THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 244, No. 19, Issue of October 10, pp. 5382-5391, 1969
Printed in U.S.A.

Genetic Control of Histidine Degradation in *Salmonella typhimurium*, Strain LT-2*

(Received for publication, May 19, 1969)

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SUMMARY

***Salmonella typhimurium* strain LT-2 produces very little histidase and urocanase upon induction by L-histidine or imidazolepropionic acid. Rare mutants were isolated that could be induced to form these enzymes. By further mutations strains specifically deficient in the ability to form histidase or urocanase and strains capable of forming these enzymes in the absence of inducer were obtained. It could be shown by transduction that the parent organism possesses the genetic information required for the production of histidase and urocanase. The genetic sites responsible for the formation and control of the enzymes are closely linked to one another and are located on the bacterial chromosome near the site responsible for galactose fermentation. The genetic site responsible for the inability of the parent strain to produce the enzymes is located between the gene for urocanase and the gene determining response to induction.**

Among the enteric bacteria, *Aerobacter aerogenes* can use L-histidine as the sole source of nitrogen or of carbon for growth. The organism is induced by histidine, urocanic acid, or imidazolepropionic acid to form a series of four enzymes catalyzing the conversion of histidine to ammonia, L-glutamic acid, and formamide by the following pathway:

$$\text{Histidine} \rightarrow \text{urocanic acid} + NH_3 \quad (1)$$

$$\text{Urocanic acid} + H_2O \rightarrow \text{imidazolonepropionic acid} \quad (2)$$

$$\text{Imidazolonepropionic acid} + H_2O \rightarrow \text{formiminoglutamic acid} \quad (3)$$

$$\text{Formiminoglutamic acid} + H_2O \rightarrow \text{glutamic acid} + \text{formamide} \quad (4)$$

The glutamic acid is further metabolized to provide the cells with energy and building blocks (1-7).

* The work was supported by Research Grants GM-07446 and CY-05210 from the United States Public Health Service and GB-265 from the National Science Foundation.

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A powerful tool for the elucidation of problems involving the regulation of enzyme synthesis is the genetic analysis of strains with mutations in the appropriate structural and regulatory genes. Unfortunately, it is not possible to use this tool for the study of the histidine-degrading enzymes in *A. aerogenes* since so far no system for the transfer of genetic material has been discovered in this organism. *Escherichia coli*, the bacterium most widely used in genetic studies, does not produce the enzymes of histidine degradation; but another enteric organism, *Salmonella typhimurium*, with genetic material which can be transferred by transduction, has been reported to possess these enzymes (8). We decided, therefore, to undertake a study of the genetic and physiological control of histidine degradation in this organism.

The strain of *S. typhimurium* most frequently used in genetic work, strain LT-2, was unable to grow in media containing L-histidine as the source of nitrogen. It was, however, possible to obtain rare mutants of this organism that had acquired the ability to use histidine as source of nitrogen. By a further mutation, the organism could acquire the ability to use histidine as sole source of carbon.

We shall describe in this paper the enzymes responsible for the utilization of histidine, the genetic and environmental control of their formation, and the reason for the inability of the prototrophic strain to metabolize histidine.

EXPERIMENTAL PROCEDURE

Chemicals—L-Histidine hydrochloride hydrate, urocanic acid dihydrate, imidazolepropionic acid (also called dihydrourocanic acid), L-glutamic acid, reduced glutathione, all of A grade, and 2-aminopurine nitrate, B grade, were obtained from the Calbiochem. Penicillin G potassium (Squibb and Son), ethylmethane sulfonate (Eastman), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich), Tris (Sigma 121), and hexadecyltrimethyl ammonium bromide (Eastman) were commercial preparations. *N*-Formimino-L-glutamic acid was synthesized as described by Tabor and Rabinowitz (9); 2-^{14}C-imidazolepropionic acid was synthesized by Dr. S. Schlesinger (6).

Cultivation of Bacteria—The strains of *S. typhimurium* used are listed in Table I. All were derived from *S. typhimurium*, strain LT-2.

The bacteria were maintained with occasional transfer on slants containing 1% Bacto-tryptone, 1.0% yeast extract, 0.5% K_2HPO_4, 0.1% glucose, and 2.0% Bacto agar, or on slants containing 1.5% Fields' tryptic digest and 2.0% Bacto agar. In the transduction experiments, the minimal Medium E of Vogel and

Bonner (10) without citrate, with trace elements (11), was used. In other experiments, a minimal medium containing 0.12 M Tris-HCl, pH 7.5; 0.08 M NaCl; 0.02 M KCl; 2.5 mM Na_2SO_4; 0.2 mM $CaCl_2$; 0.002 mM $FeCl_3$; and 0.01 M KH_2PO_4 was used. To these salt bases were added 0.2% $(NH_4)_2SO_4$ or 0.2% L-histidine HCl as source of nitrogen and 0.2% D-glucose, 0.2% sodium succinate, 0.2% D-galactose, or 0.2% L-histidine HCl as source of carbon. When required for the growth of a strain, a supplement of 20 μg of L-histidine per ml was added. In all cases, salt base, nitrogen source, carbon source, and supplement were sterilized separately at 115° for 30 min, except that urocanic acid, imidazolepropionic acid, and hexadecyltrimethyl ammonium bromide were sterilized by filtration through a bacterial filter (Millipore). In addition, we used a rich medium, LB broth, containing 1.0% Bacto tryptone, 0.5% yeast extract, and 1.0% NaCl, with pH adjusted to 7.0 with N NaOH; it was sterilized at 115° for 30 min. Agar plates were prepared by the addition of 1.5% Bacto agar to the minimal media or LB broth. Soft agar plates contained 0.8% Bacto nutrient broth powder, 0.5% NaCl, and 0.7% Bacto agar.

The cells were grown in 50-ml or 1-liter cultures contained in 250-ml or 2-liter Erlenmeyer flasks with aeration on a New Brunswick shaker at 37°. Growth was measured in a Klett-Summerson photoelectric colorimeter with a 420-mμ filter.

Enzyme Assays—For the assay of enzyme activities, the appropriate media were inoculated with sufficient cells previously grown in the same medium to give a density of approximately 15 Klett units (approximately 1×10^8 cells per ml). The cells were harvested by centrifugation at 4° after three to four mass doublings. They were washed twice with chilled 0.015 M potassium phosphate buffer, pH 7.2, and were finally suspended in approximately 8 ml of the same buffer with reduced glutathione added to a concentration of 5 mM. The cells were then disrupted by sonic oscillation for 10 min in a Raytheon 10-kc sonic oscillator cooled at 3° by an alcohol water bath, or for 1 min per ml of sample in a 60-watt ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., Spenser Street, London, S. W. 1). Cell debris was removed by centrifugation for 15 min at 20,000 rpm. The crude extract (usually 20 mg of protein per ml) was assayed immediately for histidase and urocanase but was sometimes frozen and stored before assay for formiminoglutamate hydrolase. The protein concentration of cell extracts was determined by the Lowry modification of the Folin phenol method (12). Histidase, urocanase, FGA-hydrolase,[1] and the permease for imidazolepropionic acid were assayed by previously described procedures (4–6).

Isolation of Mutants—*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was used as mutagenic agent as described by Adelberg, Mandel, and Chen (13). Mutants were also produced by exposure of cells to ultraviolet light from a germicidal lamp sufficient for 99.9% killing. In some instances, 2-aminopurine (14), ethylmethanesulfonate (15), and nitrous acid (16) were used for mutagenesis.

To obtain mutants of strain PV capable of using histidine as sole source of nitrogen, the mutagenized cells were grown overnight in a medium containing glucose, histidine, and ammonium sulfate. The cells were collected by centrifugation, washed, suspended in the salt base, and spread on agar plates containing glucose or sodium succinate as source of carbon and histidine as source of nitrogen at 10^4 to 10^5 cells per plate. Similarly, mutagenized cells of strain PV2 were spread on agar plates containing histidine and ammonium sulfate but no glucose or sodium succinate for the isolation of a mutant capable of using histidine as sole source of carbon.

[1] The abbreviation used is: FGA, formiminoglutamate.

TABLE I

Strains of Salmonella typhimurium LT-2

The wild strain PV is unable to form histidase and urocanase and cannot use histidine as a source of nitrogen; this characteristic is denoted by *hutP0*, and the gain of these abilities as *hutP*$^+$. The mutants capable of using histidine as a source of nitrogen are unable to use it as sole source of carbon; this characteristic is denoted as *hutM0* and the gain of this ability as *hutM*$^+$. The other mutant genotypes are indicated by an isolation number and in the general case by a minus sign. *hutH*, inability to produce histidase; *hutU*, inability to produce urocanase; *hutC*, constitutive synthesis of the histidine-degrading enzymes; *his*, histidine requirement; *pyrD*, pyrimidine requirement because of deficiency in dihydroorotic acid dehydrogenase; *gal*, inability to utilize galactose; *aroG*, phenylalanine requirement in the presence of tyrosine and tryptophan because of deficiency in phenylalanine-repressible deoxyarabinoheptulosonic acid-P synthetase.

Strain	Pertinent genotype	Derivation[a]
PV	*hutP0*	P. Hartman
PV1	*hutP*$^+$, *his*$^-$	UV, from PV
PV2	*hutP*$^+$, *his*$^+$	Spontaneous, from PV1
PV3	*hutM*$^+$	UV, from PV2
PV4	*hutH77*	UV, from PV3
PV5	*hutU22*	NTG, from PV3
PV15	*hutC46*	NTG, from PV3
PV20	*hut(C46, H17)*	NTG, from PV15
PV22	*hut(C46, U19)*	NTG, from PV15
PV37	*pyrD*-95	P. Hartman
PV39	*gal*-50	P. Hartman
PV87	*gal*-502, *aroG530*	D. Sprinson
PV109	*hisF6*	P. Hartman
PV110	*hutP*$^+$, *hisF6*	UV, from PV109
PV111	*hutP*$^+$, *his*$^-$	UV, from PV
PV112	*hutP*$^+$, *his*$^+$	Spontaneous, from PV111

[a] Abbreviations: UV, treatment with ultraviolet light; NTG, treatment with *N*-methyl-*N'*-nitronitrosoguanidine.

To obtain mutants capable of forming histidase in absence of inducer, the mutagenized cells of strain PV3 were grown overnight in LB broth. The cells were collected by centrifugation, washed, and grown in a liquid minimal medium with sodium succinate as source of carbon and ammonium sulfate as source of nitrogen to eliminate auxotrophs. The culture was diluted and spread on agar plates with sodium succinate as source of carbon and ammonium sulfate as source of nitrogen at approximately 300 cells per plate. The plates were incubated for 2 to 3 days. Sterile filter paper (Whatman No. 3MM) that had been cut to the size and shape of a petri dish was saturated with a sterile solution of 2×10^{-2} M histidine, 0.1 M diethanolamine at pH 9.4, and 0.025% hexadecyltrimethyl ammonium bromide. The wet paper was then picked up by sterile forceps, and, after excess liquid had been drained, the paper was pressed on top of the agar, allowing part of each colony to stick to the paper. The filter paper was then taken off the plate and placed at 37° to incubate and dry. When completely dry, the filter paper was examined over an ultraviolet lamp (General Electric, 15-watt, germicidal) with the side that had not touched the colonies facing

the lamp. A mutant colony that produces histidase constitutively is recognized by a black area due to urocanic acid which can quench the fluorescence of filter paper caused by ultraviolet light. The corresponding area of the original plate contains the mutant colony, which is then purified. At the high pH of the test solution histidase activity is optimal, wheras urocanase activity is very low and consequently urocanic acid accumulates. Urocanase-negative mutants also will be picked up by this technique since they contain high levels of histidase when grown in the succinate-ammonia medium.

To obtain mutants lacking histidase or urocanase, the mutagenized cells were first grown in LB broth, and then for two cycles in a liquid minimal medium with glucose as source of carbon and ammonium sulfate as source of nitrogen. The culture was diluted and spread on agar plates containing sodium succinate as source of carbon, histidine as source of nitrogen, and a supplement of 1% of LB broth, at 10^3 cells per plate. Neither histidase-less, nor urocanase-less *S. typhimurium* mutants can use histidine as source of nitrogen when succinate is the source of carbon. Therefore, the only source of nitrogen available to such mutants is that contained in the small supplement of LB broth; consequently, they appear as small colonies. These colonies were picked and streaked on plates containing ammonium sulfate and sodium succinate as well as a supplement of 0.01 M imidazolepropionic acid, an inducer of the histidine-degrading enzymes (see "Results"). The mutants were then tested for the presence or absence of histidase by scraping the cells from the agar plate with a wooden applicator stick and suspending them in 0.1 ml of the histidine-detergent-buffer mixture, described in the preceding paragraph, contained in a depression of a Micro-disposo-tray (Falcon Plastics). After 30 min of incubation at 37°, a drop of the suspension was placed on Whatman 3MM paper, dried, and examined over ultraviolet light. A histidase-less mutant fails to show the dark spot seen when cells of the wild strain are treated in this manner. The presence or absence of urocanase was ascertained by a similar procedure, except that the mixture in the depression of the tray contained 0.1 M potassium phosphate, pH 7.4; 2 mM urocanic acid; and 0.025% hexadecyltrimethylammonium bromide. Incubation for 30 to 60 min leads to the disappearance of urocanate when urocanase is present. Consequently, when spotted on filter paper and examined under ultraviolet light, the mixtures containing mutant cells lacking urocanase appear dark, and the others light.

Preparation of Phage Lysates—Stocks of phage P-22 were obtained from Dr. P. Hartman and Dr. H. Ozeki. A solution containing approximately 1.4×10^8 phage particles was added to 40 ml of a culture in LB broth containing approximately 10^8 cells per ml. The mixture was incubated on a shaker at 37°. After 6 to 7 hours the bacteria were removed by centrifugation at 6,000 × *g* for 15 min. The phage particles were collected from the supernatant by centrifugation at 57,000 × *g* for 1 hour and suspended in 4 ml of basal salts solution. The mixture was treated with a drop of chloroform to kill any bacteria. The phage suspension was titered by the following procedure: appropriate dilutions of the phage and the cells of strain PV were added to 2 ml of soft agar at 45°. The contents of the tubes were mixed and layered onto LB agar plates. After the soft agar layer solidified, the plates were incubated at 37° for 5 to 6 hours. The phage plaques were then counted. The titer was usually 2×10^{11} phage per ml. The phage lysates were stored at 3° in the basal salts solution in screw cap tubes.

Transduction—The recipient strain was cultivated in LB broth. A mixture containing 4 ml of a culture of exponentially growing cells (2.5×10^8 cells per ml) and phage to give multiplicity of about 5 was prepared. This mixture was incubated at 37° for 10 min, and the cells were collected by centrifugation at 6000 × *g* for 15 min and resuspended in 1 ml of basal salts solution. Appropriate dilutions of the suspension were plated onto agar plates containing the selective minimal medium, and the plates were incubated for 1 to 3 days.

The selective agar plates contained sodium succinate and histidine for the isolation of transductants with active histidase and urocanase from recipients lacking histidase, or urocanase, or both enzymes; they contained galactose and ammonium sulfate for the isolation of Gal^+ transductants from Gal^- recipients; they contained glucose and ammonium sulfate for the isolation of Pyr^+ and Aro^+ from Pyr^- and Aro^- recipients.

The transductant colonies were picked and streaked on plates of the type on which they had been selected. They were then examined for their ability to grow on various media by replica plating. They were examined for their ability to produce histidase and urocanase after growth on agar plates containing sodium succinate and ammonium sulfate by suspension in a histidine- or urocanic acid-containing solution as described in the preceding section. It was observed that mixtures containing cells with constitutive histidine-degrading enzymes and the histidine solutions formed a bright red precipitate when allowed to dry at 37°. This red color indicates the presence of breakdown products of imidazolonepropionic acid: solutions of this compound turn red upon storage in the presence of air. Cells with constitutive histidase, but lacking urocanase, do not produce the red material from histidine.

RESULTS

Histidine-utilizing Mutants—The wild type of strain LT-2 (here called strain PV) fails to grow in media containing histidine as the sole source of nitrogen. This seems to be the typical behavior of LT-2 strains, since various cultures of this organism which we obtained from several investigators (see Table I) all were unable to use histidine as a source of nitrogen.

We attempted to obtain mutants of strain PV capable of using histidine as a nitrogen source when either glucose or succinic acid was the carbon source. The mutagenic agents used were ultraviolet irradiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 2-aminopurine, ethylmethane sulfate, and nitrous acid. Although these methods readily yielded amino acid auxotrophs, in over 100 attempts only two mutants capable of using histidine as the sole source of nitrogen were isolated. Both were obtained after ultraviolet irradiation; one of them, strain PV1, was selected in a medium containing sodium succinate as its source of carbon, the other, strain PV111, was selected in a medium containing glucose as its source of carbon. Both strains could use histidine as a source of nitrogen with glucose, glycerol, or succinic acid as the source of carbon. Neither strain could use histidine as its sole source of carbon.

When the growth characteristics of strain PV111 and PV1 were examined, it became apparent that both strains required histidine for growth. The requirement for histidine could be removed by mutation. Histidine-independent mutants were isolated by placing the cells of these strains in minimal medium

containing glucose as a source of carbon and ammonium sulfate as a source of nitrogen. After several days, the cultures showing growth were spread on minimal glucose agar plates containing ammonium sulfate, and histidine-independent colonies were isolated. One revertant of each strain, PV2 and PV112, was studied further. Each of these revertants could be shown by the method of Hartman, Loper, and Šerman (17) to have regained histidine independence as a result of a reversion of the original defect. They had retained their ability to use histidine as a source of nitrogen. It appears, therefore, that the inability to produce histidine is not essential for the ability to use histidine as a source of nitrogen.

The careful mapping of the lesion responsible for the histidine requirement gave a surprising result. This mapping, carried out according to Hartman, Loper, and Šerman (17), showed that in both strains, PV111 and PV1, the lesion is located in the *hisF IX* region.[2] This region contains the site of the defect in histidine biosynthesis in strain PV109, a mutant of strain PV, from which a mutant capable of using histidine as sole source of nitrogen, strain PV110, had been obtained by Dr. A. K. Magasanik at the Institut Pasteur in Paris.[3] Although it is therefore possible that contamination of strain PV by strain PV110 is the source of strains PV1 and PV111, this is very unlikely since strain PV110 was not used in our laboratory when the isolation of strains PV1 and PV111 was undertaken. Subsequent attempts to obtain additional mutants capable of using histidine as a nitrogen source from strain PV109 were unsuccessful. We can offer no explanation for the apparent relation between a mutation at the *hisF6* site and the mutation enabling strain PV to use histidine as a source of nitrogen. The mutated genes are not closely linked, since phage P22 grown on strain PV transduced strains PV1 and PV111 to histidine independence without causing the loss of the ability to use histidine as a source of nitrogen.[4]

An attempt was next made to obtain a strain capable of growth on histidine as sole source of carbon. To this end, strain PV2, which had already acquired the ability to use histidine as a source of nitrogen, was mutagenized by ultraviolet irradiation during exponential growth in minimal glucose-ammonium sulfate medium. After intermediate culture in the same medium, the cells were harvested by centrifugation and placed in liquid medium containing histidine as sole source of carbon and nitrogen. After 60 hours of incubation, growth was evident; the cells were then spread on agar plates containing histidine as sole source of carbon and nitrogen. After 2 days of incubation colonies appeared; one, which we call strain PV3, was picked and purified for further study. This mutant resembles *A. aerogenes* in its ability to grow on histidine as sole source of carbon and nitrogen. However, in contrast to that organism, it is unable to utilize urocanic acid either as a source of carbon or as a source of nitrogen.

In order to discover whether the pathway of histidine degradation in *S. typhimurium* leads to glutamate, strain PV106, a glutamate-requiring mutant of strain LT-2, was transduced with a lysate of strain PV2 infected with phage P22, and transductants capable of growth in a medium containing glucose and a small amount of glutamic acid with histidine as the major source of nitrogen were isolated. These transductants were unable to grow in a minimal glucose-ammonium sulfate medium but could grow in such a medium when either 0.5 mg of histidine per ml or 0.5 mg of glutamic acid per ml was added. The original glutamic acid-requiring mutant could not grow when the glutamate supplement was replaced by a histidine supplement. Apparently, the acquisition of the ability to use histidine as a source of nitrogen has endowed the glutamate-requiring mutant also with the ability to use histidine specifically as a source of glutamate. Thus, the pathway of histidine degradation in *S. typhimurium*, as does that in *A. aerogenes*, leads to glutamate. The resemblance of the pathways is affirmed by the demonstration that in strain PV3 formamide accumulates as a product of histidine degradation. This was shown by growing the organism in a medium containing histidine as sole source of carbon and nitrogen until growth had come to a halt and analyzing the culture fluid for free and for alkali-labile ammonia (18). For every mole of histidine originally present in the culture fluid, 1.0 mole of free ammonia was found and an additional 0.6 mole of ammonia was produced by mild alkaline hydrolysis. The alkali-labile compound is presumably formamide; the culture fluid was free of formiminoglutamate, another degradation product of histidine that yields ammonia upon alkaline hydrolysis.

Strain PV2, although unable to grow on histidine as sole source of carbon, produces glutamate from histidine and therefore appears to possess all the enzymes required for histidine degradation. It is therefore not obvious what additional mutation has enabled strain PV3 to use histidine as sole source of carbon. This problem was further examined and will be treated in the next section dealing with the enzymatic composition of the strains capable of histidine utilization.

The derivation and the growth characteristics of the strains described in this section are summarized in Table II.

Enzymatic Composition—The findings reported in the preceding section, that the final products of histidine metabolism in *S. typhimurium* are glutamic acid and formamide, suggested that histidine is degraded in this organism by the same pathway as in *A. aerogenes*. We measured the levels of three of the four enzymes known to be required for histidine degradation *A. aerogenes* in extracts of the different *S. typhimurium* strains grown in media with and without histidine. The enzymes are histidase, urocanase, and formiminoglutamate hydrolase, catalyzing respectively the first, second, and fourth steps in the degradative pathway. The results are presented in Table III. The enzyme levels of strain PV110, derived by mutation from strain PV109, and of strains PV111 and PV1, derived similarly from strain PV (see Table I) were determined but are not presented in Table III. The levels of the enzymes in these three histidine-requiring strains did not differ significantly from the enzyme levels in strain PV2, the histidine-independent revertant of strain PV1. The enzymes appear to be inducible in the histidine-requiring strains; in media containing histidine at the low level of 20 μg per ml, or in media in which carnosine, a histidine peptide, was used to meet the histidine requirements of the mutants (1), histidase was produced at the low basal level.[5] The enzyme levels in strain PV112, the histidine-independent revertant of strain PV111, are also not shown in Table III, since they did not differ significantly from those of strain PV2.

Examination of Table III shows that the addition of histidine

[2] We are grateful to Dr. P. Hartman for help with these experiments.

[3] A. K. Magasanik, personal communication.

[4] H. K. Meiss, unpublished observation.

[5] Unpublished observation.

TABLE II

Growth of S. typhimurium strains

All strains were able to grow in a medium containing succinate, histidine, and ammonium sulfate. Those strains capable of growth in a succinate-histidine medium could also grow in a glycerol-histidine medium. Growth was rapid in media containing glucose as source of carbon and ammonium sulfate as source of nitrogen (approximately 0.7–1 generations per hour), and slower in media containing succinate as source of carbon or histidine as source of nitrogen (0.2–0.4 generations per hour).

Strain	Derived from	Growth on				
		Glucose-ammonia	Glucose-ammonia-histidine	Succinic acid-histidine	Glucose-histidine	Histidine
PV		+	+	−	−	−
PV109	PV	−	+	−	−	−
PV110	PV109	−	+	+	+	−
PV111	PV	−	+	+	+	−
PV112	PV111	+	+	+	+	−
PV1	PV	−	+	+	+	−
PV2	PV1	+	+	+	+	−
PV3	PV2	+	+	+	+	+

does affect the enzymatic composition of strain PV; there is a slight increase in the level of histidase and a more appreciable increase in the level of formiminoglutamate hydrolase. Strain PV2 differs from strain PV by producing both histidase and urocanase at a greatly increased level in response to histidine. The increased production of histidase presumably accounts for the ability of the strain to use histidine as a source of nitrogen. Strain PV2 does not differ from its parent strain PV in the response of formiminoglutamate hydrolase to histidine. The levels of the three enzymes are lower in glucose-containing media. However, the repressive effect of glucose on histidase is not nearly so strong as in *A. aerogenes* (18), and in another strain of *S. typhimurium* (19). In those organisms glucose almost completely prevents synthesis of the enzyme. The relatively weak repressive effect of glucose explains the ability of strain PV2 to use histidine as sole source of nitrogen in the presence of glucose.

Strain PV3 does not differ from strain PV2 in its ability to produce histidase and urocanase. However, in response to induction by histidine, it produces a higher level of formiminoglutamate hydrolase than does strain PV2. It is possible that the increased production of this enzyme is responsible for the ability of strain PV3 to use histidine as sole source of carbon.

The fact that the mutants of strain PV selected for their ability to produce ammonia from histidine have acquired not only the histidase essential for this purpose, but also urocanase, suggests that the mutation is in a gene that affects production of both enzymes.

We examined whether a single protein is responsible for both enzymatic activities. To this end strain PV3 was grown in a medium containing histidine, succinate, and ammonium sulfate. The cells were harvested and disrupted by sonic oscillation. The crude extract was treated with protamine sulfate (4) and centrifuged. The supernatant solution contained both histidase and urocanase activity.

A 10-ml portion of the supernatant solution (23.4 mg of protein per ml) was placed on top of a column (2.4 × 24 cm),

TABLE III

Histidine-degrading enzymes in wild strain PV and in histidine-utilizing mutants PV2 and PV3

The organisms were grown and the cell extracts were prepared as described under "Experimental Procedure." L-Histidine hydrochloride was added where indicated to give a concentration of 0.2%. Specific activity of histidase is given in millimicromoles of urocanate formed per min, of urocanase in millimicromoles of urocanate destroyed per min, and of formiminoglutamate hydrolase as millimicromoles of FGA destroyed per min per mg of protein.

Strain	Parent strain	Carbon source	Histidine addition	Specific activity		
				Histidase	Urocanase	FGA-hydrolase
PV	—	Succinate	−	12	<0.1	13
		Succinate	+	17	<0.1	55
		Glucose	−	7	<0.1	
		Glucose	+	9	<0.1	18
PV2	PV	Succinate	−	11	<0.1	10
		Succinate	+	151	27	44
		Glucose	−	12	<0.1	
		Glucose	+	51	7	12
PV3	PV2	Succinate	−	12	<0.1	18
		Succinate	+	148	23	103
		Glucose	−	9	<0.1	6
		Glucose	+	47	6	52

which was composed of DEAE-cellulose (0.96 mg per ml); a gradient elution technique was used where the eluting power of the buffer was continuously increased by the gradual addition of 300 ml of 0.25 M potassium buffer, pH 7.2, from the reservoir to 300 ml of 0.01 M potassium phosphate buffer, pH 7.2, in a mixing chamber. The flow rate was approximately 1 ml per min per cm^2. An apparatus for automatic collection was set to collect 5 ml of eluate per tube. After all the fluid had passed through the column, the tubes were assayed for histidase, urocanase, and protein.

The results of this experiment are presented in Fig. 1. It can be seen that maximal histidase and urocanase activities appeared in fractions which were 10 tubes apart. It appears, therefore, that two different proteins are responsible for the two enzymatic activities. Similar results were previously obtained in *A. aerogenes* (5).

Induction by Imidazolepropionic Acid—The failure of strain PV to form histidase and urocanase may be a reflection of its inability to concentrate histidine sufficiently well to bring about induction of these enzymes. In *A. aerogenes*, in addition to histidine, urocanic acid and imidazolepropionic acid, a nonmetabolizable analogue of urocanic acid, can serve as inducers of the histidine-degrading enzymes (5, 6). We determined, therefore, the response of strains PV and PV2 to these compounds. The results summarized in Table IV show that urocanate, even when present at a concentration of 0.1 M, fails to induce the enzymes in either strain PV or strain PV3. Imidazolepropionate at a concentration of 5 mM or higher is a good inducer of histidase and urocanase in strain PV2 but fails to induce the enzymes in strain PV. This observation militates against the hypothesis that the failure to concentrate histidine

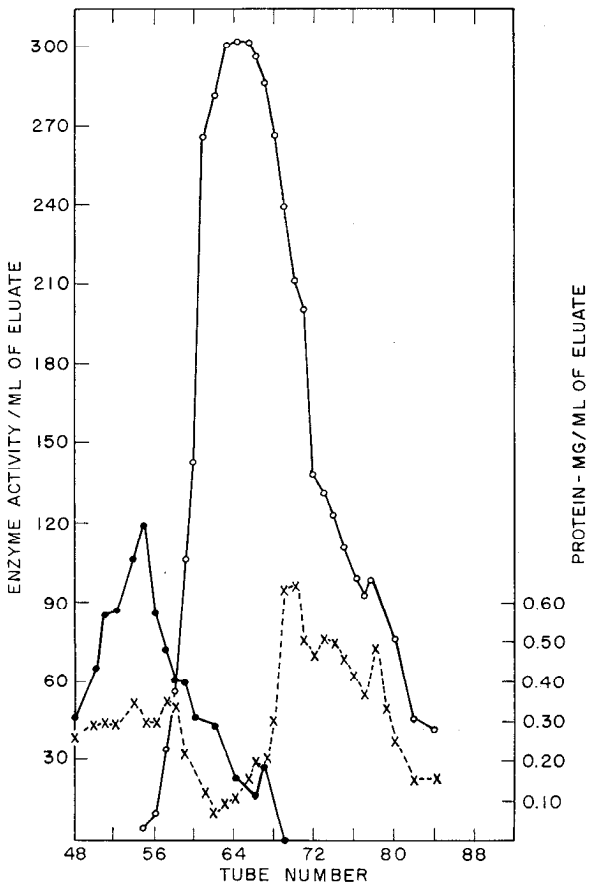

FIG. 1. Chromatographic separation of histidase and urocanase of strain PV-3 on DEAE-cellulose. Histidase activity is given in millimicromoles of urocanate produced per min per ml of eluate, O——O; urocanase activity is given in millimicromoles × 10 of urocanate destroyed per min per ml of eluate, ●——●; protein, ×- - -×.

is responsible for the inability of strain PV to produce histidase and urocanase.

It is of interest that in *A. aerogenes* 0.5 mM imidazolepropionate fully induces histidase (6), while a concentration more than 10-fold higher is required for full induction of strain PV2. It has been shown that the inducers of the histidine-degrading enzymes induced *A. aerogenes* to form a permease for imidazolepropionate and urocanate (6, 7). The experiment illustrated in Fig. 2 reveals that growth in a medium containing imidazolepropionate fails to endow strain PV2 with the ability to concentrate imidazolepropionate. The uptake of the 0.01 to 0.1 mM radioactive imidazolepropionate was very slight and was approximately the same in induced and uninduced cells of strain PV2 and in cells of strain PV. Thus, in constrast to *A. aerogenes*, *S. typhimurium* does not seem to produce a permease capable of facilitating the entry of imidazolepropionic acid. The lack of this permease may be responsible for the inability of urocanic acid to induce the enzymes in strain PV2.

Mutants Lacking Only Histidase or Urocanase—The fact that imidazolepropionic acid is capable of inducing strain PV2 raised the question whether the actual inducer is exogenously supplied histidine or urocanic acid formed endogenously from this histidine. We attempted to answer this question by isolating mutants of strain PV3 that had lost the ability to use histidine as a source of nitrogen or as a source of carbon. One of these mutants, strain PV4, produced neither histidase nor urocanase when exposed to histidine and produced urocanase, but not histidase, when exposed to imidazolepropionic acid. The levels of these enzymes in strains PV, PV3, and PV4 are compared in Table V.

TABLE IV

Response of histidase and of urocanase to inducers in strains PV and PV3

The experimental procedure is the same as in Table III.

Inducer	Concentration	PV		PV3	
		Histidase	Urocanase	Histidase	Urocanase
	M				
Experiment 1					
None		5	<0.1	10	<0.1
Urocanic acid	0.001	9	<0.1	13	<0.1
Urocanic acid	0.01	13	<0.1	12	<0.1
Urocanic acid	0.1		<0.1	18	<0.1
Experiment 2					
None		6	<0.1	14	0.8
Imidazolepropionic acid	0.0001			21	2.1
Imidazolepropionic acid	0.0005			43	4.2
Imidazolepropionic acid	0.001			69	8.4
Imidazolepropionic acid	0.005			160	24
Imidazolepropionic acid	0.01	6	<0.1	174	25
Histidine	0.01	14	<0.1	246	33

Another mutant, strain PV5, was found to be able to use histidine as sole source of nitrogen when glucose was the source of carbon but not when succinic acid was the source of carbon. The mutant could be shown by the spot test to produce histidase even in the absence of histidine or imidazolepropionic acid and not to produce urocanase under any condition. Apparently, the loss of urocanase leads, just as in the case of *A. aerogenes*, to internal induction of histidase. We shall present a more detailed examination of such a mutant in the case of *S. typhimurium*, strain 15-59, in the succeeding paper (19). The properties of these mutants show that histidine induces histidase and urocanase only by virtue of its conversion to urocanic acid.

Order of Genes Affecting Histidine Utilization—A detailed study of the genetic sites affecting the utilization of histidine was made for another strain of *S. typhimurium*, strain 15-59, and is described in the following paper (19). We did a number of transduction experiments with mutants of strain LT-2 in order to clarify the failure of strain LT-2 to produce histidase and urocanase, and in order to discover whether the genes are located on the chromosome of strain LT-2 at the same site and in the same order as on that of strain 15-59. In these experiments we used, in addition to the mutants deficient in enzyme activities, a mutant of strain PV3, strain PV15, that contains histidase and urocanase at a high level even when grown in a medium free of inducer. This constitutive mutant was identified by the spot test described under "Experimental Procedure." A more detailed description of such constitutive strains is presented in the succeeding paper (19).

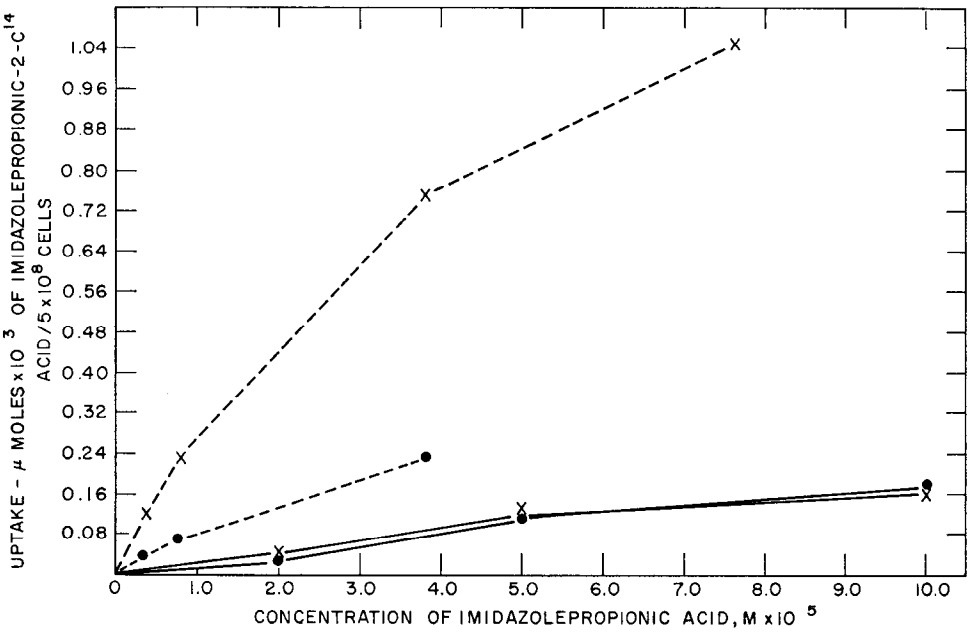

FIG. 2. The uptake of 2-^{14}C-imidazolepropionic acid by strain PV-3. Cells previously grown with histidine (×——×) and without histidine (●——●) were used. For comparison, a similar experiment with *A. aerogenes* (see Reference 6) is included. *A. aerogenes* grown with (×- - -×) and without histidine (●- - -●). The ability of Strain PV to take up 5×10^{-5} M 2-^{14}C-imidazolepropionic acid was determined, and was found to be equal to that of strain PV-3.

TABLE V

Histidase and urocanase in strains PV, PV3, and PV4

The procedures are those of Table III. Concentrations of histidine and imidazolepropionic acid were, respectively, 0.1 and 0.01 M.

Strain	Parent strain	Carbon source	Inducer	Specific activity	
				Histidase	Urocanase
PV		Succinate	None	6	<0.1
		Succinate	Histidine	21	<0.1
		Succinate	Imidazolepropionic acid	6	<0.1
		Glucose	Imidazolepropionic acid	6	<0.1
PV3	PV2	Succinate	None	12	<0.1
		Succinate	Histidine	133	22
		Succinate	Imidazolepropionic acid	40	4
		Glucose	Imidazolepropionic acid	18	2
PV4	PV3	Succinate	None	4	<0.1
		Succinate	Histidine	4	<0.1
		Succinate	Imidazolepropionic acid	5	4
		Glucose	Imidazolepropionic acid	5	1

Strain PV could be transduced for the ability to use histidine as a source of nitrogen by phage lysates of strain PV3, as well as by phage lysates of the histidase- or urocanase-negative mutants of strain PV3; in the latter instances the frequency of transduction was approximately one-tenth of that seen with lysates of strain PV3. Similarly, the histidase- or urocanase-negative mutants of strain PV3 could be transduced for the ability to use histidine as source of nitrogen by phage lysates of strain PV. These results indicate that strain PV possesses at least a portion of the genes coding for histidase and urocanase.

A number of nonselective two-factor crosses were carried out to determine whether the genes affecting histidine utilization are linked to one another. The results of these crosses, summarized in Table VI, show a high degree of linkage between *hut C46*, a marker for constitutivity, and *hut P0*, the site responsible for the failure of strain PV to produce the enzymes (Experiment 1); there is also a high degree of linkage between *hut C46*, and *hut H17* or *hut U19*, mutational sites affecting, respectively, histidase or urocanase (Experiments 2 and 3). Apparently the genes of the *hut* system are clustered on the chromosome.

The location of this cluster is indicated by crosses with mutants of the LT-2 strain defective in galactose metabolism and pyrimidine synthesis. Experiments with another strain of *S. typhimurium*, which are described in the succeeding paper, had suggested linkage between the *gal* and *hut* clusters (19). This is also evident in strain PV: when a Gal$^-$ mutant of strain LT-2 was transduced with a phage lysate of a mutant capable of utilizing galactose and histidine, approximately 60% of the Hut$^+$ transductants had acquired the ability to ferment galactose (Table VI, Experiment 4). On the other hand, there was no evidence of cotransduction between the gene cluster for histidine utilization and a gene affecting pyrimidine biosynthesis located not far from *gal* on the chromosome. The reported linkage (20) of *gal* to a gene involved in aromatic biosynthesis, *aroG*, was confirmed: when a Gal$^-$, Aro$^-$ strain was transduced with a phage lysate of a Gal$^+$, Aro$^+$ strain, 11% of the Gal$^+$ transductants were also Aro$^+$.

We next carried out a three-factor cross using the *aroG*, *gal*,

TABLE VI

Cotransduction of genetic characters

Experiment	Donor		Recipient		Selected phenotype	Transductants analyzed (number)	Unselected recombinants	
	Strain	Character	Strain	Character			Type	Percentage
1	PV15	*hut(C46, P+)*	PV	*hut(C+, P0)*	P+	144	C−	95
2	PV3	*hut(C+, H+)*	PV20	*hut(C46, H17)*	H+	144	C+	91
3	PV3	*hut(C+, U+)*	PV22	*hut(C46, U19)*	U+	144	C+	94
4	PV15	*hutP+, gal+*	PV39	*hutP0, gal-50*	P+	54	Gal+	59
5	PV15	*hutC46, pyrD+*	PV37	*hutC+, pyrD95*	Pyr+	144	C−	0

and *hut* markers, in order to locate the *hut* cluster on the chromosome with respect to the known *aroG* and *gal* markers. In this case, as shown in Table VII, an Aro−, Gal−, Hut− strain was transduced with a phage lysate of an Aro+, Gal+, Hut+ strain and Aro+, or Gal+, or Hut+ recombinants were isolated. These were then examined for the nonselected characters. It can be seen that of the four possible classes, only three were obtained when the selection was for Aro+ or for Hut+. In the former case, the class Gal−, Hut+ is missing; in the latter case the class Aro+, Gal− is missing. All four classes were obtained when the selection was for Gal+.

This result is only compatible with the order *aro, gal, hut P*. In this case when selection is for Aro+, recombinants of the Gal−, Hut+ type would only result from a rare quadruple crossover. Similarly, when selection is for Hut+, recombinants of the Aro+, Gal− type would result only from a quadruple crossover. If the order were *aro, hut, gal*, the quadruple crossover class in selection for Aro+ should be Hut−, Gal+, and in the selection for Gal+ should be Hut−, Aro+; if the order were *hut, aro, gal*, then the quadruple crossover class, in selection for Hut+, should be Aro−, Gal+, and in the selection for Gal+, should be Hut+, Aro−. As can be seen in Table VII, none of the expected results for any order other than *aro, gal, hut* was obtained. The fact that the cotransduction frequency of *aro* and *hut* is less than the cotransduction frequency of *aro* and *gal* and of *gal* and *hut* confirms the position of *gal* between *aroG* and *hutP*.

We next carried out three factor crosses between *hut* mutants. They are presented in Table VIII, and permit us to determine the order of the genetic elements of the *hut* system.

Cross 1 strongly indicates that the *P0* site is located between *C46* and *U19*. The orders placing *U19* between *C46* and *P0*, or *C36* between *P0* and *U19*, are very unlikely, for in those cases a considerable number of Hut C+ recombinants should have been found. Similarly, Cross 2 places the *P0* site between *C46* and *H17*; the order placing *H17* between *P0* and *C46* is excluded, since in this case almost all recombinants should have had the Hut C+ character; the third possible order, with *C46* between *P0* and *H17* is unlikely, since the majority of the recombinants should have been Hut C+. Cross 3 suggests the order *C46 U19, H77*. The order placing *H77* between *C46* and *U19* is excluded, for it requires the large majority of the recombinants to be Hut C−. The order placing *C46* between *H77* and *U19* is unlikely, because of the small number of Hut C− recombinants found. Similarly, the results of Cross 4 are compatible with the order placing *U22* between *C46* and *H17*; the order *C46, H17, U22* is excluded, and the order *U22, C46, H17* not likely. In summary, the order of the *hut* genes appears to be *C, P, U, H*.

TABLE VII

Transduction of strain PV87 aro−, gal−, hutP0 with phage lysate of strain PV3, aro+, gal+, hutP+

Selected phenotype	Unselected characters	
	Phenotype	Number
Gal+	Aro+ Hut+	7
	Aro+ Hut−	11
	Aro− Hut+	42
	Aro− Hut−	107
Aro+	Gal+ Hut+	6
	Gal+ Hut−	8
	Gal− Hut+	0
	Gal− Hut−	94
Hut+	Aro+ Gal+	5
	Aro+ Gal−	0
	Aro− Gal+	123
	Aro− Gal−	42

Another cross was carried out in an attempt to locate the genetic site *hutM+*, responsible for the ability of strain PV3 to use histidine as sole source of carbon. In this cross strain PV4, *hut−(P+, M+, H77)*, a histidase-less mutant of strain PV3, served as donor, and strain PV, *hut−(P0, M0, H+)* as the recipient. Recombinants capable of growth on succinate-histidine, and thus having the Hut+ phenotype, were selected: 153 had the HutM+-character of the donor, and 33 the HutM−-character of the recipient. This result is not compatible with an order placing *H77* between *P+* and *M+*, for in this case the majority of the recombinants should have been HutM−; however, the result permits no choice between the orders *M+, P+, H77*, and *P+, M+, H77*.

Finally, we attempted to orient the elements of the *hut* system with respect to *gal*. To this end the crosses analyzed in Table IX were carried out. They show that the order is *aroG, gal, hutP, hutH*. If *hutH* were located between *gal* and *hutP*, then a considerable number of Gal− recombinants should have been seen in Cross 1 of Table IX. Moreover, in such a case in Cross 2 the most common class of recombinants should be Aro−, Gal−, since all other classes require quadruple crossovers and very few recombinants of the Aro−, Gal+ class would be expected; actually, most recombinants belong to this class, and no recombinants belonged to class Aro+, Gal−. This is exactly the result expected if *hutP* is located between *gal* and *hutH*.

TABLE VIII

Transductions for ordering characters in hut system

Cross	Donor		Recipient		Selected Hut phenotype	Number of unselected recombinants	
	Strain	Character *hut*	Strain	Character *hut*		C^+	C^-
1	PV	C^+, *P0*, U^+	PV22	*C46*, P^+, *U19*	P^+U^+	0	64
2	PV20	*C46*, P^+, *H17*	PV	C^+, *P0*, H^+	P^+H^+	1	143
3	PV4	C^+, U^+, *H77*	PV22	*C46*, *U19*, H^+	U^+H^+	140	4
4	PV5	C^+, *U22*, H^+	PV20	*C46*, U^+, *H17*	U^+H^+	19	125

TABLE IX

Transductions for ordering characters of hut system with gal

Cross	Donor		Recipient		Selected Hut phenotype	Number of unselected recombinants			
	Strain	Characters	Strain	Characters		Gal^+		Gal^-	
1	PV39	*gal-50*, *hut*(*P0*, H^+)	PV20	gal^+, *hut*(P^+, *H17*)	P^+H^+	62		1	
2	PV20	aro^+; gal^+, *hut*(P^+, *H17*)	PV87	*aroG530*, *gal-502*, *hut*(*P0*, H^+)	P^+H^+	Aro^+, Gal^+: 10	Aro^+, Gal^-: 0	Aro^-, Gal^+: 90	Aro^-, Gal^-: 25

DISCUSSION

The experimental results presented in this paper show that it is possible to isolate mutants capable of using L-histidine as sole source of nitrogen from *S. typhimurium*, strain LT-2. Such a mutant converts histidine to L-glutamate and formamide by the series of four reactions previously described in *A. aerogenes* (1–7), which are summarized in the introduction.

As in the case of *A. aerogenes*, the enzymes are inducible, and urocanic acid, the product of the first reaction, and not histidine, is the actual inducer. In both organisms, mutants lacking histidase can be induced by the urocanic acid analogue imidazolepropionic acid, but not by histidine, to form urocanase and FGA-hydrolase; mutants lacking urocanase produce histidase in the absence of any added inducer.

In contrast to *A. aerogenes*, *S. typhimurium* cannot be induced by exogenously added urocanic acid. The explanation for this failure appears to be the lack of a urocanate permease. In keeping with this interpretation is the observation that a higher level of imidazolepropionic acid is required for induction in *S. typhimurium* than in *A. aerogenes* and that no active transport of imidazolepropionate could be demonstrated. We assume that imidazolepropionate can enter the *S. typhimurium* cell by free diffusion or by means of a less specific permease, while urocanate is rigidly excluded.

Although *S. typhimurium* strain LT-2 cannot use histidine as sole source of nitrogen, it is affected by the addition of histidine to the growth medium. Growth in the presence of histidine causes a slight increase in the level of histidase, previously observed by others (21), no measurable increase in the level of urocanase, and a 3-fold increase in the level of FGA-hydrolase.

The mutation which endows the organism with the ability to use histidine as sole source of nitrogen (*hutP0* to *hutP*$^+$) is reflected in a greatly increased response of histidase and urocanase to induction by histidine or imidazolepropionic acid; the response of FGA-hydrolase is not altered.

On the other hand, a further mutation which endows the organism with the ability to use histidine as sole source of carbon (*hutM0* to *hutM*$^+$) is reflected in an increased level of FGA-hydrolase not associated with any change in histidase or urocanase. The possibility that both histidase and urocanase activities are associated with the same protein moiety could be excluded by the chromatographic separation of these enzymes. It has previously been shown that in *A. aerogenes* the syntheses of histidase and urocanase are coordinated with one another but not with that of FGA-hydrolase. The fact that a single mutation in *S. typhimurium* affects both histidase and urocanase, but not FGA-hydrolase, suggests that here too the latter enzyme is controlled independently of histidase and urocanase.

The genetic studies lend strong support to the view that the parent strain PV, though unable to produce a high level of histidase and urocanase, possesses the structural genes for these enzymes. Mutants lacking urocanase or histidase can acquire the ability to use histidine as source of nitrogen by transduction with a phage lysate of strain PV (see Table VIII, Cross 1, where the donor is the wild strain, and Table IX, Cross 1, where the donor is a Gal^- mutant of this strain). The inability of strain PV to form histidase at a high level in response to histidine cannot be due to impermeability of this compound, since imidazolepropionate, which is taken up equally well by strains PV3 and PV, induces histidase in the former, but not in the latter. Moreover, histidine appears to enter cells of strain PV sufficiently well to induce FGA-hydrolase.

The genetic sites controlling histidase, *hutH*, urocanase, *hutU*, the ability to form these enzymes constitutively, *hutC*, and the ability to use histidine as sole source of carbon, *hutM*, are all closely linked to the genetic locus *hutP*, the state of which accounts for the inability of strain PV to produce histidase and urocanase at a high level. The fact that *hutP* is located between the gene determining constitutivity and the structural genes for urocanase and histidase suggests that *hutP*, like *hutC*, is a pleiotropic control gene. However, we cannot explain why it is so difficult to isolate *hutP*$^+$ mutants capable of using histidine as source of nitrogen from the LT-2 strain of *S. typhimurium*,

and why these rare mutants also carry a mutation in the *hisFIX* region.

The *hut* region is located on the *Salmonella* chromosome in the vicinity of the *gal* region; *gal* appears to be located between *hut* and *aroG*. The *hut* genes are oriented so that *hutC* is closer to *gal* than *hutH*. The sequence appears to be: *aroG*, *gal*, *hut* (*C*, *P*, *U*, *H*).

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Genetic Control of Histidine Degradation in *Salmonella typhimurium*, Strain LT-2

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J. Biol. Chem. 1969, 244:5382-5391.
