# MUTANTS OF SALMONELLA TYPHIMURIUM RESISTANT TO FEEDBACK INHIBITION BY L-HISTIDINE[1]

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AT least two mechanisms serve to control the amount of intracellular histidine available for protein synthesis in *Salmonella typhimurium*. The formation of the histidine biosynthetic enzymes is controlled by histidine in a coordinate manner (repression) (AMES and GARRY 1959; AMES and HARTMAN 1963). In addition, the activity of the first enzyme in the pathway, PR-ATP pyrophosphorylase, is inhibited by L-histidine, the end product of the pathway (AMES, MARTIN and GARRY 1961; MARTIN 1963). Thus coordinate repression acts on the protein synthesizing mechanism while feedback inhibition acts at the enzymatic level.

Recent studies indicate that feedback inhibiton may be of the noncompetitive type (MARTIN 1963) or of a complex competitive type (GERHART and PARDEE 1963; CHANGEUX 1961, 1962). Treatment of these enzyme systems with agents such as heat, mercurials, or urea results in a loss of feedback inhibition with little or no loss in enzyme activity. This suggests the possibility that such proteins have two distinct sites, a feedback inhibition site and an active site. These sites are presumed to interact with each other in some way in the presence of inhibitor to cause feedback inhibition. Thus, under appropriate conditions, it should be possible to obtain mutants capable of producing an enzyme which is resistant to feedback inhibition but enzymatically active.

MOYED (1960, 1961a,b) and MOYED and FRIEDMAN (1959) have shown that a histidine analog, 2-thiazole alanine (TA), mimics histidine in its inhibition of the compound III-synthesizing system in *E. coli* (Figure 1). Since 2-thiazole alanine thus prevents the synthesis of histidine, it inhibits the growth of wild-type cells on minimal medium. *E. coli* mutants resistant to 2-thiazole alanine (TAR) were isolated and found to have compound III-synthesizing systems which were resistant to inhibition by L-histidine as well as by 2-thiazole alanine. Histidine is excreted into the medium by these mutants, indicating that the control over the production of this amino acid is relaxed *in vivo*. MARTIN (1963) has shown that 2-thiazole alanine, like histidine, is capable of inhibiting the first enzyme in the biosynthetic sequence, PR-ATP pyrophosphorylase, in extracts obtained from wild-type *Salmonella typhimurium*.

In *S. typhimurium* the *hisG* gene controls the formation of PR-ATP pyro-

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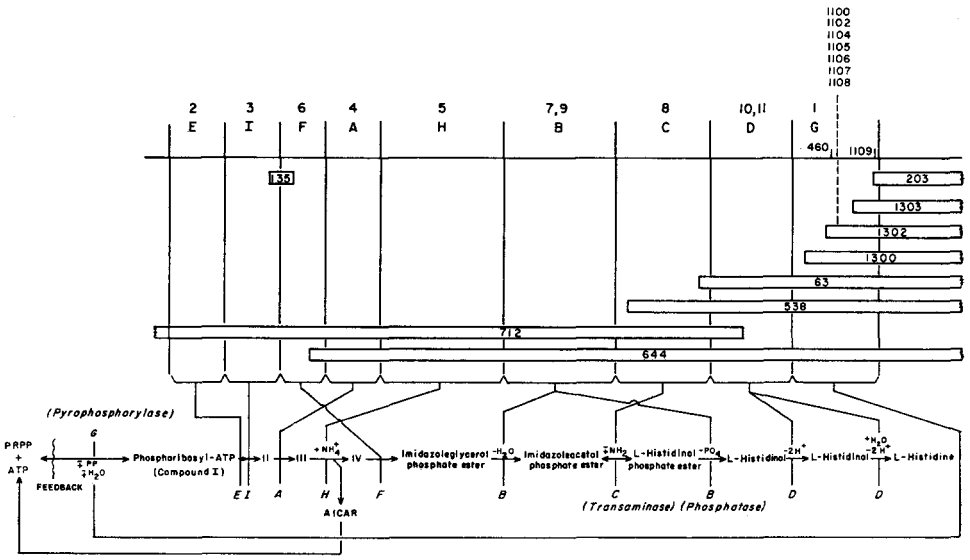


FIGURE 1.—Genetic map of the histidine region in *S. typhimurium*. The solid horizontal line represents a portion of the chromosome. The region is divided into nine genes (*E,I,F,A,H, B,C,D,G*). The 11 biochemical steps which have been assigned to the genes are shown below the map and numbered at the top. The horizontal bars below the chromosome represent "multi-site mutations" while "point mutations" are shown above the chromosome. The gene-enzyme relationships are those presented by SMITH and AMES (1964) and LOPER, GRABNAR, STAHL, HARTMAN and HARTMAN (1964).

phosphorylase (AMES, MARTIN and GARRY 1961; HARTMAN, LOPER and ŠERMAN 1960) (Figure 1). Resistance to 2-thiazole alanine, therefore, might represent a mutation in gene *G* resulting in the production of a PR-ATP pyrophosphorylase structurally altered such that it is resistant to feedback inhibition by 2-thiazole alanine and L-histidine. This study was designed to test such a hypothesis for the mechanism of 2-thiazole alanine resistance.

## MATERIALS AND METHODS

*Media:* Difco nutrient broth and nutrient agar were used as complete media. E medium (VOGEL and BONNER 1956) made 0.02 M with respect to glycerol or 0.5 percent with respect to dextrose was normally used as minimal medium. When desired, a histidine supplement of 0.13 mM was added to the E medium. Histidinol supplement was 1.0 mM in minimal agar and 0.05 mM in liquid medium.

*Chemicals:* 5-phosphoribosyl-1-pyrophosphate was obtained from Pabst Laboratories; ATP from Schwarz Bioresearch Inc.; yeast inorganic pyrophosphatase from Worthington Biochemical Corporation; penicillin from Parke, Davis, and Co.; 2-aminopurine and 5-bromouracil from California Biochemical Corporation; Sephadex gels from Pharmacia, Inc. D,L-2-thiazole alanine was secured from DR. R. G. JONES, Eli Lilly Company. L-Histidinol phosphate was generously supplied by DR. FRANK VASINGTON.

*Bacterial strains:* All of the histidine-requiring strains used throughout this work were obtained from the collection of DR. PHILIP E. HARTMAN, The Johns Hopkins University.

*Selection of 2-thiazole resistant mutants:* A Salmonella typhimurium strain LT-2 wild-type

culture was streaked on a nutrient agar plate to obtain single colonies. Isolated colonies were then picked and resuspended in nutrient broth containing 200 $\mu$g 2-aminopurine/ml. These cultures were allowed to grow to $2 \times 10^9$ bacteria/ml at 37°C, centrifuged, and the cells resuspended to their original volume in 0.85 percent saline. The viable count of the resuspended cultures was reduced to about 3 percent by ultraviolet irradiation from a 15w General Electric germicidal lamp. Then 0.05 ml of the cell suspension was added to a tube containing one ml of liquid minimal agar containing $10^{-3}$ M D,L-2-thiazole alanine. The resulting suspension was poured into one quadrant of a 5 cm sterile petri dish, allowed to harden, and incubated 24 hr at 37°C. Colonies which arose from cells resistant to TA were picked, resuspended in a 0.85 percent saline solution and replated on minimal agar with a background of $10^7$ *hisE,I,F,A,H,C, D712* cells. Those TAR colonies which were surrounded by halos of *hisE,I,F,A,H,C,D712* growth (presumably caused by histidine excretion) were picked, purified by restreaking on minimal medium, and placed on nutrient agar slants for storage. Six independent TAR halo-producing mutants were obtained by this procedure (*hisG1104,1105,1106,1107,1108,1109*). *his G1102* was a TAR halo-producing mutant obtained from DR. BRUCE AMES. *his G1100* was a halo producing strain which arose in a nitrogen mustard treated culture that also contained an extended multisite mutation (AMES, HARTMAN and JACOB 1963).

*Selection of a* hisI,F135 *histidinol⁻ double mutant:* A saturated culture of *hisI,F135* was prepared on nutrient broth medium. Approximately 100 cells from this culture were added to each of several tubes of broth that contained 200 $\mu$g 5-bromouracil/ml and were allowed to grow to $2 \times 10^9$ bacteria/ml. The cultures were centrifuged and washed with saline and resuspended to their original volumes. Then 0.1 ml of the cell suspension was added to 3.0 ml of minimal medium which contained 1 $\mu$mole L-histidinol/ml, 50 units of penicillin/ml, and an excess of all common amino acids except histidine (LEDERBERG 1950). After a 24 hour incubation appropriate saline dilutions were plated on minimal agar plates containing 0.13 mM L-histidine. Colonies which grew on minimal plus histidine were then replica-plated onto plates containing 1.0 mM L-histidinol (LEDERBERG and LEDERBERG 1952). Those colonies which did not grow on histidinol were picked from the histidine containing plate, purified by single-colony isolation and stored on slants. Strains mutant in the histidine *D* gene cannot grow on histidinol (HARTMAN, LOPER and SERMAN 1960). Thus these secondary mutations were tentatively assumed to be located in the *D* gene.

*Transduction and mapping procedures:* PLT22 phage suspensions were prepared, assayed, and stored as described by HARTMAN (1956). Nutrient broth-grown recipient bacteria were centrifuged, resuspended, and stored in T2 buffer (HERSHEY and CHASE 1952) at 4°C for up to two weeks. One-tenth ml aliquots of phage and bacteria were spread together directly on minimal agar medium (spread plate technique). Controls consisted of separate platings of phage and bacteria. When transduction tests were used to map TAR mutants, a pour plate technique was utilized. Transducing phage and recipient bacteria were mixed in a Wasserman tube. The mixture was allowed to stand at room temperature for 10 min to permit phage adsorption. A 0.10 ml aliquot of the mixture was pipetted into 10 ml of liquid minimal agar at 45°C. The resulting suspension was then poured into a petri dish containing a thin layer of minimal agar, allowed to harden, and incubated 48 hr at 37°C. Halo-producing TAR colonies were scored at 3× magnification using a dissecting microscope.

*Growth of bacteria:* All cultures were maintained on nutrient agar slants at 4°C. Subcultures were prepared by inoculating minimal medium containing 0.13 mM L-histidine and allowing growth to proceed to saturation. Subcultures were diluted to 1/50 into minimal medium which had been made 0.05 mM with respect to L-histidinol. This concentration of L-histidinol allowed a generation time of about 7 to 8 hours and resulted in a 10 to 20-fold increase in the specific activities of the histidine biosynthetic enzymes in extracts of histidine-requiring mutants (AMES and GARRY 1959; AMES, GARRY and HERZENBERG 1960; AMES, MARTIN and GARRY 1961). Cultures were allowed to grow for 35 to 48 hours under aeration at 37°C to a final optical density of about 250 Klett units. The cells were then chilled with ice and harvested in a Lourdes continuous flow centrifuge CFR 1 or by batch centrifugation.

*Preparation of cells for enzyme assay:* Cells were suspended in buffer containing 0.01 M

Tris, pH 7.5, 0.3 M NaCl, $10^{-3}$ M mercaptoethanol(MET), $2 \times 10^{-3}$ M $MgCl_2$, and $10^{-3}$ M ATP (Buffer A). Cell disruption was obtained by treatment for 3¼ minutes at 1.4 to 1.7 amperes in an MSE Ultrasonic disintegrator, Model 60W (Measuring and Scientific Equipment, Ltd.). Cell debris was removed by a 45-min centrifugation at 25,000 × *g*. Protein was determined by the LOWRY procedure (LOWRY, ROSEBROUGH, FARR and RANDALL 1951). Protein concentration in crude sonicates varied from 16 to 25 mg/ml.

*Assay of ATP-PRPP pyrophosphorylase:* ATP-PRPP pyrophosphorylase, the first enzyme in the histidine biosynthetic pathway, catalyzes the condensation of ATP and PRPP to yield phosphoribosyl-ATP (Figure 1) (AMES, MARTIN and GARRY 1961). The assay system was essentially that described by AMES, MARTIN and GARRY (1961). The rate of formation of PR-ATP was determined by following the increase in optical density at 290 mμ for 4 minutes. The assay is proportional to enzyme concentration up to an optical density change of 0.3. Twenty units of yeast inorganic pyrophosphatase was added to the experimental vessel to maintain a linear reaction rate when partially purified preparations were used, as suggested by MARTIN (1963).

*Heat inactivation of ATP-PRPP pyrophosphorylase:* In order to remove reagents present during sonic disintegration, enzyme preparations were passed through a column of Sephadex G50 (PORATH 1960) which had been equilibrated with 0.10 M Tris, pH 8.5. In some cases the Sephadex G50-treated extract was then made $10^{-2}$ M with respect to MET before heat inactivation was begun. Addition of MET was necessary because the enzyme activity of certain mutants was reversibly lost upon removal of MET. The extract was then placed in a water bath at the appropriate temperature and 20 or 50 μl aliquots were taken at designated time intervals and assayed immediately.

*Assay of L-histidinol phosphate phosphatase:* L-histidinol phosphate phosphatase (phosphatase) catalyzes the reaction: L-histidinol phosphate + $H_2O$ → L-histidinol + inorganic phosphate. The assay system used was that described by AMES, GARRY and HERZENBERG (1960) (Figure 1).

*Assay of imidazoleacetol phosphate transaminase:* Imidazolacetol phosphate transaminase (transaminase) catalyzes the reaction: Imidazoleacetol phosphate + L-glutamate → L-histidinol phosphate + α-ketoglutarate. The assay system used was that described by AMES, GARRY, and HERZENBERG (1960) (Figure 1).

## RESULTS

*Halo production:* The nature of halo production by certain of the TAR mutants was analyzed by means of cross-feeding experiments with auxotrophic strains of Salmonella which had various amino acid requirements.

Minimal medium pour plates were prepared which contained a mixture of about $10^7$ cells of a particular amino acid auxotroph and about 100 cells of a TAR strain. As a control, wild-type cells were used in place of TAR cells. The plates were incubated at 37°C until colonies were formed. Halo formation surrounding a colony is attributed to the excretion by the cells of that colony of some metabolite required by the auxotrophic background cells for growth.

Table 1 shows the results of these cross-feeding experiments.

When either of the two histidine auxotrophs is used as background, only the TAR mutants *hisG1100* and *hisG1106* produce halos. Thus these two TAR halo-producing mutants appear to be excreting greater amounts of histidine into the surrounding medium than does the wild-type strain. That this is not a general lytic or excretion phenomenon is supported by the observation that the TAR halo-producing mutants do not differ from wild type in their ability to produce halos except when plated on a histidine auxotrophic background.

TABLE 1

*Halo formation by mutants resistant to 2-thiazole alanine*

| Background auxotroph | Halo-forming strain | | |
|---|---|---|---|
| | *hisG1100* | *hisG1106* | wild type |
| leucine39 | no halo | no halo | no halo |
| cysteine20 | large distinct halo | large distinct halo | large distinct halo |
| threonine9 | slight halo | slight halo | slight halo |
| *hisI,F135* | large distinct halo | large distinct halo | no halo |
| *hisH,B22* | large distinct halo | large distinct halo | no halo |

Two mechanisms were investigated which could account for the excretion of histidine into the surrounding medium by the TAR resistant mutants. (a) The enzyme levels in the histidine biosynthetic pathway might have become derepressed, thus permitting a more rapid production of histidine than required for protein synthesis. (b) The first enzyme in the pathway, PR-ATP pyrophosphorylase, for which feedback inhibition by histidine has been demonstrated (MARTIN 1963), might have become resistant to feedback inhibition, thus permitting the synthesis of histidine to proceed unchecked.

*Transaminase and phosphatase levels in TAR mutants:*

Cultures of four TAR mutants were prepared on minimal medium and crude cell extracts were prepared by sonic disintegration. Specific activities (units of enzyme/mg protein) were determined for two enzymes in the extracts, transaminase and phosphatase, whose levels are known to be regulated by histidine (AMES and GARRY 1959). Controls were extracts from wild-type bacteria grown on minimal medium and *hisI,F135* bacteria grown on 0.05 mM L-histidinol to achieve conditions of derepression (AMES and GARRY 1959; AMES, MARTIN and GARRY 1961; AMES, GARRY and HERZENBER 1960).

Table 2 shows the results of these assays. It should be noted that the enzyme levels in *hisG1100*, *1102*, *1106*, and *1108* do not differ appreciably from wild-type levels and are 10 to 20-fold lower than the enzyme levels found in *hisI,F135* grown under conditions of derepression. Thus histidine excretion in the TAR strains cannot be attributed to increased enzyme levels.

TABLE 2

L-*histidinol phosphate phosphatase and imidazoleacetol phosphate transaminase levels in wild-type and mutant strains of* Salmonella typhimurium

| Strain | Units of enzyme per mg protein | |
|---|---|---|
| | phosphatase | transaminase |
| wild type | 1.4 | 0.48 |
| *hisG1100* | 0.67 | 0.33 |
| *hisG1102* | 0.85 | not assayed |
| *hisG1106* | 2.00 | 0.55 |
| *hisG1108* | 0.93 | 0.41 |
| *hisI,F135** | 15.0 | 8.8 |

* Grown on 0.05 mM histidinol to achieve conditions of enzyme derepression.

*Genetic locus of TAR resistance:* If the ability of TAR resistant colonies to excrete histidine into the medium represents a genetically altered property of the pyrophosphorylase which renders it resistant to feedback inhibition, the property should map in the *G* gene which controls the formation of that enzyme (AMES, MARTIN and GARRY 1961; HARTMAN, LOPER and ŠERMAN 1960). Figure 1 is a map of the histidine region. The biochemical steps involved in the pathway as well as the position of certain "multisite mutations" are indicated (HARTMAN, LOPER and ŠERMAN 1960; AMES, HARTMAN and JACOB 1963). The positions of the TAR mutants indicated on the map were determined from the data presented in Table 3.

If, in a cross between a TAR mutant and a multisite mutant, recombinant colonies arise which are nonhalo-forming (i.e. wild-type), then the genetic locus of the TAR mutant is presumed to lie outside the genetic region encompassed by the multisite mutation. However, if no wild-type recombinant colonies arise in a reasonable number of tests, the TAR mutant is presumed to lie within the genetic region encompassed by the multisite region. The data indicate that *hisG1100*, *1102*, *1104*, *1105*, *1106*, *1107*, and *1108* all are located in gene *G* between the ends of *hisG1302* and *hisG1303*. In contrast, *hisG1109* lies somewhere within the region covered by *hisG203*.

*Construction of* hisI,F135 $G^{TAR}$ *double mutants:* If pyrophosphorylase from TAR mutants is resistant to feedback inhibition by histidine, it should be possible to demonstrate this in an *in vitro* enzyme assay system. However, pyrophosphorylase activity is very difficult to measure quantitatively in extracts prepared from repressed cultures using the assay system described in METHODS; furthermore, the enzyme activity is unstable in extracts and conditions for stabilization were not found. Thus, it was not feasible to attempt to analyze the enzyme in detail unless the TAR strains could be grown under conditions which would yield derepressed levels of pyrophosphorylase. Since the TAR strains were able to grow on minimal medium, it was not possible to create conditions of derepression without altering the genetic background.

TABLE 3

*Genetic location of mutants resistant to 2-thiazole alanine*

| | Recipient bacteria | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *hisC,D,G63* | | *hisF,A,H,B, C,D,G644* | | *hisE,I,F,A, H,B,C,D712* | | *hisG203* | | *hisG1303* | | *hisG1302* | | *hisG1300* | |
| Donor phage | wild type | TAR | wild type | TAR | wild type | TAR | wild type | TAR | wild type | TAR | wild type | TAR | wild type | TAR |
| *hisG1102* | 0 | 771 | . | . . . | 80 | 525 | 35 | 1118 | 16 | 1025 | 0 | 900 | 0 | 1858 |
| *hisG1100* | 0 | 1141 | 0 | 806 | 26 | 808 | 31 | 663 | 29 | 1135 | 0 | 604 | 0 | 763 |
| *hisG1104* | 0 | 1792 | 0 | 488 | 19 | 965 | 12 | 416 | 4 | 1436 | 0 | 840 | 0 | 1050 |
| *hisG1105* | 0 | 1928 | 0 | 396 | 67 | 791 | 17 | 379 | 14 | 834 | 0 | 872 | 0 | 975 |
| *hisG1106* | 0 | 1264 | 0 | 496 | 22 | 776 | 7 | 253 | 12 | 876 | 0 | 992 | 0 | 1060 |
| *hisG1107* | 0 | 1776 | 0 | 468 | 13 | 887 | 10 | 310 | 7 | 825 | 0 | 832 | 0 | 940 |
| *hisG1108* | 0 | 2352 | 0 | 744 | 38 | 1048 | 11 | 481 | 8 | 1312 | 0 | 864 | 0 | 1430 |
| *hisG1109* | 0 | 3776 | 0 | 840 | 54 | 984 | 0 | 3716 | 0 | 3700 | 0 | 1092 | 0 | 1800 |

A *hisI,F135 D*⁻ double mutant was obtained as described in METHODS. P22 transducing phage, prepared on the TAR strains, was used to transduce the *G* gene of the TAR strains into the *hisI,F135 D*⁻ double mutant. The crosses were performed on minimal agar plates containing 1.0 mM L-histidinol. Figure 2 is a diagrammatic representation of the cross and the recombi-

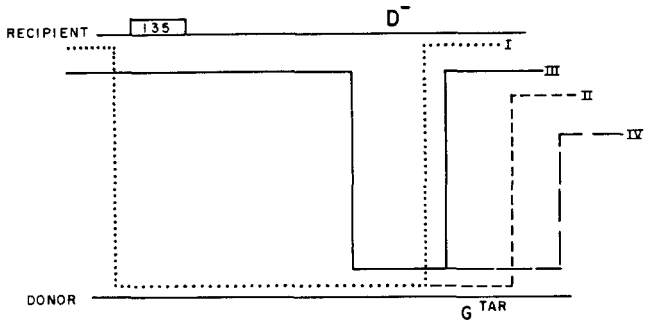


FIGURE 2.—Schematic representation of a cross between a TAR strain upon which transducing phage has been grown and a *hisI,F135 D*⁻ recipient strain. The cross was performed on a minimal agar plate containing 1.0 mM L-histidinol. Recombinants which require histidinol for growth (*135*⁻ *D*+) will form small colonies on a histidinol-containing plate while wild-type recombinants (*135*+ *D*+) will form large colonies. The four possible classes of recombinants that will form colonies are shown.

| *Class* | *Genotype* | *Phenotype* |
|---|---|---|
| I | *135*+ *D*+ *G*+ | large colony |
| II | *135*+ *D*+ $G^{TAR}$ | large colony with halo |
| III | *135*⁻ *D*+ *G*+ | small colony, requires histidinol for growth |
| IV | *135*⁻ *D*+ $G^{TAR}$ | small colony, requires histidinol for growth |

nant classes expected. Since bacteria growing on histidinol form smaller colonies than do wild-type recombinants, Classes I and II can be distinguished from Classes III and IV on the plate (HARTMAN, LOPER and SERMAN 1960). Since genes *D* and *G* are closely linked (Figure 1), one expects many more recombinants in Class IV than in Class III. A small colony (capable of growth on histidinol) was picked from each cross, purified on a plate containing histidinol, and stored on a slant. The presence of the TAR mutation in these strains was tested by transducing each *hisI,F135* $G^{TAR}$ double mutant with transducing phage prepared on *hisC,D,G63*. All of the recombinant colonies which arose from these crosses had the halo-forming property, indicating that the TAR mutation was present in all of the newly constructed *hisI,F135* $G^{TAR}$ double mutants: The presence of the *hisI,F135* marker was confirmed by recombination in tests with other histidineless mutants. By allowing strains *hisI,F135* $G^{TAR}$ to grow on histidinol, it is possible to obtain cultures which are derepressed and thus contain high pyrophosphorylase activity.

*Histidine inhibition of ATP-PRPP pyrophosphorylase from TAR strains:* Figure 3 shows the extent of inhibition of pyrophosphorylase from TAR mutant and wild-type extracts at various histidine concentrations.

Wild-type enzyme is 50 percent inhibited at an L-histidine concentration of about $5 \times 10^{-4}$M. In contrast, no appreciable inhibition of pyrophosphorylase from TAR mutant extracts is observed at $10^{-3}$M L-histidine or below. The inhibition, if any, of *G1105* is not appreciable below $10^{-3}$ M, or even at $10^{-3}$ M. In

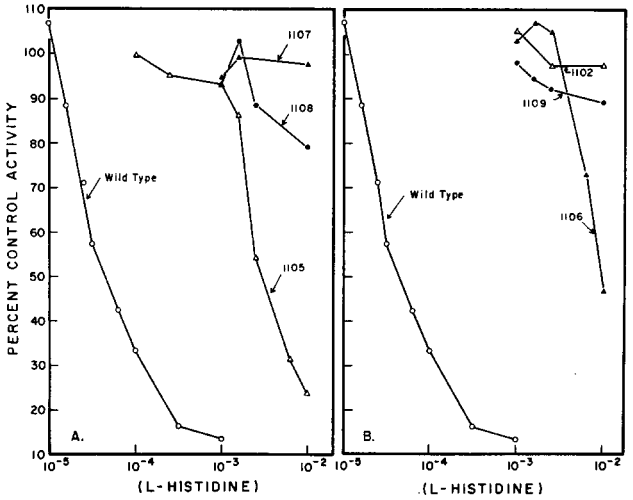


FIGURE 3.—Feedback inhibition by histidine of ATP-PRPP pyrophosphorylase. Wild-type pyrophosphorylase was obtained from a depressed culture of *hisI,F135*. Mutant pyrophosphorylases were obtained from derepressed cultures of *hisI,F135* $G^{TAR}$. Control activity for each extract was determined in the absence of histidine. All of the extracts tested were crude extracts except *hisI,F135 G1109* and *hisI,F135 G1108* which were partially purified 7 to 10-fold above the derepressed level.

extracts of *hisI,F135 G1102* and *hisI,F135 G1107* no appreciable inhibition is observed even at $10^{-2}$ M L-histidine. It was not possible to determine the effect of histidine on pyrophosphorylase from extracts of *hisI,F135 G1100* since the crude extract contained very low enzyme levels even under conditions of derepression. Extracts of *hisI,F135 G1104* were not tested.

*Partial purification of ATP-PRPP pyrophosphorylase:* Pyrophosphorylase activity was partially purified by passage of crude cell extracts through a Sephadex G200 column (PORATH 1960) previously equilibrated with Buffer A. Fractions containing the major portion of enzyme activity were pooled, and the protein was concentrated by precipitation with ammonium sulfate. The protein was then resuspended in Buffer A and stored in 3 to 5 ml aliquots at —60°C. In various experiments this procedure resulted in a 7 to 17-fold purification.

*$K_m$ determinations:* $K_m$ and $V_{max}$ for pyrophosphorylase from four TAR mutants and *hisI,F135* are presented in Table 4. There is no appreciable difference between the values obtained for enzyme extracts from TAR mutants and *hisI,F135*.

*Treatment with mercaptoethanol:* MARTIN (1963) has observed that pyrophosphorylase from *hisI,F135* cells loses its sensitivity to feedback inhibition upon aging. Incubation of enzyme with MET partially restored feedback inhibition. It was therefore of interest to determine whether MET treatment would increase the histidine sensitivity of pyrophosphorylase from TAR mutants.

The partially purified enzyme extract was passed through a Sephadex G50 column equilibrated with 0.01 M Tris, pH 8.5 to remove NaCl, $MgCl_2$, MET, and ATP. Sensitivity of this

TABLE 4

*$K_m$ and $V_{max}$ for mutant and wild-type ATP-PRPP pyrophosphorylase*

| Strain | $K_m$ATP | $K_m$PRPP | Change in optical density at 290 mμ (4 minutes) $V_{max}$ATP | $V_{max}$PRPP |
|---|---|---|---|---|
| *hisI,F135* | $2.56 \times 10^{-4}$M | $6.67 \times 10^{-5}$M | 0.244 | 0.333 |
| *hisI,F135 G1109* | $1.92 \times 10^{-4}$M | $8.0 \times 10^{-5}$M | 0.238 | 0.356 |
| *hisI,F135 G1108* | $2.27 \times 10^{-4}$M | $5.55 \times 10^{-5}$M | 0.244 | 0.204 |
| *hisI,F135 G1107* | $2.03 \times 10^{-4}$M | $6.05 \times 10^{-5}$M | 0.278 | 0.323 |
| *hisI,F135 G1106* | $2.78 \times 10^{-4}$M | $7.15 \times 10^{-5}$M | 0.322 | 0.444 |

The values were obtained from standard LINEWEAVER and BURK (1934) double reciprocal plots.

Sephadex G50-treated preparation (NO MET) to various concentrations of histidine was determined (Figure 4). Then to 0.87 ml of the Sephadex G50-treated extract were added 10 μmoles of $MgCl_2$, 5 μmoles ATP, and 329 μmoles MET. The final volume was 1.0 ml. The tubes were then tightly stoppered and incubated for 30 minutes at 37°C. The precipitate formed was removed by centrifugation at 19,000 × *g* for 15 minutes, and the supernatant was assayed for sensitivity to increasing concentration of histidine (MET) (Figure 4).

*hisI,F135 G1106* and *hisI,F135 G1109* show a definite increase in feedback inhibition following MET treatment; in contrast, *hisI,F135 G1108* and *hisI,F135 G1107* do not show an increase.

*Effect of pH on feedback inhibition by histidine:* MARTIN (1963) has also observed a decrease in feedback inhibition of pyrophosphorylase from *hisI,F135* with an increase in pH. The resulting titration curve indicated a pK of 9.2. Similar titration curves with pyrophosphorylase from TAR mutants indicate a definite decrease in feedback inhibition with increasing pH for those mutants

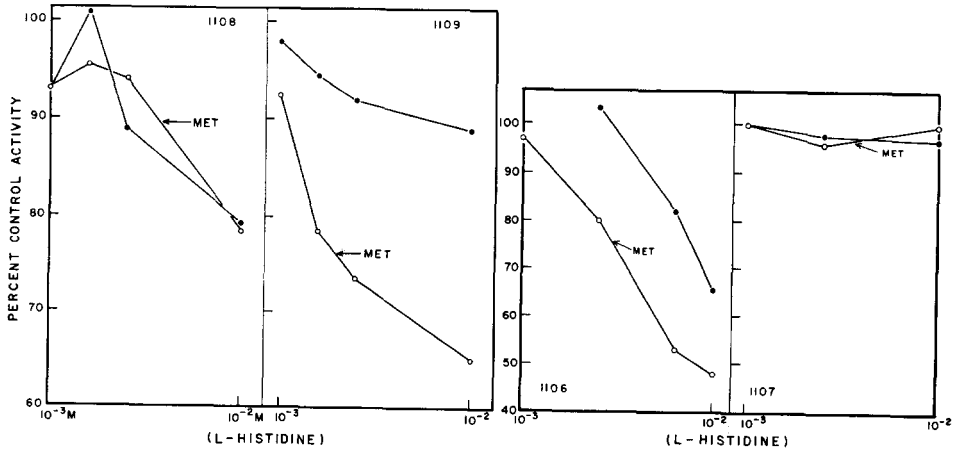


FIGURE 4.—Effect of mercaptoethanol (MET) incubation of feedback inhibition by histidine. Extent of feedback inhibition before (●) and after (○) MET incubation was determined. Control activity was determined before and after MET incubation in the absence of histidine.

showing a significant feedback inhibition at pH 8.5. The pK's obtained are shown in Table 5.

TABLE 5

*pK of feedback inhibition by histidine*

| Strain | pK |
|---|---|
| *hisI,F135* | 9.6 |
| *hisI,F135 G1105* | 9.2 |
| *hisI,F135 G1106* | 9.2 |
| *hisI,F135 G1109* | 8.9 |

*Heat inactivation:* Heat inactivation kinetics were determined for partially purified preparations of pyrophosphorylase from *hisI,F135* and *hisI,F135* $G^{TAR}$ strains. The results are shown in Figure 5. The thermal lability of the pyrophosphorylase from mutant *hisI,F135 G1109* appears to be quite similar to that of the pyrophosphorylase from *hisI,F135* at 45°C (Figure 5A). Pyrophosphorylase from *hisI,F135 G1106* and *hisI,F135 G1107* are definitely more thermolabile (Figure 5B). Pyrophosphorylase from *hisI,F135 G1108* is extremely thermolabile at 40°C, losing more than 95 percent of its activity in less than 10 minutes.

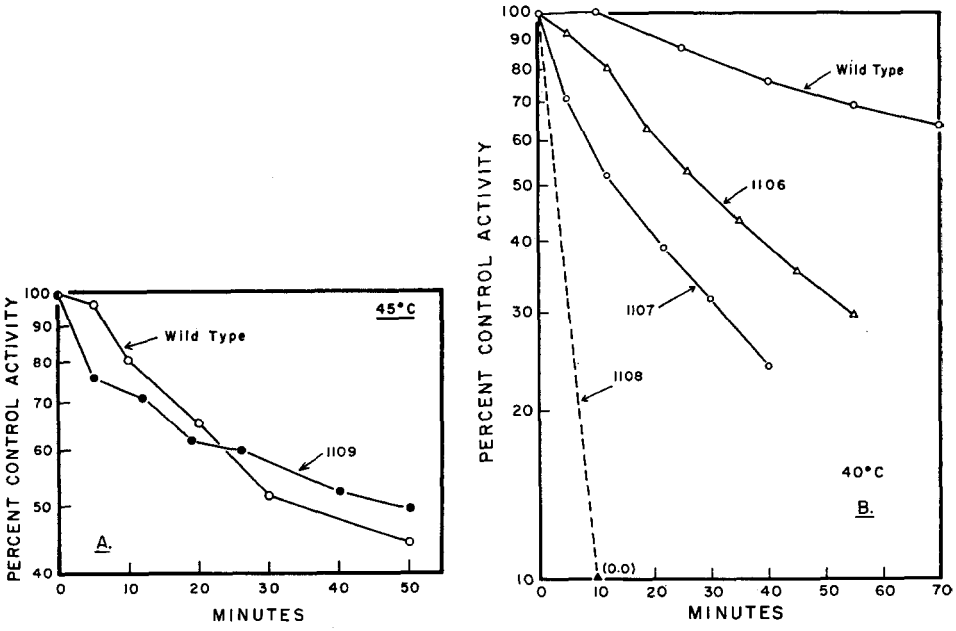


FIGURE 5.—Heat inactivation kinetics of partially purified ATP-PRPP pyrophosphorylase. Wild-type pyrophosphorylase was obtained from a derepressed culture of *hisI,F135*. Mutant pyrophosphorylases were obtained from derepressed cultures of *hisI,F135* $G^{TAR}$. Control activity for each extract was determined at the start of each heat inactivation experiment. Extracts examined at 40°C contained $10^{-2}$ M MET. Extracts examined at 45°C contained no MET.

## DISCUSSION

The mechanism observed here for histidine excretion by the Salmonella mutants resistant to 2-thiazole alanine is similar to that observed by MOYED and FRIEDMAN (1959) and MOYED (1960, 1961a,b) in a TAR mutant of *E. coli*. The Compound III synthesizing system in the *E. coli* TAR mutant was found to be at the repressed level, and resistant to feedback inhibition by L-histidine.

The mapping data indicate that all of the Salmonella histidine excreting strains isolated in the current study are located at two widely separated positions within the structural gene for pyrophosphorylase, gene *G*. Seven of the eight mutants are grouped in a cluster (Figure 1). Although a sufficient number of mutants have not been collected to preclude the possibility of other mutational regions which could give rise to feedback resistant pyrophosphorylase, the existence of a cluster suggests that there may be only a few regions within the genome which can be altered to permit the formation of an enzymatically active, feedback resistant enzyme. Of 29 *hisG*$^-$ point mutants tested (LOPER, GRABNAR, STAHL, HARTMAN and HARTMAN 1964), only one, *hisG460*, is located between the ends of *hisG1303* and *hisG1302* (Figure 1).

The enzyme from *hisI,F135 G1109*, appears to have been altered in its feedback property without an accompanying alteration in its thermal stability. Thus, the genetic information contained in the region between the ends of *hisG1302* and *hisG1303* may determine an area of the protein critical for thermal stability as well as feedback inhibition. Enzymes from three mutants (*hisI,F135 G1106*, *hisI,F135 G1107*, *hisI,F135 G1108*) of this cluster showed greater heat lability than wild-type enzyme from *hisI,F135* (Figure 5). Furthermore, the enzyme from *hisI,F135 G1108* was much more heat labile than the enzymes from *hisI,F135 G1106* and *hisI,F135 G1107*. Mutant *hisI,F135 G1100* contains an unstable pyrophosphorylase with low activity. Figure 3 indicates that there is a wide range of relative resistance to feedback inhibition for the various TAR mutants. In particular, the enzymes from *hisI,F135 G1106* and *hisI,F135 G1107*, which had similar heat inactivation properties, are distinguished on the basis of sensitivity to histidine. Of the seven TAR mutants within the cluster, only the enzymes of *hisI,F135 G1102* and *hisI,F135 G1107* have similar properties. Thus most of the mutations within the cluster represent distinct mutational changes which can lead to the formation of different mutant proteins with properties different from one another.

MARTIN (1963) has observed that the extent of feedback inhibition with wild-type enzyme from *hisI,F135* decreased as the pH was increased. The pK obtained from such a titration was 9.2. Since the $\alpha$-amino group of histidine has a pK of 9.2, it was suggested by MARTIN that the interaction of histidine with the enzyme molecule was a function of its charge or the charge of certain groups on the protein. However, the possibility that pH may influence the conversion of an "enzyme-histidine" complex to feedback inhibited enzyme cannot be eliminated. The similarity in the pK of feedback inhibition between wild-type and mutant enzyme (Table 5) would suggest that mutation to feedback resistance in the

case of *hisI,F135 G1105*, *hisI,F135 G1106*, and *hisI,F135 G1109* has either altered the affinity of the enzyme for histidine or the ease with which some configurational change follows histidine binding without altering the basic mechanism of inhibition.

Values for $K_m$ and $V_{max}$ were the same for mutant and wild-type proteins. This is in contrast to *in vitro* modified aspartate transcarbamylase studied by GERHART and PARDEE (1963). They observed a twofold increase in $K_m$ and $V_{max}$ upon selective destruction of feedback inhibition. CHANGEUX (1962) also has observed an alteration in substrate kinetics after destruction of the feedback inhibiting effect of isoleucine on threonine deaminase. In both the above cases, however, native enzyme exhibits a complex competitive type inhibition of feedback inhibitor with substrate, indicating a profound interaction of the feedback inhibitor with the active site. In contrast, MARTIN (1963) has shown a noncompetitive type of inhibition of ATP-PRPP pyrophosphorylase activity by histidine. This observation, as well as our results, suggest that the feedback site and the active site of ATP-PRPP pyrophosphorylase are sufficiently distinct such that a mutational alteration at the feedback site need not affect the function of the active site.

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## SUMMARY

The mechanism responsible for histidine excretion by 2-thiazole alanine resistant (TAR) mutants of *Salmonella typhimurium* has been examined.

Mutations to 2-thiazole alanine resistance were mapped in two distinct regions of the pyrophosphorylase (*G*) gene. One of the regions contained a cluster of seven of the eight mutants described. Pyrophosphorylases extracted from TAR mutants clustered in one region were more thermolabile than wild-type pyrophosphorylase. Pyrophosphorylase extracted from the mutant in the other mutational region was indistinguishable from wild-type pyrophosphorylase in thermal lability.

Pyrophosphorylases extracted from TAR mutants were at least 100-fold more resistant to feedback inhibition than was the wild-type enzyme; in two mutants the feedback resistance appeared to be complete. Feedback inhibition could be partially restored by mercaptoethanol incubation with two of the four mutant enzymes tested. An increase in pH of the reaction buffer decreased feedback inhibition of both wild-type and mutant enzymes. This suggests, as one mechanism for feedback resistance, an alteration in the affinity of the mutant enzyme for histidine without an alteration in the basic mechanism of feedback inhibition.

Similarity of $K_m$ and $V_{max}$ between wild-type and mutant enzymes indicated no apparent alteration in the function of the active site as a result of mutation to feedback resistance.

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