent restriction on their universality would be the tolerance of the organism to a high osmotic pressure. In fact, a mutant which might be classified as "osmotic-remedial" has been reported for *Neurospora crassa* (KUWANA 1961). This was a temperature-sensitive nutritionally irreparable mutant which responded to either a lowered incubation temperature (23°C) or an increased osmotic pressure (0.4 M KCl) at 27°C. The osmotic response was assumed to be a manifestation of the phenotype of this mutant which was postulated to have an altered cell membrane or transport system. However, in view of the coincidence of the osmotic response with temperature-sensitivity observed for the yeast mutants, it seems likely that this is a characteristic of the mutant protein and not of the physiological defect.

SUMMARY

The designation "osmotic-remedial" is proposed for a class of nutritional mutants which will grow without the usual supplement when the minimal medium has been modified by the addition of solutes such as KCl, glycerol, or sorbitol to raise the osmotic pressure. About one in seven (36/231) of the nutritional mutants of *Saccharomyces cerevisiae* have this property. From the variety of osmotic-remedial mutants obtained thus far (7 adenine, 4 histidine, 2 isoleucine, 1 threonine, and 1 tyrosine genes are represented), it appears that this is a generalized phenomenon which will be useful in classifying alleles.

The osmotic-remedial mutants are interpreted as missense mutants which form an abnormally folded protein as a consequence of a single amino acid substitution. Observations which support this contention are: (1) Osmotic-remediability and susceptibility to the super-suppressors appear to be mutually exclusive properties. Alleles with the latter characteristic are postulated to be nonsense mutants. (2) For genes where intragenic complementation has been demonstrated, the osmotic-remedial alleles are found mainly in the complementing class, which suggests the formation of a complete polypeptide chain under normal culture conditions. (3) Many of the osmotic-remedial mutants are also temperature-sensitive, another property thought to be associated with misssense mutations.

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