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Mutants of *Salmonella typhimurium* Able to Utilize D-Histidine as a Source of L-Histidine

KRYSTYNA KRAJEWSKA-GRYNKIEWICZ, W. WALCZAK, AND T. KŁOPOTOWSKI
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 12, Poland

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Secondary mutants able to utilize D-histidine, *dhu*, were isolated in histidine auxotrophs of *Salmonella typhimurium*. Mutations of one class (*dhuA*) are closely linked with the *hisP* locus which codes for a component of histidine permease. The specific activity of L-histidine permeation was estimated as increased two- to sevenfold in *dhuA* mutants. The *dhuB* mutants which have not been mapped also had elevated specific activity of L-histidine permeation. The uptake of D-histidine, barely detectable in the parental strains, was prominent in *dhuA* mutants and showed an apparent Michaelis constant about 1,000-fold higher than that observed with L-histidine. No change was detected in the kinetics of L-histidine permeation. D- and L-histidine competed in the uptake process. Tertiary mutants which lost the ability to grow on D-histidine were isolated by ampicillin counter-selection in *dhuA his*$^-$ strains. All of them mapped in the *dhuA hisP* region. Most of them had all known properties of *hisP* mutants. It is inferred from these data that the *dhuA* mutations increase synthesis of components critical to D- and L-histidine permeation.

Mutants of *Salmonella typhimurium* unable to synthesize histidine require exogenous L-histidine for growth (1). The requirement cannot be satisfied by D-histidine. However, histidine auxotrophs plated on agar medium containing D-histidine gave rise to a number of colonies composed of cells apparently able to utilize D-histidine as a source of histidine (B. N. Ames, *personal communication*). There are no reports on the uptake, racemization, or utilization of D-histidine by bacterial cells.

In the present study we show that mutants of *S. typhimurium* utilize D-histidine. Some of the mutations (*dhuA*) are located at the locus for histidine permease and lead to an apparent derepression of this enzyme activity. D-Histidine uptake utilizes components of the L-histidine permease. The components are encoded in the *hisP* gene which has been described by G. F. Ames (3, 4, 9).

MATERIALS AND METHODS

Organisms. Bacterial strains used in the present study are *S. typhimurium* LT-2. Mutants L4 of phage P22 was isolated by H. O. Smith (10). All these organisms were obtained from the collections of P. E. Hartman, The Johns Hopkins University, Baltimore, Md., and B. N. Ames, University of California, Berkeley.

Media. The basic minimal medium was medium C of Vogel and Bonner (11). It contains 0.2% citrate as the only organic compound. For liquid cultures it was supplemented with 0.5% glucose. Other additions will be specified. Solid minimal medium contained C salts, 2% agar, and 2% glucose. The supplemented agar media contained, in addition: 0.1 mM L-histidine, 0.1 mM D-histidine, 0.01 mM L-histidine plus 2 mM L-tryptophan, 0.01 mM L-histidine plus 0.5 mM L-arginine plus 0.5 mM L-tryptophan, 0.1 mM adenine plus 0.01 mM thiamine, and 0.1 mM L-histidine plus 0.1 mM adenine plus 0.01 mM thiamine. The inorganic medium C-C of G. F. Ames was devoid of citrate and magnesium, but contained a mixture of rare elements (2). Medium E-C was prepared by supplementing medium C-C with 0.5% glucose. Broth medium contained 1% dried broth and 0.5% sodium chloride. It was solidified by addition of 2% agar. All liquid media were sterilized by standard autoclaving and supplemented when applicable with glucose autoclaved separately as a 50% solution.

Bacterial cultures. Liquid cultures were incubated at 37 C with horizontal shaking at about 100 cycles per min. Growth rates were measured in sterile Spekol spectrophotometer tubes. The optical density at 420 nm was converted to micrograms (dry weight) per milliliter with the use of a standard calibration curve. With low cell density, cultures readings were taken in a Unicam SP500 spectrophotometer.

Phage cultures. Logarithmic bacterial cultures in broth medium were infected with phage at a multiplicity of 0.1. After 8 to 18 hr at 37 C with shaking, the cells were discarded by centrifugation. Phage was collected by 50 min of centrifugation at 16,000 rev/min. The phage pellets were soaked for 2 hr in C medium and resuspended with vigorous shaking. The phage sus-

pensions were emulsified with 1/20 volume of chloroform and stored in a refrigerator.

Selection of mutants. Positive selection of mutants was made on appropriate solid agar medium after 3 days of incubation at 37 C. 2-Hydrazino-3-[4(5)-imidazolyl] propionic acid-resistant mutants (*hisP*) were isolated from inhibition zones. In the selection of auxotrophs, washed cell suspensions were mutagenized by incubation for 1 hr in medium C saturated with diethylsulfate. After growth in broth medium, the cells were diluted and spread on minimal medium plates with supplements required by the strain used, and 0.4 ml of the broth medium. After 2 days at 37 C, small colonies were picked and tested for additional growth requirements (B. N. Ames, *unpublished data*). All newly isolated mutants were passed twice through single-colony isolation on unselective medium before use in physiological or genetic experiments.

Transduction. An 0.1-ml amount of an overnight bacterial culture in liquid broth medium was spread together with at least 10^9 phage particles on selective agar medium. The result was scored after 2 days of incubation at 37 C. When *dhu* was the selective marker on D-histidine plates, the incubation was continued for at least 2 full days. Unselective markers were scored by standard replica plating procedures.

Assay of histidine permease activity. The two procedures of G. F. Ames (2) were used. The only modification was that, for the protein incorporation procedure, cells were grown overnight in C medium and transferred to E medium 1 hr before the assay. This avoided long lags before growth started. However, the *hisP* mutants grew only after long lag periods.

Uptake of ^{14}C-D-histidine. The procedure used with growing cells was that of G. F. Ames (2), except for the substitution of D-histidine for L-histidine. D-Histidine uptake was calculated from the difference of cell radioactivities between the 20th and 10th min of incubation with the substrate, to minimize errors due to the contaminating radioactive L-histidine, since traces of L-histidine would be exhausted before the 10th min.

Radioactivity counting. The radioactive cells collected on membrane filters (Biomed, Warsaw) and glued to aluminum planchets were counted, after drying, in a Nuclear-Chicago gas-flow counter, model Biospan.

Chemicals. L-2-Hydrazino-3-[4(5)-imidazolyl] propionic acid hydrochloride was obtained by courtesy of L. H. Sarrett, Merck, Sharp and Dohme Research Laboratories, Rahway, N.J. A sample of the D-isomer of this compound from the same source was kindly supplied by G. F. Ames, University of California, Berkeley. Both isomers were used interchangeably because of comparable physiological effects in *S. typhimurium* (see footnote in reference 9). Both isomers are referred to in the present report as hydrazinoimidazolyl propionic acid.

All other chemicals were obtained commercially. 2-^{14}C-L-histidine hydrochloride (specific radioactivity of 57.8 mCi/mmole) was the product of Radiochemical Centre, Amersham, Bucks, England; 2-^{14}C-D-histidine (14.5 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. Other chemicals were: L-histidine hydrochloride (Chemapol, Prague, and Sigma Chemical Co., St. Louis, Mo.), D-histidine hydrochloride (Hoffman-La Roche, Paris, and Calbiochem, Los Angeles, Calif.), L-arginine and L-β-imidazolelactic acid (Sigma Chemical Co.), L-tryptophan (Reanal, Budapest), imidazolepyruvic acid (Calbiochem), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), and diethylsulfate (Aldrich Chemical Co., Milwaukee, Wis.). Dried broth was a product of Biomed, Warsaw.

RESULTS

Isolation and some properties of D-histidine-utilizing mutants. Colony formation by histidine auxotrophs was noted after 3 days of incubation on agar plates supplemented with D-histidine. These colonies were formed by mutant cells apparently able to take up D-histidine and convert it into L-histidine. The *his* strains tested for the appearance of *dhu* mutants included a set of deletions covering the entire length of the histidine operon and adjacent regions. This result indicated that no gene in the *his* operon or its vicinity was necessary for the occurrence of the *dhu* character.

The *dhu* mutants could be isolated in any *S. typhimurium his* auxotroph and in many multiply marked strains, except for those which had mutations in the histidine permease gene *hisP* or required arginine or lysine for growth.

The mutational origin of the *dhu* character was evidenced by using mutagens. Histidine deletion mutants were used in these experiments to rule out the appearance of revertants to prototrophy. Both NTG and diethylsulfate considerably increased the number of colonies on D-histidine plates inoculated with *his*$^-$ cells.

The ability of the *dhu his* mutants to grow on plates containing 0.1 mM D-histidine was a stable property. However, direct positive selection for *dhu*$^+$ revertants has not been available to rigorously estimate the reversion frequency.

The ability of various mutants to utilize D-histidine as an alternative source of L-histidine has been quantitatively compared in growth experiments. Low concentrations of L-or D-histidine in liquid medium were used to get clear-cut differences of growth rates and total yields of cell mass per micromole of histidine isomer. Tables 1 and 2 present data obtained in these experiments. The mutant designations *dhuA* and *dhuB* result from genetic classification which will be presented later.

Table 1 shows that *dhuA* mutants grew on 0.03 mM D-histidine at rates which were the same, or reduced not more than 50%, as those obtained on 0.03 mM L-histidine. At 0.1 mM concentrations of either D- or L-histidine, the growth rates of *dhuA his*$^-$ mutants were equivalent.

The *dhuB his*$^-$ mutants appeared to be rather poor utilizers of D-histidine: the respective growth rates were at least three times lower as compared with 0.03 mM L-histidine cultures. At 0.01 mM D-histidine, growth of *dhuB his*$^-$ mutants was

TABLE 1. *Growth rates of dhu his⁻ mutants in the presence of* D- *or* L-*histidine*

Mutant	Growth rate[a]			
	L-Histidine (0.03 mM)	D-Histidine		
		0.01 mM	0.03 mM	0.1 mM
hisAF645	1.25	<0.05	<0.05	<0.05
hisBH22	1.25	<0.05	<0.05	<0.05
dhuA1 hisAF645	1.25	0.50	0.59	1.25
dhuA4 hisHAFIEgnd2327 purE801 trpA8[b]	1.25	0.35	0.55	
dhuA21 hisHA134	1.25	0.67	0.67	
dhuA22 hisBH22	1.20	0.59	0.77	
dhuA31 hisIE659	1.00	0.67	1.25	
dhuA45 hisBH22	1.00	0.50	1.00	
dhuB12 hisDCBHAFI52	1.25	<0.05	0.18	0.63
dhuB17 hisF41	1.15	<0.05	0.13	0.33
dhuB20 hisHA134	1.00	0.08	0.40	0.67
dhuB23 hisBH22	0.91	<0.05	0.22	0.49
dhuB32 hisIE659	0.91	<0.05	0.07	0.60
dhuB33 hisBH22	1.25	0.08	0.46	1.00
dhuB40 hisHAFIEgnd2327 purE801 trpA8[b]	1.00	<0.05	<0.05	0.55

[a] Growth rates are expressed as divisions per hour. At 0.1 mM L-histidine they were essentially the same as at 0.03 mM L-histidine.

[b] Media for these strains were supplemented with 0.1 mM L-tryptophan, 0.2 mM adenine, and 0.02 mM thiamine.

TABLE 2. *Bacterial mass yields after growth in the presence of* L- *or* D-*histidine of dhu his⁻ mutants*

Mutant	Bacterial mass yield[a]	
	L-Histidine[b]	D-Histidine[b]
hisAF645	12.0	<0.05
dhuA1 hisAF645	8.4	8.5
hisHAFIEgnd2327 purE801 trpA8[c]	12.0	<0.05
dhuA4 hisHAFIEgnd2327 purE801 trpA8[c]	9.0	10.0
hisDCBHAFI52	17.4	<0.05
dhuB11 hisDCBHAFI52	17.0	8.5
dhuB12 hisDCBHAFI52	11.4	16.2
hisF41	13.0	<0.05
dhuA16 hisF41	12.5	13.5
hisBH22	17.5	<0.05
dhuA22 hisBH22	16.8	15.0
dhuB33 hisBH22	11.4	12.8

[a] Saline-washed broth culture bacteria were used at an initial cell density of 8 μg (dry weight) per ml. After 16 and 20 hr at 37 C, the optical density at 420 nm was measured to assure that growth was stopped and that contaminating organisms were not present. Bacterial mass yield was corrected for that present in control cultures without histidine. It is expressed as milligrams (dry weight of cells) per micromole of histidine.

[b] Histidine was present at 0.03 mM concentration.

[c] Media for growing these strains were supplemented with 0.1 mM L-tryptophan, 0.2 mM adenine, and 0.02 mM thiamine.

barely detectable. At 0.1 mM D-histidine (Table 1), they grew at rates not lower than 25% of the rate at the same L-histidine concentration.

The cell mass yield per micromole of D-histidine was slightly lower than that with L-histidine (Table 2). This could simply mean that after 24 hr of incubation the uptake process was still going on in the D-histidine cultures at extremely slow rates. Cell mass yields per micromole of L-histidine were slightly higher in the original histidine auxotrophs than in their *dhu* derivatives. The significance of this observation has not been established.

The ability of the *dhu his⁻* strains to utilize other L-histidine analogues was examined by spot tests. One micromole of imidazolepyruvate supported growth of all *dhuA his⁻* and a majority of *dhuB his⁻* mutants but not the parental strains (Fig. 1). When greater amounts of imidazolepyruvate were applied, growth was visible even with *dhu⁺ his⁻* strains, but the diameters of the growth zones were consistently smaller than with *dhu his⁻* mutants. L-Imidazolelactate was not utilized as a source of histidine by any of these strains.

A total of 60 spontaneous *dhu* mutants isolated on D-histidine plates were studied. Only a fraction of them grew well enough on these plates to give normal-sized colonies after 2 days of incubation. Almost all of these appeared to be of the *dhuA* type when tested genetically.

Uptake of D-histidine by dhu mutants. Two procedures for following D-histidine uptake were used. One procedure consists of incorporation of exogenous amino acid into cell proteins of growing bacteria (2). The principle of the procedure requires that the growth rate during assay must not depend on substrate concentration. Therefore, *dhu his⁻* mutants were first tranduced to histidine prototrophy. The mutant *hisAF645* transduced to histidine independence with phage grown on strain LT-2 was used as a wild-type control.

Figure 2 shows the results of an experiment in which the protein incorporation procedure was used to assay uptake of radioactive D-histidine. Though radioactivity was rapidly incorporated into cells of all these strains during the first few minutes of incubation, this radioactivity could be accounted for by contaminating radioactive L-histidine. The *dhuA1* mutant was able to take up considerable amounts of D-histidine and incorporate it into trichloroacetic acid-insoluble material. The wild-type strain was much less active in this respect: after 2 hr of growth in the presence of radioactive D-histidine, it contained about one-tenth of radioactivity as compared with the *dhuA1* mutant. The three *hisP* mutants, which are defective in uptake of L-histidine, had the lowest ability to take up D-histidine from the medium. It seems probable that all the radioactivity in *hisP* cells can be accounted for by contaminating L-histidine. There was no difference between the *hisP1661* (*dhu⁺*) strain and the two double *dhuA1 hisP* mutants. This indicates that the *hisP* mutations completely nullify the in-

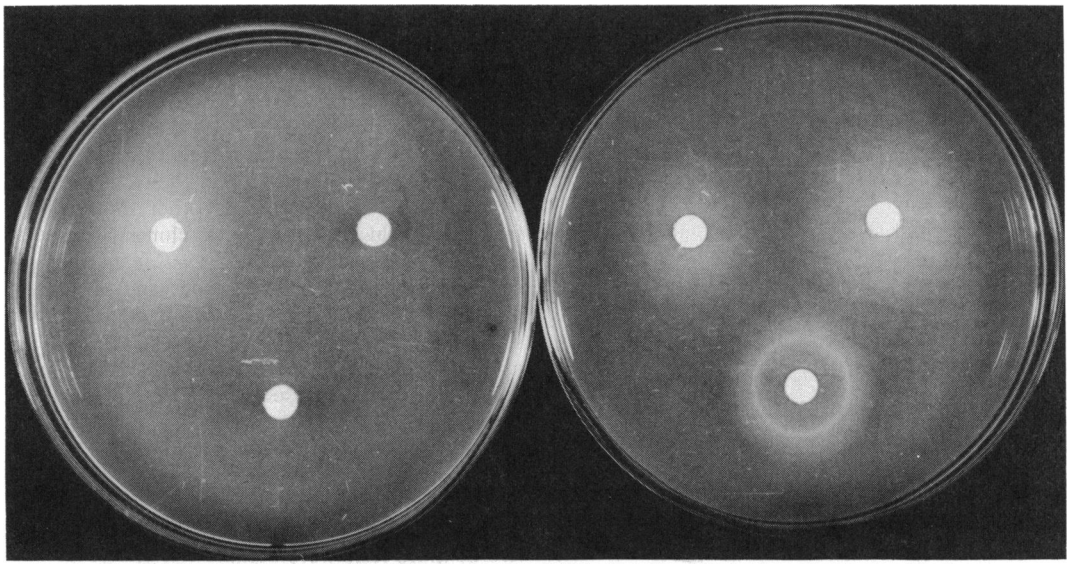

FIG. 1. *Spot tests for the utilization of* L-*histidine,* D-*histidine and imidazolepyruvate. The bacteria: hisAF645 (left) and dhuA1 hisAF645 (right) were layered in 0.6% agar on minimal Vogel-Bonner medium with 2% glucose. The paper discs contained: 0.1 μmole of* L-*histidine (upper left), 0.1 μmole of* D-*histidine (upper right), and 1 μmole of sodium imidazolepyruvate, pH 7, (lower).*

creased ability of the *dhuA1* mutant to take up D-histidine.

An apparent Michaelis constant of 20 μM for D-histidine uptake by growing *dhuA1* cells was calculated from the experiment shown in Fig. 3.

Uptake of L-histidine by dhu mutants. Two assay procedures of G. F. Ames (2) for L-histidine transport velocity were used in these experiments. Besides the protein incorporation procedure, the assay method with energy-starved cells was used.

Table 3 shows a summary of L-histidine transport activities in various *dhu* mutants estimated by the protein incorporation procedure. All *dhuA* and some *dhuB* mutants have L-histidine permease activity elevated by a factor of 2 to 3.

An attempt was made to compare K_m values for wild-type strains and *dhu* mutants. This was complicated by the fact that Lineweaver-Burk plots obtained by the protein incorporation procedure give two-component curves reflecting saturation of cellular protein biosynthesis by exogenous L-histidine (2).

Figure 4 presents Lineweaver-Burk plots for two *dhu* mutants and their isogenic wild-type strain. All K_m values fall in the range 0.02 to 0.04 μM. In other *dhu* mutants with higher specific activity of histidine permease, protein synthesis can be saturated with radioactive L-histidine at lower exogenous L-histidine concentration. This affects the slope of the Lineweaver-Burk plot in the sense of apparently lower K_m value. Average K_m values are presented in Table 4. The wild-type strains had a K_m value of 0.04 μM. In the *dhu* mutants it was about 0.02 μM. These data showing only a twofold difference argue against a concept of a qualitative change after *dhu* mutation.

This conclusion was checked with the use of the other assay procedure for histidine permease activity. Figure 5 presents the time course of L-histidine uptake by starved cells. Cells of the mutant *dhuA1 hisAF645* transported L-histidine at a faster rate than did *dhu*$^+$ *hisAF645* cells. In addition, the *dhuA1* mutant was apparently able to attain a much higher intracellular concentration of L-histidine.

Figure 6 presents Lineweaver-Burk plots obtained by using the starved cells assay. Data are plotted for several *dhu hisBH22* mutants. The specific activity of L-histidine uptake at any substrate concentration was higher by factors of 5 to 7 in *dhuA* mutants than in the parental *dhu*$^+$ strain. The Michaelis constant of L-histidine permease in the original strain was 0.4 μM, and those of the *dhu* mutants were from 0.3 to 0.6 μM.

The increased specific activity of L-histidine transport without significant change of K_m value argues against the possibility of changed specificity of histidine permease in *dhuA* mutants. If this were the case, decreased affinity to the natural histidine stereoisomer should have been found. Rather, these results support an alternative idea,

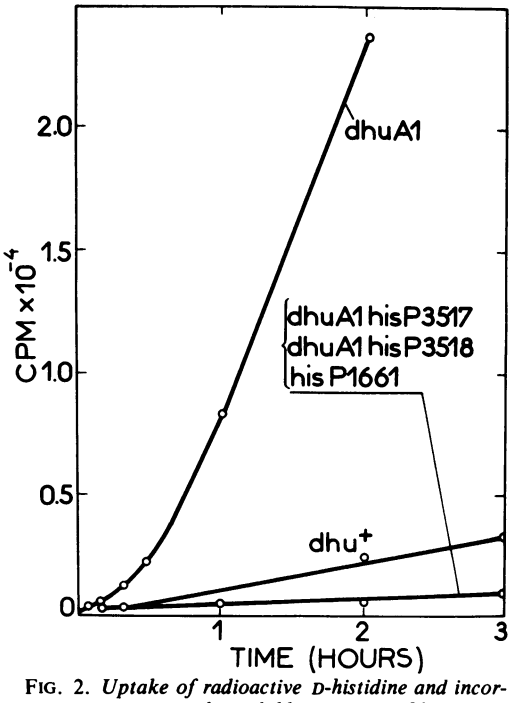

FIG. 2. *Uptake of radioactive* D-*histidine and incorporation into material insoluble in 5% trichloroacetic acid by wild-type (dhu⁺) bacteria, a* D-*histidine fast-growing mutant (dhuA1), and by three strains with genetic lesions in histidine permease (hisP).*

namely, that the *dhu* character relies on an increased specific activity of a permease component which has affinity for both histidine isomers.

To examine this further, experiments were done in which the effect of one unlabeled histidine stereoisomer upon the uptake of the other, radioactive, stereoisomer was studied. In these studies the protein incorporation assay procedure was used. At equal concentrations of each amino acid, the uptake of D-histidine in strain *dhuA1 his⁺* was completely inhibited by L-histidine. At 0.05 μM L-histidine, D-histidine present at 100-fold excess inhibited the uptake of the natural stereoisomer by 57%. These results are compatible with the concept that the two stereoisomers compete with each other for the same active site of a permease component.

The competitive character of D-histidine inhibition of L-histidine uptake is shown in Fig. 7. The K_m value in this experiment was 0.04 μM. The K_i value for D-histidine was calculated to be 20 μM.

Genetic mapping of dhuA mutations. If a change in L-histidine permeation allows *S. typhimurium* to utilize D-histidine, at least some *dhu* mutants should map in or near the *hisP* locus. This locus has been shown to code for components of histidine permease and is cotransducible with the *purF* gene (3, 4, 9). To test this point, the mutant *purF145 hisA3500* was made by diethylsulfate mutagenesis of *purF145* and was used as recipient in crosses on L-histidine-containing minimal agar plates with phage grown on *dhu* donors. The *pur⁺ hisA3500* transductants were then scored for the *dhu* character. In crosses with some *dhu* donors, about 50% of the transductants were *dhu hisA3500*, whereas other *dhu* donors failed to yield any *pur⁺ dhu* clones. The *dhu* mutations cotransducible with *purF* (e.g., *dhu-1*, *-4*, *-5*, *-13*, *-16*, *-21*, *-22*, *-31*, *-45*, and *-47*) were named *dhuA*. About one-fourth of all randomly isolated *dhu* mutants belong to this class. The majority, named for convenience *dhuB*, could constitute several classes with different localizations.

The value of 50% cotransduction was close to that reported for *hisP-purF* linkage (4). To put the *dhuA* marker into the proper place among the genes of this region (*hisP purF hisT aroD*), two or more tests were made.

The first used *aroD5 hisT1529 hisC2326* as recipient. Some 1 to 3% of the *aro⁺* transductants were *dhuA*. Linkage of *hisP* with *aroD5* is about 0.3% (4). This result indicates that the gene order is *aroD hisT purF* (*dhuA hisP*) and that *dhuA* is very closely linked to *hisP*.

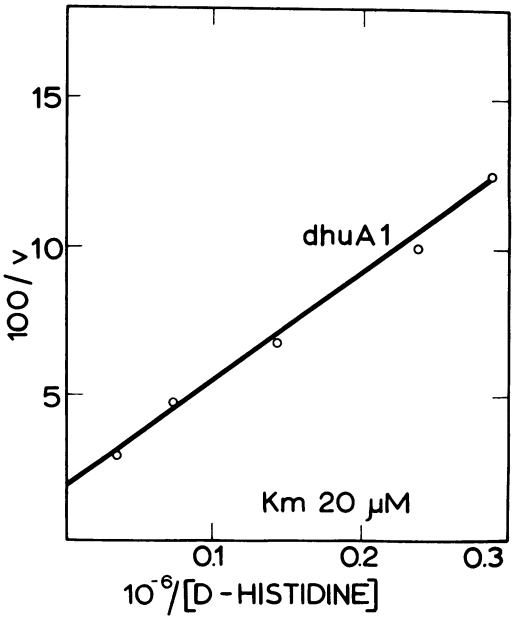

FIG. 3. *Lineweaver-Burk plot of* D-*histidine uptake. The protein incorporation procedure was used. The* D-*histidine concentrations are given in moles per liter. Reaction velocity (v) is expressed in counts per minute per microgram (dry weight of cells) per 10 min of incubation. A* D-*histidine-utilizing mutant (dhuA1) was used.* D-*histidine uptake by wild-type (dhu⁺) strains was too low to obtain data.*

TABLE 3. *Histidine permease specific activity as assayed by protein incorporation procedure*

Strain	No. of assays	Histidine permease[a]
Wild type[b]	17	0.17
dhuA1	4	0.50
dhuA4 purE801 trpA8[c]	2	0.43
dhuA5 hisT1504	2	0.53
dhuA21	3	0.43
dhuA47	2	0.29
dhuB20[d]	4	0.38
dhuB24[d]	3	0.27
dhuB49[d]	2	0.28
dhuB23[e]	2	0.18
dhuB25[e]	2	0.13

[a] Specific activity is expressed as picomoles of L-histidine taken up per microgram (dry weight of cells) per minute at 0.02 μM L-histidine initial concentration.

[b] As required by the assay procedure, all strains were histidine prototrophs made by transduction. There were no significant differences in histidine permease specific activity among the parental strains of the *dhu* mutants used. Therefore, their specific activities were averaged.

[c] Assay done in the presence of adenine, thiamine, and L-tryptophan.

[d] Derived from the best D-histidine utilizers among *dhuB his*⁻ strains.

[e] Poor D-histidine utilizer.

The second experiment aimed to more precisely localize the *dhuA* marker involved a three-point transduction test. The same recipient strain *purF145 hisA3500* was used. A *hisP* mutation was introduced into the donor *dhuA1 his*⁺ by selecting an NTG-induced mutant resistant to hydrazinoimidazolyl propionate. Phage grown on the resulting strain *dhuA1 hisP3561* (TK 549) was used as donor. The cross was made on L-histidine plates. Among the *pur*⁺ *hisA3500* transductants, only 5%, instead of 50%, grew on D-histidine plates, since all *dhuA1 hisP3561 hisA3500* recombinants were unable to utilize D-histidine. A total of 278 *pur*⁺ *hisA3500* recombinants obtained from L-histidine plates were scored by replica plating for their content of the unselected *dhuA* and *hisP* mutations. Recombinants printing on D-histidine plates were *dhuA1 hisP*⁺ *hisA3500*. Those which did not print on plates containing 0.01 mM L-histidine plus 2 mM L-tryptophan were *hisP3561 hisA3500* with *dhu*⁺ or *dhuA1*. These 104 recombinants were streaked on broth agar plates, and single colonies were picked from each to inoculate broth liquid medium. The cultures were used as recipients for crosses on D-histidine plates with phage grown on the strain *dhuA*⁺ *hisP*⁺ *purF145 hisA3500*. The appearance of colonies able to utilize D-histidine indicated that the given recipient harbored the *dhuA1* mutation. Of the 104 recombinants, only two appeared to be *dhuA*⁺. The remaining class of recombinants, *dhuA*⁺ *hisP*⁺ *hisA3500*, was unequivocally scored as those which did not grow on D-histidine but grew on plates containing 0.01 mM L-histidine plus 2 mM L-tryptophan.

The result of the three-point cross (Table 5) shows that the rarest recombinant class was *hisP dhuA*⁺. It would have resulted from quadruple crossovers if the mutation order were *hisP3561 dhuA1 purF145*. The cotransducibility values with *purF145* calculated from these data were: for *dhuA1*, 41.0%, and for *hisP3561*, 37.4%. They are in agreement with the order deduced from the rarest recombinant class.

Relationship between dhuA and hisP loci and functions. The preceding evidence indicates that

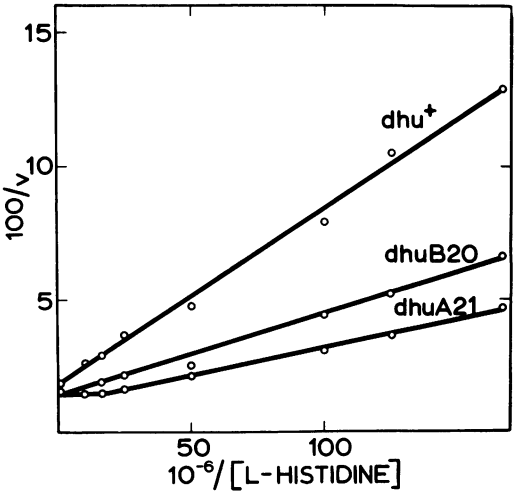

FIG. 4. *Lineweaver-Burk plots of L-histidine uptake by wild-type (dhu⁺) strain and by two D-histidine-utilizing mutants, dhuB20 and dhuA21. All these strains were derived from the mutant hisHA134. The protein incorporation procedure was used. The L-histidine concentrations are given in moles per liter. Reaction velocity is expressed in counts per minute per micrograms (dry weight of cells) per minute of incubation.*

TABLE 4. *Michaelis constant values of histidine permease as estimated by protein incorporation assay procedure*

Strain	No. of estimates	Michaelis constant (μM)
Wild type	6	0.040
dhuA1	7	0.018
dhuA4 purE801 trpA8	1	0.015
dhuA21	1	0.020
dhuB20	2	0.021

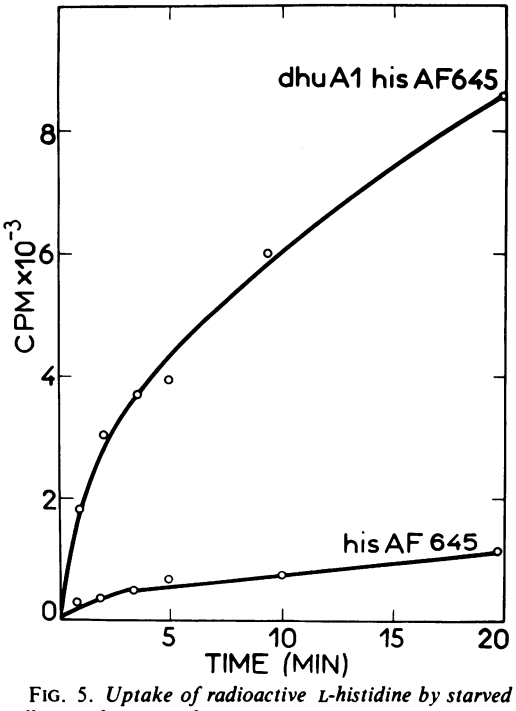

FIG. 5. *Uptake of radioactive* L-*histidine by starved cells as a function of time.*

the introduction of *hisP* mutations prevented a *dhuA* mutation from expressing its phenotype.

To pursue this further, *dhu* mutants were sought in strain *hisP1661 hisBH22* (TA 242). No spontaneous or mutagen-induced mutants able to grow on D-histidine plates could be found. This result indicates that all classes of *dhu* mutants require *hisP*$^+$ function. At the same time, it rules out mutational changes in other permease systems as a mechanism of D-histidine utilization by any *dhu*$^-$ mutants including *dhuB*.

It was not clear whether the *dhuA* mutations map in a locus which is separate from the *hisP* locus or, alternatively, whether mutations causing the *dhuA* phenotype are in the same locus as *hisP* mutations. The latter alternative would argue against the conclusion stemming from biochemical experiments that the *dhu* character results from increased production and not from changed specificity of histidine permease.

Therefore, we attempted to isolate additional double *dhu hisP* mutants. Two strains were used in this experiment: *dhuA1 hisAF645* (TK 51) and *dhuA4 hisHAFIEgnd2327 purE801 trpA8* (TK 54). Mutants of these strains were sought which had lost the ability to utilize D-histidine. Cells mutagenized with diethylsulfate were subjected to penicillin selection in the presence of D-histidine (also supplemented with adenine and tryptophan for TK 54). Mutants unable to grow on D-histidine plates were isolated. They were crossed with one another by transduction on D-histidine plates. Thirteen of those which recombined with at least one other mutant were retained and studied in more detail. Upon transduction on D-histidine plates with phage grown on the strain *dhu*$^+$ *his-AF645*, each of them gave some *dhuA his*$^-$ colonies among the recombinants. This indicated that none was a true *dhuA*$^+$ revertant. When they were used as donors in transductions with a *purF145 hisA3500* recipient on L-histidine plates, only 5% of the *purF*$^+$ *hisA3500* recombinants were able to grow on D-histidine plates. On the basis of this result, all 13 apparent *dhuA* revertants were classified as *dhuA hisP* double mutants. Their locations in respect to the *dhuA* mutations were similar to one another and to the gene order of *dhuA1* and *hisP3561* described above. If any one of these *hisP* mutations were localized between *dhuA* and *purF*, four crossovers would be required to give a recombinant able to grow on D-histidine plates. As can be deduced from the data in Table 5, the frequency of such recombinants in the case of reversed positions of *hisP* and *dhuA* mutations should be less

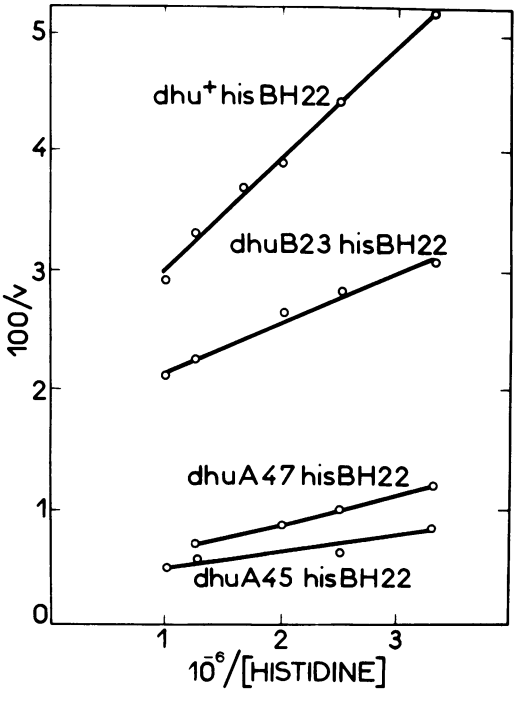

FIG. 6. *Lineweaver-Burk plots of histidine permease activity. Bacteria were starved in medium lacking any assimilable carbon and energy source and incubated afterwards with radioactive* L-*histidine as described by G. F. Ames (2).* L-*Histidine concentration and reaction velocity are expressed as in the Fig. 3 and 4. Cells were incubated with substrate for 2 min.*

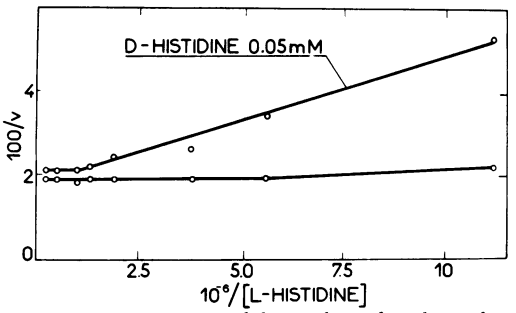

FIG. 7. *Competitive inhibition by* D-*histidine of* L-*histidine uptake. Strain dhuA1 and the protein incorporation assay procedure were used.* D-*Histidine was added at 0.05 mM concentration 10 min before adding radioactive* L-*histidine to minimize errors which could result from contamination of* D-*histidine with* L-*histidine.* L-*Histidine concentrations are expressed as in Fig. 4. Reaction velocity is expressed in counts per minute per microgram (dry weight of cells) per 2 min of incubation with* L-*histidine. Michaelis constant is 0.04 μM (Table 4 and text). K_i for* D-*histidine is 0.02 mM.*

than 1%. This result indicates that there is no overlapping of the chromosome segments in which *dhuA* and *hisP* mutations occur.

It has been reported that histidine is transported in *S. typhimurium* by at least two permeases (4). The permease most specific for L-histidine is that coded for by the *hisP* locus. The aromatic permease has a high affinity for L-tryptophan, L-tyrosine, and L-phenylalanine and a low affinity for L-histidine. Mutants completely lacking the specific histidine permease are unable to grow on plates containing low levels of L-histidine and high levels of L-tryptophan (4). Some of our *dhuA1 hisP hisAF645* mutants are able to grow on these plates. Unlike the tight *hisP* mutants, the tryptophan-resistant *dhuA hisP* mutants have a histidine permease with a K_m of 0.02 μM, but its activity is greatly reduced. Because tight *dhuA hisP* mutants completely lack activity of this histidine permease, the leaky *dhuA hisP* mutants could have missense mutations in *hisP* or polar mutations affecting histidine permease activity. Mapping of these mutations has not yet been attempted.

Isolation of dhu mutants in the absence of D-histidine. It has been found that the specific histidine permease is sensitive to L-arginine (G. F. Ames, *personal communication*).

We reasoned that, on plates containing low L-histidine and high L-arginine and L-tryptophan concentrations, only mutants derepressed or altered in either histidine or aromatic permease activity would be able to grow.

We first showed that the only *his*$^-$ strains able to grow on these plates were those with *dhuA* or some *dhuB* mutations. This ability to overcome the inhibitions caused by L-arginine and L-tryptophan supported the idea that *dhu* mutants have derepressed activity of histidine permease.

Further evidence for this idea was obtained by isolation of *dhu* mutants on plates which contained low L-histidine and high L-arginine and L-tryptophan concentrations. The strain *hisBH22* was spread on these plates, and a few crystals of NTG were put in the center. After 4 days of incubation, there was only residual growth except for a circle of small colonies at a distance around the mutagen application point. Eight colonies were picked, and single colonies were isolated three successive times on unselective broth agar plates. Five of the eight strains tested were able to grow on D-histidine agar plates. By genetic crosses with *purF145 hisCBHAFIE3501* recipient, it was found that four were *dhuA* (*-71*, *-72*, *-73*, and *-74*) and the fifth was classified as *dhuB75*.

This result suggests that the *dhuA* gene and the gene in which mutation *dhuB75* occurred may regulate the synthesis of the arginine-sensitive component of the histidine transport system.

It appears that a genetic change near the gene for the histidine-specific permease causes the formation of a permease system which is both resistant to arginine inhibition and able to utilize D-histidine. A derepression of the histidine permease is the simplest common denominator of the two characters.

DISCUSSION

This study confirms and extends the observation made by B. N. Ames (*personal communication*) that histidine auxotrophs of *S. typhimurium* can acquire, by mutation, the ability to utilize D-histidine for growth.

At the beginning of this study, two concepts

TABLE 5. *Genetic mapping of dhuA by three-point cross*[a]

Nonselected markers		No. of cross-overs[b]			No. of recombinants	Per cent of recombinants
dhuA	*hisP*	A	B	C		
+	+	2	2	2	162	58.3
+	3561	2	2	4	2	0.7
1	+	2	4	2	12	4.3
1	3561	2	2	2	102	36.7

[a] In the transduction cross, *purF*$^+$ was the selective marker. The cross was made with donor phage grown on strain *dhuA1 hisP3561* and recipient cells of strain *hisA3500 purF145*. The cross was performed on minimal agar plates supplemented with L-histidine.

[b] Designations of possible gene order are: A, *dhuA purF hisP;* B, *dhuA hisP purF;* C, *hisP dhuA purF.*

were considered to explain D-histidine utilization by *dhu* mutants. The first was that the *dhu* mutations bring about an ability to racemize D-histidine. However, we have not found any difference in the specific activity of histidine racemization enzymes found in wild type and the *dhu* mutants tested (*unpublished data*).

The second possibility assumed that the acquired ability to utilize D-histidine was due to changed specificity of a permease. The L-histidine-specific permease appeared as the most probable site of such a change. It was expected that this would result in a loss of permease affinity for its normal substrate, L-histidine. Such a loss should be detectable as an increase in the Michaelis constant, and the mutations should map in structural genes of the permease components.

The finding that the best utilizers of D-histidine mapped very close to the locus coding for histidine permease (*hisP*) supported the permease idea. The values found in transductional three-point tests for *purF* linkage with *dhuA* and *hisP* mutations were 41.0 and 37.4%, respectively. This suggested that the two mutations could be within one gene length of each other. However, the specific activity of histidine permease with the L-stereoisomer as substrate was increased in all *dhuA* mutants studied; a result contrary to that had been expected on the basis of the concept of changed specificity.

The results of genetic mapping and histidine permease activity determinations suggest that a derepression of histidine permease enables *S. typhimurium* to take up exogenous D-histidine faster and concentrate it within cells to a higher level. It has been reported that *gltC* mutants of *Escherichia coli* which had derepressed levels of glutamate permease were able to take up the amino acid from the medium at faster rates, and were also able to attain higher intracellular concentrations (5). We assume that an elevated intracellular concentration of D-histidine is necessary for the racemization process to produce L-histidine at rates adequate to its requirement in protein synthesis.

The assumption that histidine permease did not undergo any qualitative change has been supported by the fact that apparent Michaelis constant value of histidine permease was virtually the same in *dhuA* mutants and the original strains, whatever the assay procedure.

The evidence that D- and L-histidine are transported by the same permease comes from a number of experiments. Competition experiments have shown that, at equimolar concentrations of both stereoisomers, uptake of the D-stereoisomer was completely blocked. When D-histidine was present at a 100-fold excess, uptake of L-histidine was inhibited by about 60%.

Further evidence supporting the conclusion that both isomers of histidine are transported by the same permease comes from genetic experiments. The *hisP* mutations produce a disappearance of the histidine-specific permease (i.e., component curve in Lineweaver-Burk plot with lowest Michaelis constant for L-histidine) or reduction of its V_{max} value. They also nullify the uptake of D-histidine and the ability to utilize D-histidine for growth of cells carrying a *dhu* mutation. The simplest explanation for this pleiotropic effect is that all these abilities depend on at least one common protein.

The derepression of histidine permease in *dhuA* and some *dhuB* mutants suggests that a genetically controlled system of regulating this permease exists in *S. typhimurium*. Because of the close proximity of *dhuA* and *hisP* loci, they could constitute one operon with the *dhuA* gene as an operator.

Support for this concept was provided by Ames and Lever (3). They found that the *dhuA1* mutant has a fivefold increase in the activity of the histidine-binding protein *J*. The protein, in addition to the *hisP* product, is a component of the histidine transport system. The *hisJ* gene is linked to *dhuA* and *hisP*, but the gene order is not known.

The three *dhuB* mutants (*-20*, *-24*, and *-49*) which do not map at *hisP*, but do have elevated histidine permease activity, may have changed functions not related to the operator region. This suggests the possibility of the existence of unlinked regulatory gene(s) for the *dhuA-hisJ-hisP* functional unit.

There are only a few reported cases of regulation of amino acid permease systems. Leucine-binding protein, an apparent component of a leucine permease of *E. coli*, undergoes repression when the cells are grown in the presence of leucine (7). The glutamate permease regulatory system of *E. coli* consists of three genes: the operator and structural genes are linked together and are separated from a regulator gene (6). Derepression of glutamate permease activity enables *E. coli* mutants to use L-glutamate as a sole source of carbon. As with *dhu* mutations, a quantitative change in glutamate permease activity confers an apparently qualitative change in bacterial physiology.

We have been unable to detect induction or repression of the histidine permease by growing bacteria in the presence or absence of L- or D-histidine. In addition, a *hisS* mutation, known to decrease the concentration of histidyl-transfer ribonucleic acid and cause derepression of the histidine operon (8), had no effect on histidine

permease activity (*unpublished data*). The failure of these attempts does not rule out the possibility that regulation of the histidine permease exists. It is also possible that *dhu* mutations affect a promoter, and there may not be a means of physiologically regulating the uptake system.

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