Electrolyte Effects on the Activity of Mutant Enzymes in Vivo and in Vitro†

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ABSTRACT: All temperature-sensitive histidine auxotrophs of *Salmonella typhimurium* tested are corrected by addition of neutral salts to their growth medium. The correctability seems to result from direct electrolyte effects on mutant protein stability since several of the mutant proteins are also salt correctable in vitro.

A large body of data exists concerning the effects of electrolytes on the function and stability of proteins (von Hippel & Schleich, 1969; von Hippel et al., 1973; von Hippel, 1975). Although the bases of these effects are not entirely understood, it is likely that their explanation will also apply to electrolyte effects on mutants and may explain a variety of genetic phenomena. Conversely, some of the genetic findings may shed light on the nature of these electrolyte effects.

A few studies have been published on the phenomenon of "osmotic remedial" or "salt correctable" conditional mutants. These mutants show the defective phenotype in standard growth medium and are at least partially corrected by a high concentration of salt in the medium. Such mutants have been studied most extensively in fungi where a large percentage of missense mutants show some degree of salt correction (Hawthorne & Friis, 1964). Very few data are available to explain the basis of this correctability. In bacteria, many salt-correctable mutants have been described (Good & Patteen, 1970; Russel, 1972; Bilsky & Armstrong, 1973; Steinberg, 1974). Many of these are temperature-sensitive mutants affecting DNA replication or cell division (Siccardi et al., 1971). This has frequently been cited as evidence that the protein involved acts in association with membranes (Siccardi et al., 1971; Russel, 1972; Bilsky & Armstrong, 1973). Recently Fincham & Baron (1977) reported that in *Neurospora* an osmotically repairable glutamine dehydrogenase mutation results in the substitution of a polar residue for a hydrophobic one. This observation led to speculation that osmotic repair is due to a change in the interaction of protein and water in the area of the substitution.

To investigate the nature of salt-correctable mutants in bacteria, we have studied a series of such mutations of the histidine operon of *Salmonella typhimurium*. Our results suggest that correctability reflects direct electrolyte effects on the mutant protein. Furthermore, there is a perfect correlation between temperature-sensitive mutations and those which are correctable by increased concentrations of salt in the growth medium.

Materials and Methods

(a) Bacterial Strains. All *Salmonella* strains used are derived from strain LT2. Strains carrying the regulatory mutation *his01242* and various histidine temperature-sensitive mutants were constructed by P22-mediated transductional crosses using TR5576 (*his01242 hisD2565*) as recipient and various his_{ts} donors at 30 °C. A list of the histidine temperature-sensitive, cold-sensitive, and osmotic remedial mutants used is in Table III.

(b) Bacteriophage Strains. The phage used for mutagenesis and transduction was *int-4*, a nonintegrating mutant of P22 (Smith & Levine, 1967). Phage sensitivity tests were done with P22-H5, a clear (C_2) mutant of P22, on green indicator medium (Levine & Curtiss, 1961). All phage were stored in T2 buffer (Hershey & Chase, 1952).

(c) Growth Media. Difco nutrient broth was used as a maximally supplemented liquid medium, to which Difco agar (1.5%) was added for use as solid medium. The E medium of Vogel & Bonner (1956) was used as minimal salt medium which was always supplemented with 2% glucose. For identifying phage-free transductants, green indicator medium was used (Levine & Curtiss, 1961). Phage-free transductants and stable lysogens will form a light green colony on this medium; colonies in which P22 is growing are dark green.

(d) Chemicals. Neutral salts and glycerol were purchased from Mallinckrodt Chemical Co. L-Histidinol was purchased from Cyclo Corp. and DPN, grade III, from Sigma.

(e) Transductional Crosses. The nonlysogenizing P22 mutant, *int-4*, was used in all transductional crosses. Plates were spread with 1×10^9 phage particles and 2×10^8 recipient cells. Transductant colonies were scored after 48–72-h incubation at 20, 30, 37, or 42 °C.

(f) Transducing Phage Mutagenesis. Transducing phage lysates were mutagenized with hydroxylamine by the method of Hong & Ames (1971). Mutagenized phage were stored in T2 buffer (Hershey & Chase, 1952) at 4 °C with a few drops of chloroform.

(g) Mapping of Mutants. Histidine heat- or cold-sensitive, osmotic remedial mutants were mapped by transductional crosses and by complementation tests. Complementation tests were performed by using an *Escherichia coli* F′*his* episome carrying different *his*⁻ mutations; F′*his* mutants were selected and characterized by G. Fink & J. Roth (unpublished results). Complementation tests were performed by spot tests selecting for transfer of known F′*his*⁻ episomes into the new *his* mutant. Transductional crosses used *his* deletion and point mutants characterized by Hartman et al. (1971).

(h) Tests of Salt Correctability. Osmotic remediability of *his* auxotrophs was examined initially by dropping crystals of NaCl onto a lawn of cells in a soft agar layer. Bacteria were grown at 37 °C in nutrient broth to 2×10^9/mL. An aliquot of the culture was mixed with 3.0 mL of minimal soft agar at 45 °C and then plated on minimal plates. Crystals of NaCl were dropped at the edge of the plates, and the plates were incubated at 20, 30, 37, or 42 °C for 1–3 days. When a bacterial strain is salt-correctable, a ring of growth can be seen surrounding the NaCl.

(i) Enzyme Assay. Histidinol dehydrogenase was measured as described by Martin et al. (1970). The dehydrogenase assay

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Table I: Relative Effectiveness of Various Salts in Correcting Histidine Temperature- or Cold-Sensitive Mutants at 42 or 20 °C[a]

strain	← more effective less effective →
hisD6585$_{ts}$	NaCl >> LiCl > NH_4Cl > CsCl > $(NH_4)_2SO_4$ > KCl > $MnCl_2$ > $MgCl_2$ NaCl >> Na_2SO_4 > $NaCH_3COOH$
hisB8538$_{cs}$	NaCl >> LiCl, KCl, CsCl > $(NH_4)_2SO_4$, Na_2SO_4

[a] Relative effectiveness of various salts in correcting a temperature- or cold-sensitive mutant was determined by measuring cell-doubling time in various media at 42 °C for the temperature-sensitive mutants or 20 °C for the cold-sensitive mutants. A final concentration of 0.2 M salt was used for monovalent ions, and 0.08–0.1 M salt was used for divalent ions.

was done at room temperature. All assays were done in duplicate or quadruplicate with sonicated cell-free extracts. Protein concentration was determined by the microbiuret method (Zamenhof, 1962) standardized with bovine serum albumin.

(j) Enzyme Preparation for in Vitro Salt Correction. Cells were grown in 600 mL of E medium containing 0.1 mM histidine at 30 °C. Cells of logarithmically growing cultures, at OD_{650} of 1.0, were harvested by centrifugation, and cells were resuspended in 10 mL of 0.05 M Tris-HCl buffer (pH 7.5). The resuspended cells were chilled and disrupted by sonication. After disruption, cell-free extracts were prepared by centrifugation at 25000*g* for 40 min. The insoluble residue was discarded. Ammonium sulfate was added to the supernatants to 33% saturation. After stirring 20 min at room temperature, the treated extracts were centrifuged at 25000*g* for 30 min and the precipitate was discarded. The supernatant was then brought to 55% saturation by addition of solid ammonium sulfate and stirred for 20 min at room temperature. The precipitate was collected by centrifugation at 25000*g* for 30 min and the pellet was resuspended in 5 mL of 0.05 M Tris-HCl buffer (pH 7.5). The resulting protein solution was extensively dialyzed against 1000 mL of the same buffer at 4 °C. This dialyzed fraction was used for thermal denaturation tests. It is purified about fivefold over the initial crude extract.

(k) Thermal Denaturation. Thermal denaturation was performed in a water bath maintained at 68 °C (±1.0 °C). The sample tube contained 100 μL of enzyme fraction, 500 μL of 0.05 M Tris-HCl (pH 7.5), and salt at a final concentration of 1 M. After various times, enzyme activity was assayed at 30 °C.

Results

(a) Salt Correctability. Figure 1 shows that the auxotrophic mutants *hisD6585*$_{ts}$ and *hisB8538*$_{cs}$ can grow on medium supplemented with either NaCl or histidine. The white zone indicates growth of cells after 24-h incubation. The data in Figure 2 indicate that a concentration of 0.2 M NaCl gives optimal growth stimulation. The effectiveness of various salts in stimulating growth of these two mutants is shown in Table I. For all mutants tested, NaCl is most effective in stimulating cell growth. Nonelectrolytes, such as sucrose and glycerol, and divalent cations ($CaCl_2$, $ZnCl_2$, and $BaCl_2$) are ineffective in stimulating growth of histidine temperature-sensitive mutants. The order of effectiveness of various salts in stimulating mutant growth determined by such in vitro tests is not similar to the Hofmeister series of salts. Hofmeister ordered salts according to their relative effectiveness in reducing solubility of proteins (Hofmeister, 1888; von Hippel, 1975). However, a positive response in our test may require that a salt both enter the cell and also affect protein structure. Thus, we may be scoring both of these processes simultaneously. To test the effect of various salts on the protein itself, in vitro salt correction was examined (see section h).

(b) All Previously Known his$_{ts}$ *and his*$_{cs}$ *Mutants Are Salt-Correctable.* Over 100 of the available histidine auxotrophs of *Salmonella typhimurium* have been screened for

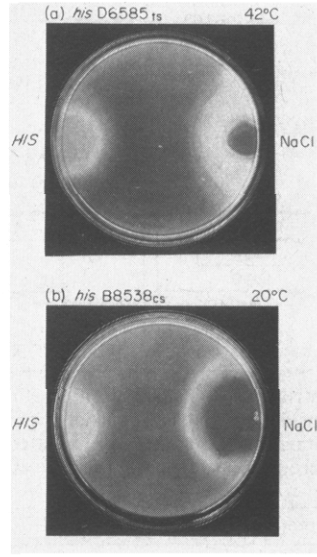

FIGURE 1: Salt correctability of histidine auxotrophs; method is described in the section of Methods and Materials. (a) The response of a temperature-sensitive mutant, *hisD6585*, to histidine and NaCl at 42 °C after 48-h incubation. (b) The response of a cold-resistant mutant, *hisB8538*, to histidine and NaCl at 20 °C after 56-h incubation. White zone indicates the growth of cells.

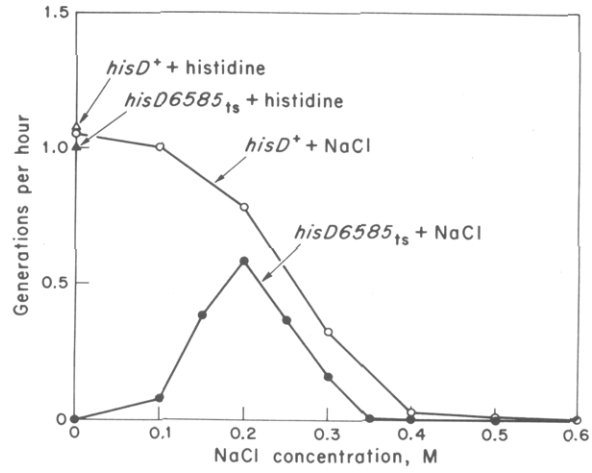

FIGURE 2: Optimum salt concentration for correction of *hisD6585*; all strains carry the *his01242* regulatory mutation. Fresh overnight cultures were diluted 1:100 into E medium supplemented with 0.3 mM methionine and the indicated concentration of NaCl (+ NaCl on the figure). Cultures were incubated at 42 °C on a shaker. At intervals of 30 min, optical density of 650 nm was measured to determine growth rates. For comparison the *hisD*$^+$ and *hisD6585*$_{ts}$ strains show growth rates of 1.08 and 1.00 generation per h when grown with excess histidine.

phenotypic suppression by high salt concentration. It was found that all histidine heat-sensitive mutants are salt-correctable (out of 34 tested). Mutants tested included *hisB59*, *hisC15*, *hisC163*, *hisD141*, *hisD6500*, and 29 *his*$_{ts}$ mutants which were isolated by Hong & Ames (1971). A cold-sensitive mutant, *hisC588*, is also salt-correctable. None of the nonsense and frameshift mutants tested is salt-correctable. This suggests

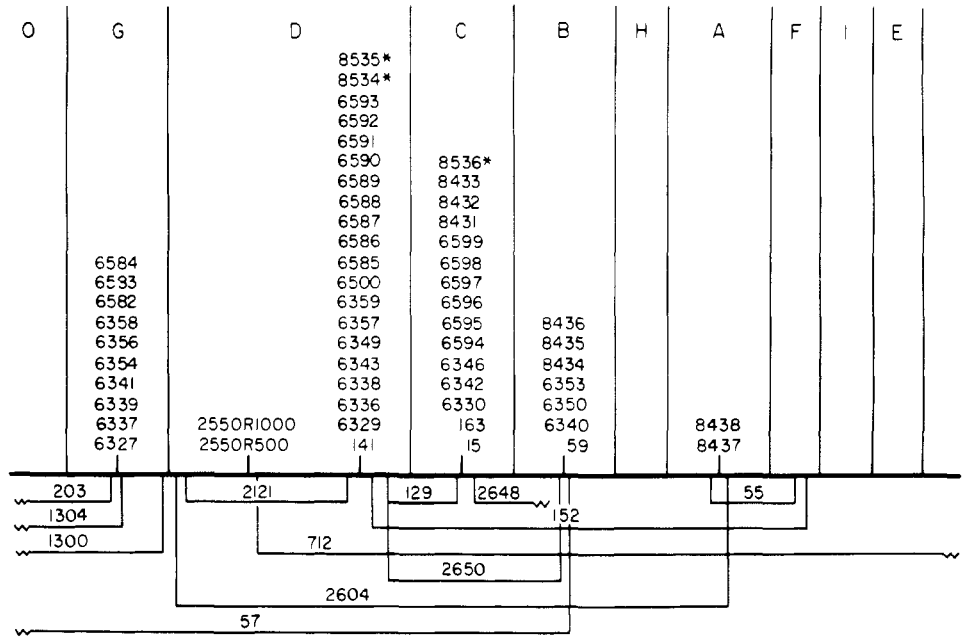

FIGURE 3: Map distribution of histidine temperature-sensitive mutations; the ts mutations are presented above the horizontal line. Vertical lines demark regions defined by end points of various deletions. Point mutations within each column have not been tested for recombination with each other. Starred (*) mutants were isolated as salt-correctable mutants at 42 °C. Standard deletions, characterized by P. Hartman and co-workers, were used in mapping; these deletions are presented below the heavy horizontal line.

Table II: Salt-Correctable Histidine Heat-Sensitive Mutants[a]

hisG: 6327[b], 6337[b], 6339[b], 6341[b], 6354[b], 6356[b], 6358[b], 6582, 6583, 6584

hisD: 141[d], 6329[b], 6336[b], 6338[b], 6343[b], 6349[b], 6357[b], 6359[b], 6500, 6585, 6586, 6587, 6588, 6589, 6590, 6591, 6592, 6593, 8535,[c] 2550 R500, 2500 R1000,

hisC: 15[d], 163[d], 6330[b], 6342[b], 6346[b], 6594, 6595, 6596, 6597, 6598, 6599, 8431, 8432, 8433, 8536[c], 8537

hisB: 59[d], 6340[b], 6350[b], 6353, 8434, 8435, 8436

hisA: 8437, 8438

[a] Except for strains designated below, all of the above mutants were isolated by the method of Hong & Ames (1971). [b] These strains were isolated by and obtained from J. Hong & B. N. Ames. [c] These strains were isolated at 42 °C as salt correctable mutants. [d] These strains were obtained from P. E. Hartman and have been described previously (Hartman et al., 1971).

Table III: Salt-Correctable Cold-Sensitive Histidine Mutants

hisG: 8581, 8582, 8583, 8625[a]

hisC: 588, 8585, 8586, 8587, 8588, 8589, 8590

hisB: 8538, 8584, 8591, 8592, 8593, 8594, 8596, 8597[a], 8598, 8604

hisH: 8580, 8599, 8600, 8601, 8602, 8603, 8606

hisA: 8607, 8609, 8610[a]

hisF: 8611, 8612, 8613, 8614, 8615, 8616, 8617, 8618, 8619, 8620, 8621, 8622, 8623, 8624

not mapped: 8605, 8606

[a] Phage resistant.

that the type of protein alteration which leads to heat or cold sensitivity may be correctable by increasing electrolyte concentration and that correction is probably not due to miscoding induced by the added salts (see section f).

(c) All Newly Isolated Histidine Heat-Sensitive Mutants and Cold-Sensitive Mutants Are Salt-Correctable. Large scale isolation of histidine heat- or cold-sensitive mutants was done to see whether the correlation between temperature sensitivity and salt correctability is generally true. Mutants were isolated by the method of Hong & Ames (1971) and examined for salt correctability. It was found that all are phenotypically correctable by NaCl (Tables II and III).

(d) Mapping of his_{ts} Salt-Correctable Mutants. Salt-correctable *his* heat-sensitive mutants were classified by genetic mapping and complementation testing. The results are presented in Figure 3. Mutations of this type are found in many *his* genes, but in only one region of each gene (two exceptions, *hisD2550R500* and *hisD2550R1000*, will be discussed in a later section). The affected proteins include the *hisA* gene product, a single protein chain (Margolies & Goldberger, 1967), *hisC* and *hisD* proteins, dimers of identical subunits (Yourno et al., 1970; Henderson & Snell, 1973), *hisG* protein, with six identical subunits (Voll et al., 1967), and the bifunctional *hisB* enzyme with a complex subunit structure (Houston, 1973). These results suggest that salt correctability is not limited to a particular type of protein. It also indicates that the effect is not limited to membrane-bound enzymes since there is no evidence for a membrane association of any histidine enzyme (Martin et al., 1970). Furthermore, these mapping data show that the mutational sites are not evenly distributed across the genetic map of the histidine operon. Mutations in a particular restricted region of each gene give rise to temperature-sensitive proteins. This suggests that alterations of the corresponding region of the protein can have striking effects on the thermostability of the protein.

(e) Mapping of his_{cs} Mutants. The histidine cold-sensitive salt-correctable mutations were mapped to six of the nine *his* genes by complementation tests and deletion mapping (Table III). Like the heat-sensitive mutations, cold-sensitive mutations are not limited to particular types of proteins. It has been suggested that mutations causing cold sensitivity usually affect subunit interactions of multimeric proteins. In this regard, it is interesting that some of our cold-sensitive mutations affect the *hisA* protein, which is monomeric (Margolies & Goldberger, 1967).

(f) Salt Correction Is Not Due to Misreading of the Mutant Message. Since neither nonsense nor frameshift mutations

Table IV: Effects of Streptomycin and a *strA* Mutation on Salt Correction[a]

strain	growth at 42 °C			
	min.	min. + NaCl	min. + str	min. + his
hisC537$_m$	–	–	+	+
hisC537$_m$ *strA*	–	–	–	+
hisD6585$_{ts}$	–	+	±	+
hisD6585$_{ts}$ *strA*	–	+	–	+
his 6475$_n$	–	–	+	+
his 6475$_n$ *strA*	–	–	–	+
hisB6480$_{fs}$	–	–	–	+
hisB6480$_{fs}$ *strA*	–	–	–	+

[a] A *strA* mutation was transduced by P22-mediated transduction from strain TR2246 (HfrK5, met⁻ *strA* recA1) into various mutant recipients. The mutants were scored for correction of their histidine requirement by exogenous histidine, by streptomycin, or by NaCl. Streptomycin, NaCl, or histidine was spotted on the center of a minimal plate seeded with various strains. Response was scored as a ring growth around center spot. A sign of + indicates growth; a sign of – indicates no cell growth; ± indicates weak growth. Mutant types are designated as: m, missense; ts, heat sensitive; n, nonsense; and fs, frameshift.

were found to be salt correctable, it seemed likely that salt induction of miscoding is not involved in suppression of the mutant phenotype. This was further tested using a mutation (*strA*) which reduces ribosomal miscoding (Gorini, 1970). Strains were constructed which carry a *strA* mutation and various nonsense, frameshift, and temperature-sensitive mutations. Salt correctability was examined for each of these strains (Table IV). A missense mutant, *hisC537*, is phenotypically correctable by streptomycin, while the *hisC537 strA* double mutant is not corrected. This demonstrates the restrictive effect of *strA* mutations on streptomycin-induced miscoding (Gorini, 1970). Salt correctability of a histidine heat-sensitive mutation, *hisD6585*$_{ts}$, is not affected by the *strA* mutation (Table IV). As presented in Table IV, neither nonsense mutations (*his-6475*) nor frameshift mutations (*his-6480*) are salt correctable either with or without a *strA* mutation in the strains. These results suggest that the salt effect is not due to ribosomal misreading of message induced by the electrolyte.

(g) Mutant Enzyme Is Unstable at 42 °C in Vivo but Is Stabilized by Addition of Salt to the Medium. To check the stability of mutant enzyme in vivo, histidinol dehydrogenase assays were performed on *hisD* temperature-sensitive mutants grown under various conditions. Mutant *his01242 hisD6585*$_{ts}$ was grown at various temperatures on medium containing histidine either with or without 0.2 M NaCl; growth was followed by monitoring OD_{650}. Each culture was harvested at OD_{650} = 0.8 (5 × 10^8 cells/mL), and crude extracts were prepared and assayed for histidinol dehydrogenase activity. Figure 4 shows the levels of *hisD* enzyme in this mutant and in a wild type strain grown at various temperatures, either with or without NaCl. Strain *hisD6585*$_{ts}$ grown at 25 °C with no added salt has 40% of histidinol dehydrogenase activity found in a similarly grown wild type (*hisD*$^+$) strain. At 40 °C, the mutant has only about 3% of the wild type level. This loss of activity is prevented when the mutant is grown in medium with a high salt concentration. This suggests that NaCl can act to stabilize this mutant enzyme in vivo.

(h) Salt Correction Is Due to Electrolyte Effects on the Mutant Proteins. If salt corrects mutant phenotypes by stabilization or activation of a defective gene product, it should be possible to demonstrate the effect of neutral salt on the thermal stability of partially purified histidinol dehydrogenase.

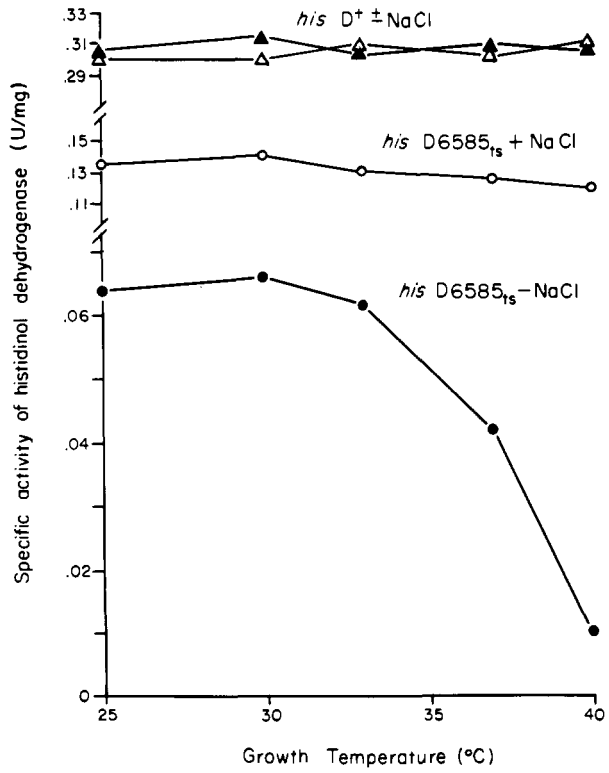

FIGURE 4: Effect of growth temperature and NaCl on the production of histidinol dehydrogenase by *hisD6585*$_{ts}$ and wild type (*hisD*$^+$) strain. Both the *hisD*$^+$ strain and mutant *hisD6585*$_{ts}$ carry the regulatory mutation (*his01242*). Both strains were grown on medium supplemented with histidine and methionine with (+) or without (–) added NaCl (0.2 M). See text for the preparation of crude extract and assay of the enzyme. (△—△) *hisD*$^+$ with NaCl; (▲—▲) *hisD*$^+$ without NaCl; (○—○) *hisD6585*$_{ts}$ with NaCl; and (●—●) *hisD6585*$_{ts}$ without NaCl.

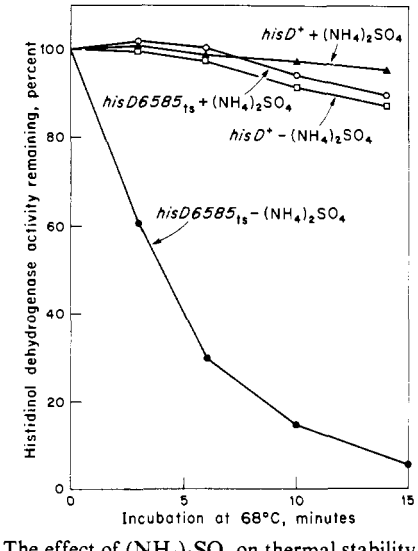

FIGURE 5: The effect of $(NH_4)_2SO_4$ on thermal stability of histidinol dehydrogenase derived from wild type (*hisD*$^+$) and *hisD6585*$_{ts}$. Enzyme preparation and thermal stability tests were done as described in text. Both strains carry the *his01242* regulatory mutation. (▲—▲) *hisD*$^+$ with $(NH_4)_2SO_4$; (□—□) *hisD*$^+$ without $(NH_4)_2SO_4$; (○—○) *hisD6585*$_{ts}$ with $(NH_4)_2SO_4$; and (●—●) *hisD6585*$_{ts}$ without $(NH_4)_2SO_4$.

Enzyme from wild type and from three *hisD* temperature sensitive mutants was analyzed. Figure 5 shows that the enzyme derived from wild type is stable, but that of the mutant, *hisD6585*, is heat sensitive. In addition, Figure 5 shows that the thermal sensitivity of this mutant enzyme is reversed by addition of $(NH_4)_2SO_4$, one of the salts capable

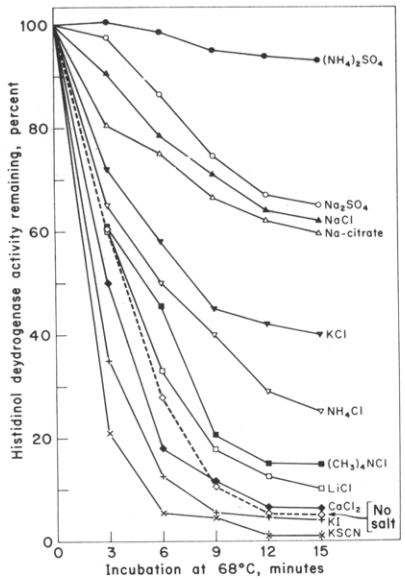

FIGURE 6: The effect of various neutral salts on the thermal stability of histidinol dehydrogenase derived from *hisD6585*$_{ts}$. Experiments were done as described in text. The dashed line indicates the mutant histidinol dehydrogenase activity without any added salt. Various salts used are indicated at the right side of the figure. Added salts were used at a concentration of 1.0 M.

of correcting the mutant phenotype in vivo. Little effect of $(NH_4)_2SO_4$ on wild type enzyme activity is seen. Figure 6 shows data on the ability of a variety of neutral salts to stabilize thermosensitive *hisD* enzyme. Salts effective in "salting-out" seem to correct the thermal sensitivity of mutant enzyme. The order of effectiveness of salts is quite different from the order seen in vivo. The in vitro order is more similar, but not identical, to the order of salts in the Hofmeister series. All three mutant enzymes tested behave similarly. The differences between the effectiveness of salts in our tests and the tests described by von Hippel may reflect some salt effects which are specific for particular proteins.

(i) Salt Permits Detection of a New Class of Revertants of Frameshift Mutation, hisD6610. A frequent class of revertants of frameshift mutants carries a second frameshift mutation near the site of the first. Revertants of this type have a sequence of improper amino acids between the two mutational sites. These proteins are expected to be abnormal. If a particular reversion event gives rise to an abnormal protein which can only function in the presence of high salt concentration, then this revertant type can only be detected if revertants are selected on medium containing a high salt concentration. A case of this type has been seen in frameshift mutant *hisD6610*. It was found that the apparent reversion rate of this frameshift mutant is stimulated about 50-fold by high salt concentrations. These results are presented in Figure 7. More than 90% of the revertants on medium of high salt concentration are found to be dependent on salt for their His^+ phenotype. This suggests that reversion on high salt medium makes possible the detection of a particular common type of revertant which cannot be detected on standard medium. This particular revertant has an altered protein which is either stabilized or activated by the high concentration of salt.

(j) Effect of Glycerol on hisD2550 R1000$_{ts}$ *in Vivo and in Vitro.* Glycerol is widely used for stabilization of proteins. An attempt was made to see if glycerol causes in vivo correction of any of our mutants. Among more than 50 histidine temperature-sensitive mutants of *Salmonella typhimurium*, only one was found to be phenotypically suppressed by addition of glycerol to the growth medium. This mutant (*hisD2550* R1000$_{ts}$) was isolated as a His^+ revertant of the well-studied frameshift mutation, *hisD2550* (Yourno et al., 1971). It was found that about 10% of the His^+ revertants of *hisD2550* are heat sensitive (as is *hisD2550* R1000$_{ts}$). The glycerol effect can also be seen in vitro (Table V). It appears that glycerol stabilizes the thermosensitive histidinol dehydrogenase of *hisD2550* R1000$_{ts}$. The role of glycerol in protein stabilization is complex and not well understood. Glycerol corrects many of the osmotic remedial mutants of yeast (Hawthorne & Friis, 1964) and *Neurospora* (Metzenberg, 1968), while available reports indicate that no osmotic remedial mutants of *E. coli* are affected by glycerol (Russel, 1972; Bilsky & Armstrong, 1973). Among *Salmonella his* mutants, glycerol-correctable mutants are extremely rare. It is possible that glycerol causes increased salt uptake by fungi and thereby causes correction by indirectly affecting the electrolyte level. This does not seem to be the case in bacteria.

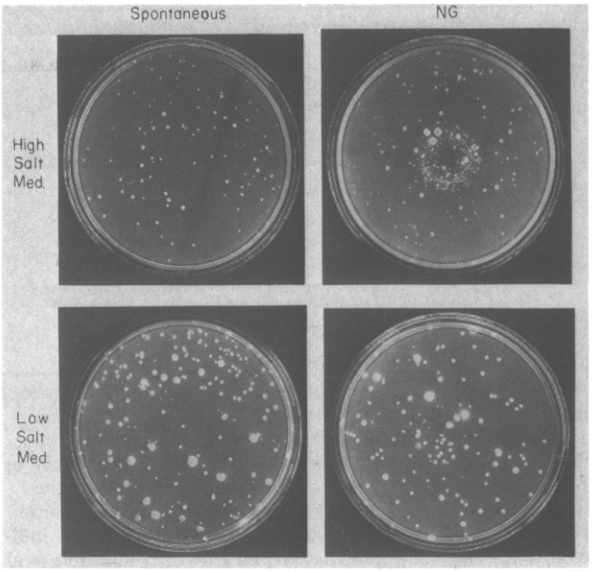

FIGURE 7: High salts medium increases apparent reversion frequency of the frameshift mutation, *hisD6610*. A frameshift mutation *hisD6610* was tested for reversion induced by nitrosoguanidine (NG). Tests were performed on high salt medium (0.2 M final concentration of NaCl) and on low salt medium (minimal E medium without addition of NaCl). Each plate contains minimal medium with sufficient histidine (5 μM) to permit all cells to divide several times. The plates were seeded with 2×10^8 cells of a fresh culture of *hisD6610*. NG (20 μL of 1 mg/mL solution) was placed on the center of the plate. Plates were incubated for 5 days at 33 °C.

Table V: Effect of Glycerol on the Stability of Histidinol Dehydrogenase Activity in a Temperature-Sensitive Mutant[a]

addition to crude extract	wild type		*hisD2550* R1000	
	30 °C	58 °C	30 °C	68 °C
none	13.2	13.8	2.1	<0.001
3.0 M glycerol	13.5	14.1	2.3	2.4

[a] Cells were grown at 30 °C in E medium containing histidine. Crude extracts from wild type (*his01242*) and mutant strain (*his01242 hisD2550* R1000$_{ts}$) were prepared by sonication and centrifugation to remove cell debris. The crude extract either with or without added glycerol (3 M) was incubated for 15 min at 30 or 68 °C. After 15-min incubation, specific activity (U/mg) of histidinol dehydrogenase was measured at room temperature.

Discussion

(a) Osmotic Remedial vs. Salt-Correctable Mutants. The results reported here indicate that the temperature-sensitive phenotype of all mutants tested is correctable by addition of salt to the growth medium. Various neutral salts (NaCl, KCl,

NH_4Cl, etc.) permit mutant growth at the nonpermissive temperature. Sucrose, which does not enter the cell, and glycerol, which enters the cell freely (Hayashi & Lin, 1965), have no corrective effect. (One exceptional glycerol-correctable mutant is described).

Because of these results, we feel that the salt correctability is likely to be due to electrolyte effects on mutant proteins. Osmotic pressure alterations do not seem sufficient to cause correction. The term "osmotic remedial" was first used by Hawthorne & Friis (1964) to describe a class of mutants of yeast. Both polar and nonpolar compounds causing osmotic pressure changes (KCl, NaCl, sucrose) and compounds capable of penetrating the cell (glycerol and glucose) suppress most temperature-sensitive mutations in yeast (Hawthorne & Friis, 1964; Bassel & Douglas, 1970) and *Neurospora* (Metzenberg, 1968). On the other hand, in *E. coli* (Bilsky & Armstrong, 1973; Russel, 1972), *Bacillus* (Steinberg, 1974), and *Staphylococcus aureus* (Good & Pattee, 1970), only electrolytes are effective. In the work described here, only neutral salt affected the growth of the mutants. We think it likely that all of the cases described above result from direct correction of mutant protein by electrolytes. Changes in electrolyte levels may be caused indirectly in some cases by addition of various substances to the medium.

(b) Mechanisms of Salt Repair. Several possible mechanisms for salt repair can be suggested. (1) The mutant gene product is bound to a membrane and can only function if the membrane is conformationally altered in a medium of high salt concentration. (2) Salt-correctable mutants are defective in cell wall structure and can grow only in a medium of high osmotic pressure. (3) The altered protein of a salt-correctable mutant is not required at high salt concentrations. (4) High salt concentrations induce alteration of the protein synthesis apparatus to cause misreading of message in the mutant thereby suppressing the defect. (5) The high salt concentration causes, either directly or indirectly, a structural alteration of the mutant protein which makes it functional. (6) The mutant proteins may retain enzymatic activity but be sensitive to proteolysis. Loss of enzyme activity from the mutant cell may depend on this degradation. Exogenous salt could reduce the proteins' sensitivity to degradation or could inhibit protease activity.

The first possibility could be true for some osmotic remedial mutants. Membrane-bound enzymes have been implicated in salt correction of thermosensitive mutants of *E. coli* (Siccardi et al., 1971; Bilsky & Armstrong, 1973; Russel, 1972). The second possibility may well operate for the cases of Good & Pattee (1970) and Matsuzawa et al. (1969). However, this is probably not a universal explanation for salt correction. The third possibility seems unlikely since some auxotrophic mutants (Hawthorne & Friis, 1964) and some mutants having alterations of an essential enzyme (such as aminoacyl-tRNA synthetase) (Steinberg, 1974; Russel, 1972) are salt-correctable. The fourth possibility seems less likely since a *strA* mutation, which is known to reduce miscoding, does not impair the salt correctability of other mutations (see Table IV). Also, the finding that protein harvested from cells grown in salt is still defective and is stabilized in vitro by salt argues against the correction being due to informational suppression. We feel that the fifth and sixth possibilities ties are the most likely explanation of the phenomenon of salt correctability. Since enzymes are susceptible to conformational changes induced by the ionic environment, it is not unreasonable to suggest that a change in the salt concentration of the medium can result in stabilization of a mutant protein. This stabilization could permit increased activity or it could reduce susceptibility to proteolytic degradation. It is possible that some of the effects noted are due to secondary intracellular changes in the electrolyte level induced by the added salt. We have no data pertaining to this point, but fluctuations in intracellular putrescine levels have been shown to occur in response to exogenous salt (Munro et al., 1972). Effects such as these could be involved in the phenomenon described here.

(c) Correlation between Thermostability and Salt Correction. A strong relationship between temperature sensitivity and salt correctability is indicated from the following two results: (1) all histidine-requiring, heat- or cold-sensitive mutants are phenotypically suppressed by a large number of neutral salts; (2) when mutants are isolated at high temperature as salt-correctable mutants, it is found that all are temperature sensitive and produce a functional protein only at low temperature (data not shown). This indicates that the protein alterations which cause thermal instability are frequently the type of change which is corrected by increasing electrolyte concentration. Von Hippel & Schleich (1969) demonstrated a relationship between temperature and salt concentration when protein thermostability was studied in the presence of a number of neutral salts at various concentrations. Their study supports the fifth possible mechanism of salt repair, in which ionic compounds in the cell are responsible for stabilizing the mutant protein.

(d) Salt Affects Many Mutant Proteins and Is Not Limited to Membrane-Bound Enzymes. Genetic mapping of salt-correctable mutations in the histidine operon of *Salmonella typhimurium* indicates that salt correction is not limited to particular types of proteins. Many different proteins are affected, including a monomer (*hisA* gene product), a hexamer (*hisG* gene product), a bifunctional tetrameric enzyme (*hisB* gene product), and enzymes composed of two identical subunits (*hisD* and *hisC* gene products). None of these proteins show evidence of membrane association. This suggests that the salt effects on mutant growth noted here are due to a very general effect of salt on the stability of many types of proteins.

(e) "Hot Spots" for Temperature-Sensitive Mutations. Temperature-sensitive *his* mutations map in only one restricted region of each gene. This distribution indicates that the mutations affect only particular, restricted regions of the protein. This result is surprising since it is difficult to imagine that only one small region of the protein exists in which an amino acid substitution can affect thermal stability of the enzyme. Our observation may be due to the mutant isolation method used. The temperature-sensitive mutants described here must carry "tight" mutations with very little residual function at the nonpermissive temperature. This is due to two related properties of the histidine system. First, even very low levels of activity are still sufficient to permit histidine-independent growth of *Salmonella*. Second, when growth of *Salmonella* is limited by the presence of a "leaky" *his* mutation, the cell responds by derepressing the *his* operon, thereby elevating the level of the rate-limiting enzymes. The above two facts combine to prevent detection of mutants with only a partial loss in enzyme activity. We are therefore looking at a stringently selected collection of mutants which have particularly low levels of activity at nonpermissive temperature.

(f) In Vitro Salt Correction. Figure 4 shows that the mutant strain $hisD6585_{ts}$ has higher histidinol dehydrogenase activity when it is grown in minimal medium supplemented with NaCl than when it is grown without NaCl at 40 °C. This suggests that the mutant enzyme is less stable than that of wild type and that the thermal instability is corrected in medium of high

ionic strength. This interpretation is supported by the finding that histidinol dehydrogenase activity of the mutant is stabilized in vitro by salt (see Figure 4). Increased ionic strength will also stabilize a temperature-sensitive *E. coli* valyl-tRNA synthetase, but only in the presence of its substrate (Anderson & Neidhardt, 1972). Hawthorne & Friis (1964) concluded that their "osmotic remedial" yeast mutants contained missense mutations causing abnormally folded proteins which could be stabilized by direct interaction with osmotically active compounds. This hypothesis was favored by Bassel & Douglas (1970) and Metzenberg (1968), who demonstrated that the penetrating agent glycerol was effective for in vivo osmotic remediability of yeast and *N. eurospora*, respectively. Studies of enzyme levels in osmotic remedial mutants revealed that the level of mutationally altered threonine deaminase or galactose-1-phosphate uridylyltransferase activity of these mutants increased with the osmolarity of the medium in which the cells were grown. However, no significant increase in the mutant transferase activity was observed when extracts prepared from cells grown in the absence of osmotic agents were assayed in their presence (Bassel & Douglas, 1970). Similarly, Steinberg (1974) was not able to demonstrate in vitro salt repair of a mutant form of lysyl-RNA synthetase in *Bacillus subtilis*. It is possible that proteolysis destroyed any protein which might have been renaturable in the presence of salt.

Our results show that the salt correction of mutant protein in vitro is similar to the effect of neutral salt on protein stability described by von Hippel and collaborators (reviewed by von Hippel, 1975). The order of effectiveness of various salts in stabilizing mutationally altered *hisD* protein in our experiments is not identical with the Hofmeister series. This difference may be due to specific effects of particular salts on the protein being assayed. Such effects could cause minor differences in the order of effectiveness depending on the protein studied. In a recent series of publications (von Hippel et al., 1973), von Hippel and collaborators presented evidence that salts bind directly to the polypeptide chain and exert their effects on hydrophobic interaction through the ordering of water molecules in the vicinity of the binding site. These results suggest a possible mechanism for the stabilization of mutant proteins by salts.

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