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Histidine Regulatory Mutants in *Salmonella typhimurium*

II. Histidine Regulatory Mutants having Altered Histidyl-tRNA Synthetase

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(1) The *hisS* gene of *Salmonella typhimurium* is the structural gene for histidyl-tRNA synthetase. Mutants in the *hisS* gene have an altered histidyl-tRNA synthetase and constitute one of the four classes of histidine regulatory mutants.

(2) Mutant *hisS1520* has a synthetase with a 50-fold decreased affinity for histidine. This mutant has a markedly decreased growth rate on minimal medium and is de-repressed for the biosynthetic enzymes of the histidine operon. Both of these properties appear to reflect the decreased amount of charged histidine-specific tRNA due to the mutant synthetase. The amount of histidyl-tRNA can be increased in this mutant by swelling its internal pool of histidine, and this swelling results in restoration of the normal growth rate and repression of the operon. These results support the idea that histidyl-tRNA is more directly connected with the repression of the histidine operon than histidine itself.

(3) Levels of the histidyl-tRNA synthetase are unaffected by de-repression of the histidine operon.

1. Introduction

Mutations in the *hisS* gene, which maps far from the histidine operon on the *Salmonella typhimurium* chromosome, result in high levels of the histidine biosynthetic enzymes. This de-repression of the histidine operon is caused by a reduction in the activity of histidyl transfer-RNA synthetase (Roth, Antón & Hartman, 1966). The work to be reported here shows that the *hisS* gene is the structural gene for the histidyl transfer-RNA synthetase, since *hisS* mutants have synthetases with altered catalytic properties. One such mutant studied in detail was found to have a synthetase with greatly decreased affinity for histidine. The results lead us to suggest that charged histidine transfer-RNA is concerned more directly with repression than free histidine.

2. Materials and Methods

(a) *Bacterial strains*

Mutant *hisS1520* was obtained in *S. typhimurium* strain LT-2 by selection for resistance to triazolealanine plus aminotriazole (Roth *et al.*, 1966*a*). A mutation in the histidine-specific permease (*hisP1656*) was introduced into *hisS1520* by selection for resistance to

α-hydrazinoimidazole propionic acid (Shifrin, Ames & FerroLuzzi-Ames, 1966). A feedback-resistant derivative of *hisS1520*, the double mutant *hisS1520 hisG1924*, was selected as a thiazolealanine-resistant mutant (Sheppard, 1964). Mutant *hisS1210* is described in Roth *et al.*, 1966*a*).

(b) *Growth of cells and preparation of extracts*

Bacteria were grown in the 300-l. fermenter by D. Rogerson at the National Institutes of Health. Wild-type cells and *hisS1210* cells were grown on minimal-salts medium (Vogel & Bonner, 1956) with 0·5% glucose. Mutant *hisS1520* was grown on this medium supplemented with 0·1 mM-histidine. Cells were suspended and sonicated (Silbert, Fink & Ames, 1966) in buffer containing the following: 40 mM-Tris–HCl (pH 7·5); 0·1 M-NaCl; 1·4 mM-β-mercaptoethanol; 0·5 mM-EDTA. After sonication, extracts were centrifuged at 30,000 *g* for 50 min to remove cell debris.

(c) *Enzyme assays*

Histidyl-tRNA† synthetase was assayed in two ways; by the complete transfer of [^{14}C]histidine to tRNA, and by the histidine-dependent exchange of [^{32}P]pyrophosphate into ATP. The conditions of aminoacyl-tRNA formation were as previously described (Roth *et al.*, 1966*a*) with the addition of 4 units (0·5 μg) or inorganic pyrophosphatase (Worthington) per assay. Transfer RNA was prepared from *S. typhimurium* using the method of Holley *et al.* (1961), as modified by Silbert *et al.* (1966).

The histidine-dependent [^{32}P]pyrophosphate–ATP exchange was assayed in a 0·35-ml. reaction mixture containing the following: 40 μmoles Tris–HCl buffer (pH 8·0); 2 μmoles $MgCl_2$; 1 μmole ATP; 1·4 μmole, β-mercaptoethanol; 1 μmole [^{32}P]sodium pyrophosphate (100 to 200 cts/min/mμmole). The histidine concentration is indicated in the text. Reactions were started by the addition of enzyme and terminated by the addition of 12 mg of charcoal (Norit A) in 0·5 ml. of 10% trichloroacetic acid. The charcoal was collected on a membrane filter (Millipore Filter Co.), washed with 5 ml. of 5% trichloroacetic acid and 80 ml. of water. Filters were dried, cemented to planchets upside down, and radioactivity assayed in a gas-flow thin-window counter.

The specific activity of histidinol phosphate phosphatase was assayed as an index of the degree of de-repression of the histidine operon. The toluenized-cell assay (Ames, Hartman & Jacob, 1963) was used. (The assay description has 0·22 ml. of substrate; this is a misprint for 0·02 ml.)

(d) *Column chromatography*

Crude extracts were fractionated on diethylaminoethyl-cellulose 70 (Schleicher and Schuell, Inc.) using a 400-ml. linear salt gradient from 0·05 to 0·5 M-NaCl made up in the buffer described for the sonication, except that the concentration of Tris–HCl was reduced to 20 mM and the initial NaCl concentration was 0·05 M. Approximately 5 mg of protein/1 ml. of column volume were applied and elution proceeded at 0·5 ml./min.

(e) *Chemicals*

[^{14}C]L-Histidine (35 mC/m-mole) was obtained from the Nuclear-Chicago Corp., Des Plaines, Illinois. [^{14}C]L-Histidine (200 mC/m-mole) was obtained from the New England Nuclear Corp., Boston, Massachusetts. Unlabeled L-histidine was obtained from the Sigma Chemical Company. The histidine analogues, thiazolealanine and triazolealanine, were obtained through the courtesy of R. G. Jones, Eli Lilly Company. The herbicide 3-amino-1,2,4-triazole (Amitrole) was obtained from the Aldrich Chemical Company. The α-hydrazinoimidazolepropionate was obtained through the courtesy of Merck and Company.

3. Results

In the assay previously used for histidyl-tRNA synthetase (transfer of [^{14}C]histidine to tRNA) the *hisS* mutants examined had about 10% of the wild-type activity (Roth *et al.*, 1966*a*). These mutants did not have an imparied growth rate on minimal medium. In this investigation we have concentrated our attention on a new *hisS*

† Abbreviations used: tRNA, transfer RNA; DEAE-cellulose, diethylaminoethyl-cellulose.

mutant, *hisS1520*, which shows no detectable synthetase activity (less than 0·2% of the wild-type activity) in this same assay. The growth rate of the mutant on minimal medium is much lower than that of wild type, presumably because of the shortage of charged histidyl-tRNA for protein synthesis. It seemed probable that in order to grow, the mutant ought to have *some* histidyl-tRNA synthetase activity, and we have been able to show this by means of the [^{32}P]pyrophosphate exchange assay in which a much higher histidine concentration is used. The following experiments indicate that mutant *hisS1520* has an altered synthetase enzyme with a very low affinity for histidine.

(a) *Chromatography of histidyl-tRNA synthetase*

Histidyl-tRNA synthetase from the *hisS1520* mutant and from the wild type were eluted from DEAE-cellulose columns as single peaks at the same salt concentration. The elution patterns for the two activities are compared in Fig. 1. In the transfer

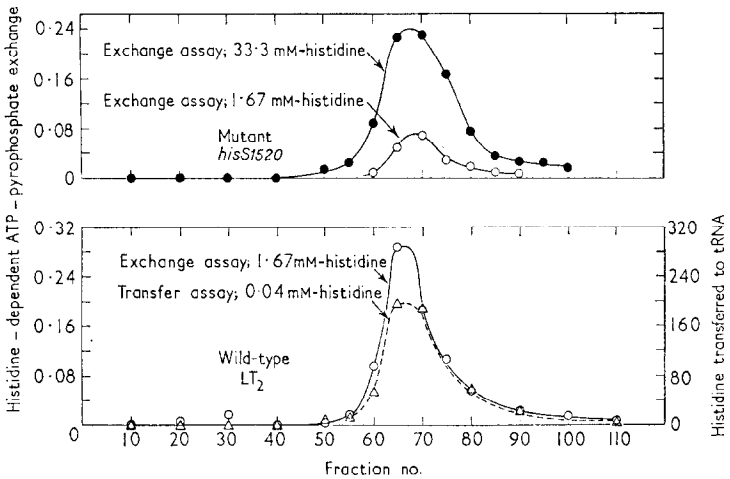

FIG. 1. DEAE-cellulose column chromatography of histidyl-tRNA synthetase from mutant *hisS1520* and wild type LT-2.

Protein is distributed from tubes 10 to 110. Pooling fractions 60 to 80 yields approximately fivefold purification with 80% recovery of activity. Exchange activity is presented as mμmoles [^{32}P]pyrophosphate exchanged per min by 1 μl. of fraction. Transfer activities are presented as cts/min [^{14}C]histidine (35 mc/m-mole) incorporated into tRNA per min by 1 μl. of fraction.

assay (0·04 mM-[^{14}C]histidine, — △ —— △ —), the wild-type enzyme shows a peak of activity, but the mutant enzyme showed no detectable activity (not shown in Figure), its peak tube having less than 0·2% of the wild-type activity. In the [^{32}P]pyrophosphate exchange assay at 1·67 mM-histidine (—○—○—), the mutant enzyme has a peak coinciding with the peak of the wild-type enzyme but of only about one-third the activity. In the exchange assay at high histidine concentration (33·3 mM, —●—●—) the mutant-enzyme peak is approximately the same as that of the wild type. The wild-type enzyme peak at 33·3 mM-histidine is not shown, but was the same as at 1·67 mM.

Because it was apparent that the mutant enzyme had a low affinity for histidine, an attempt was made to show some activity of the mutant enzyme also by the transfer assay. Upon increasing the [^{14}C]histidine concentration in the transfer assay

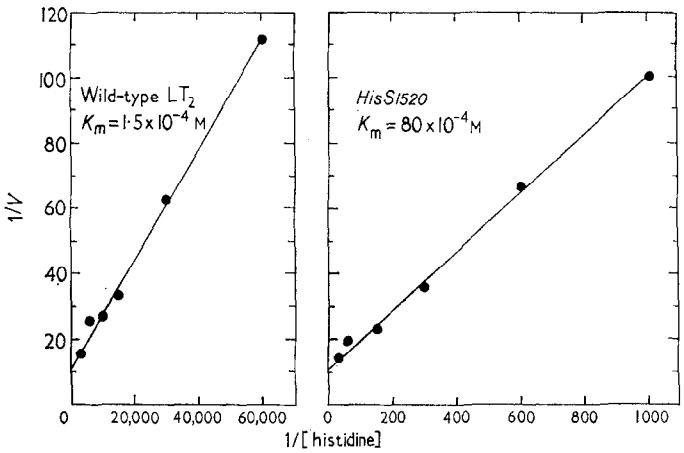

FIG. 2. Dependence of pyrophosphate exchange reaction on histidine concentration. Velocity (V) is mμmole [^{32}P]pyrophosphate exchanged per min. Enzyme used is from peak fractions of DEAE-cellulose columns; 11 μg of mutant protein and 14 μg of wild-type protein were used.

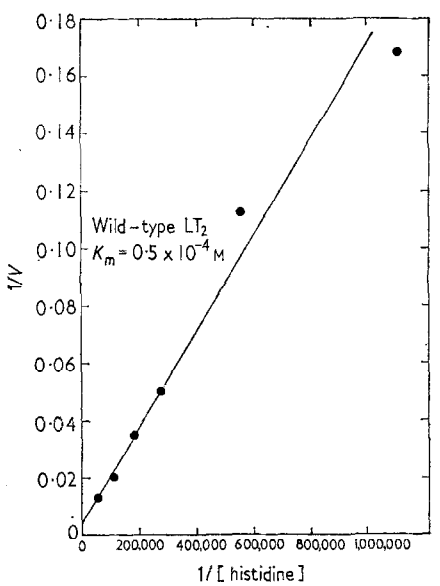

FIG. 3. Dependence of transfer activity on histidine concentration. Activity is cts/min [^{14}C]histidine (222 mc/m-mole) transferred per min. Enzyme is 7 μg of wild-type protein from peak DEAE-cellulose fraction.

20-fold, the peak tube of the mutant enzyme showed an activity 2·5% of that of the wild type. It was difficult to go higher in [^{14}C]histidine concentration due to technical reasons.

(b) *Affinity of the synthetase for histidine*

The affinity of histidine for the histidyl-tRNA synthetase from both wild type and the *hisS1520* mutant was measured (Fig. 2). Use of the pyrophosphate exchange assay showed a 50-fold difference between the K_m values for the two enzymes, the wild-type enzyme having a K_m of $1{\cdot}5\times10^{-4}$M and the mutant enzyme 80×10^{-4}M. Both enzymes showed about the same maximal reaction velocity.

The K_m value for histidine of the wild-type enzyme using the transfer assay is presented in Fig. 3. Technical difficulties were encountered in determining the K_m for histidine of the mutant enzyme using this assay, because of the high concentrations of [^{14}C]histidine required. However, the activity of mutant enzyme was found to increase linearly up to a concentration of 0·6 mM-histidine, suggesting that the K_m value for histidine is considerably greater than 6×10^{-4}M, or at least 12-fold greater than the K_m of wild-type enzyme.

Another *hisS* mutant, *hisS1210*, was also investigated. Histidyl-tRNA synthetase from this mutant had a K_m value for histidine of 26×10^{-4}M when assayed by pyrophosphate exchange. This value is intermediate between the values found for wild type ($K_m = 1{\cdot}5\times10^{-4}$ M) and *hisS1520* ($K_m = 80\times10^{-4}$ M) enzymes. Thus two mutations, *hisS1210* and *hisS1520*, both mapping in the same region of the chromosome, affect the K_m value for histidyl-tRNA synthetase to differing extents.

(c) *Role of histidine in repression and growth stimulation of* hisS *mutants*

Mutant *hisS1520* grows more slowly (doubling time 185 minutes) on minimal medium and its histidine biosynthetic enzymes are de-repressed 17 times more than wild type (Table 1). However, when excess histidine is added to the growth medium, the mutant has a normal doubling time (48 minutes) and repressed levels of the histidine biosynthetic enzymes (Table 1). The data in Table 1 show that addition of histidine overcomes the damaging effects of the *hisS* mutation.

Our interpretation of these findings is that both growth of *hisS1520* and repression of the histidine operon are limited by the supply of charged histidyl-tRNA, and that

TABLE 1

Growth and de-repression of hisS1520

Strain	Additions to minimal medium	Level of de-repression†	Doubling time (min)
LT-2 (wild type)	0	1·0	53
	0·5 mM-L-histidine	1·0	53
hisS1520	0	17·3	185
	0·05 mM-L-histidine	1·3	48
	0·5 mM-L-histidine	1·3	52

† De-repression is defined as the specific activity of histidinol phosphate phosphatase relative to the specific activity in repressed wild-type cells. (The wild type had an actual specific activity of 2·7 in the assay described in Ames *et al.*, 1963.)

addition of histidine allows increased synthesis of histidyl-tRNA by the mutant enzyme. A more detailed analysis is presented in the following sections.

(i) *The histidine pool and feedback inhibition*

The size of the histidine pool, in the wild type growing on minimal medium, is limited by feedback inhibition by histidine of the first enzyme of the pathway. The K_i value of that first enzyme (Martin, 1963) is about the same as the concentration of histidine in the amino acid pool of *Salmonella*, which is about $1{\cdot}5 \times 10^{-5}$ M (Ferro-Luzzi-Ames, 1964). This histidine pool can be increased in several ways: (i) addition of histidine to the minimal medium increases the pool about fivefold (FerroLuzzi-Ames, 1964); (ii) de-repression of the histidine biosynthetic enzymes in the various classes of histidine regulatory mutants, increases the histidine pool; thus several of the regulatory mutants tested appear to have a pool of histidine large enough to excrete histidine and feed a histidine-requiring mutant. *HisS1211*, for example, has a slight histidine excretion, is slightly resistant to the histidine analogue hydrazino-imidazole propionate (Shifrin, Ames & FerroLuzzi-Ames, 1966) and has a lower [^{14}C]histidine incorporation rate into protein than wild type, indicating dilution of the [^{14}C]histidine; (iii) mutants having a first enzyme insensitive to histidine feedback inhibition have a greatly increased histidine pool, as shown by their excretion of histidine into the growth medium (Sheppard, 1964).

Our interpretation of the data of Table 1 is that the slow growth of mutant *hisS1520* on minimal medium is due to the mutant synthetase ($K_m = 8 \times 10^{-3}$ M) which cannot work at the rate needed for a maximum growth rate with the histidine pool of the mutant. The external histidine can swell the pool sufficiently so that the synthetase can make enough histidyl-tRNA for maximum protein synthesis. It also appears that when the histidine tRNA is charged, then the histidine operon is repressed, and when it is not charged the operon is de-repressed.

(ii) *Increasing the histidine pool by endogenous histidine*

The partial block in the *hisS1520* mutant of histidyl-tRNA synthesis can be overcome also by an increased pool due to endogenously synthesized histidine in a feedback-resistant mutant. A double mutant was constructed which carries both the *hisS1520* mutation and a mutation conferring feedback resistance, *hisG1924*. This strain excretes histidine and thus presumably has an increased internal pool of endogenously synthesized histidine. Consequently, this strain has a repressed histidine operon and a normal doubling time even on minimal medium (Table 2).

(iii) *Entry of external histidine into the pool*

In *Salmonella*, histidine enters the cell by means of two permeases, a specific histidine permease ($K_m = 10^{-7}$ M), and a general aromatic permease ($K_m = 10^{-4}$ M for histidine) (FerroLuzzi-Ames, 1964). The specific histidine permease is controlled by the *hisP* gene (Shifrin *et al.*, 1966). In order to determine if it is necessary for external histidine to enter the cell through the specific permease in order to repress, a second double mutant was constructed which carries both the *hisS1520* lesion and a histidine-specific permease mutation, *hisP1656*. In this double-mutant strain, *hisS1520 hisP1656*, histidine enters the cell only *via* the general aromatic permease. As shown in Table 2, under these conditions exogenous histidine can still repress the histidine operon. When excess aromatic amino acids is added, preventing use of the

TABLE 2

De-repression of hisS1520*-derivative strains*

Strain	Additions to minimal medium	Level of de-repression†	Doubling time (min)
hisS1520 hisG1924 (feedback-resistant)	None	1·0	49
	0·05 mM-L-histidine	1·1	51
hisS1520 hisP1656 (permeaseless)	None	11·5	135
	0·05 mM-L-histidine	1·4	48
	0·05 mM-L-histidine 3·0 mM-phenylalanine 3·0 mM-tyrosine 3·0 mM-tryptophan	11·8	70
hisS1520 (parent strain)	None	17·3	185
	0·05 mM-L-histidine	1·3	48
	0·05 mM-L-histidine 3·0 mM-phenylalanine 3·0 mM-tyrosine 3·0 mM-tryptophan	1·3	52

† See footnote to Table 1.

aromatic permease, the exogenous histidine is no longer taken into the cell and de-repression results (Table 2).

(d) *Mapping of the* hisS *gene*

The mutation *hisS1520* is co-transducible (14%) with the *strB* locus (far removed from the histidine region of the *Salmonella* chromosome), as are six other *hisS* mutants that have been examined previously (Roth *et al.*, 1966*a*). Mutations in the *strB* locus confer low-level streptomycin resistance and simultaneous requirements for thiamine and nicotinic acid (Demerec *et al.*, 1960). Phage P22 grown on *hisS1520* can be used to transduce a *strB* mutant to prototrophy by selection for growth on minimal medium. The *hisS1520* mutation is received by 14% of the recombinants from such a cross. Furthermore, phage P22, grown on *hisS1520*, can transduce a normal histidine operon into long deletion mutations covering all of the histidine operon. Such recombinants fail to receive the growth and de-repression characteristics of the donor *hisS1520* strain. Thus the mutation *hisS1520* is not co-transducible with the structural genes for the histidine biosynthetic enzymes, but is co-transducible with the *strB* locus.

(e) *Properties of* hisS *mutants*

The *hisS* mutants we have isolated were obtained because they had high levels of histidine biosynthetic enzymes, which conferred resistance to the inhibitory selective agent, triazolealanine. We would not have isolated any *hisS* mutants which simultaneously required histidine, since there was no histidine on the selection plates. (Histidine counteracts the triazolealanine inhibition.)

Of seven *hisS* mutants isolated, only *hisS1520* has a very slow growth rate on minimal medium. However, two others, *hisS1210* and *hisS1209*, which have normal

doubling times, are also repressed by exogenous histidine. One of these, *hisS1210*, was further examined and found to have an altered synthetase the K_m value of which was intermediate between that of wild type and *hisS1520*. Mutants *hisS1211*, *1213*, *1219* and *1259* are only partially repressed by exogenous histidine, and we suspect that these mutants may have a synthetase which is altered in its affinity for tRNA or in its V_{max}.

(f) *Control of the histidyl-tRNA synthetase*

Synthesis of histidyl-tRNA synthetase is not regulated co-ordinately with the enzymes of the histidine operon. This conclusion stems from three lines of evidence. (1) Three classes of histidine regulatory mutants, *hisR*, *hisT* and *hisO*, have five- to tenfold de-repressed levels of the histidine biosynthetic enzymes, whereas the level of their histidyl-tRNA synthetase is unaffected (Roth *et al.*, 1966*a*). (2) Physiological de-repression of the histidine operon does not change the synthetase levels, as shown

TABLE 3

Constitutivity of histidyl-tRNA synthetase

Histidine requiring mutant	Growth supplement	Histidyl-tRNA synthetase		De-repression of histidine operon†
		Assay method	Specific activity	
hisH107	0·1 mM-histidine	[^{14}C]histidine transfer	0·25	1
	0·4 mM-adenine	[^{14}C]histidine transfer	0·25	18
hisE11	1·0 mM-histidine	[^{32}P]pyrophosphate exchange	0·84	1·5
	0·03 mM-formylhistidine	[^{32}P]pyrophosphate exchange	0·96	17

† See footnote to Table 1.

[^{14}C]Histidine transfer activity is expressed as mμmoles histidine transferred/mg protein/min. [^{32}P]Pyrophosphate exchange activity is expressed as mμmoles pyrophosphate exchanged/mg protein/15 min. All activities are measured in crude cell extracts.

The wild type has essentially the same specific activity as the mutants in both assay methods.

in Table 3. This conclusion had been previously reached by Ames & Garry (unpublished data, summarized by Ames & Hartman, 1962). Histidine-requiring mutants were grown under conditions of limiting histidine (Silbert *et al.*, 1966). Regardless of the extent of de-repression of the operon, the specific activity of histidyl-tRNA synthetase remained essentially unchanged. (3) The large deletion mutant *his63* carrying a deleted operator region has no detectable histidine biosynthetic enzymes and normal levels of synthetase. This suggests that no subunit of the synthetase is coded for by a gene in the histidine operon.

4. Discussion

(a) *Histidyl-tRNA is more directly concerned with repression than histidine*

The first clear evidence that an aminoacyl-tRNA is more directly involved in repression than the amino acid came from the work of Schlesinger & Magasanik

(1964). They showed that in *Aerobacter* and in *Escherichia coli*, the histidine biosynthetic enzymes were de-repressed and histidine accumulated upon addition of α-methyl histidine to the bacterial culture. Since this analogue inhibited the histidyl-tRNA synthetase, they explained their finding by suggesting that the pool of histidyl-tRNA and not the pool of free histidine is responsible for repression. This conclusion was strongly supported (for the enzymes of valine biosynthesis) by the work of Eidlic & Neidhardt (1965) on temperature-sensitive valyl-tRNA synthetase. The work on the *hisS* mutants previously reported (Roth, 1965; Roth *et al.*, 1966*b*; also discussed in Ames & Hartman, 1963; and Ames, 1965) and the present investigation give more direct support for this conclusion.

The *hisS* mutants studied were isolated as histidine regulatory mutants. The fact that a partial block in histidyl-tRNA synthesis can cause de-repression strongly suggests that this product is important in the regulation of the histidine operon. This conclusion is also reinforced by the finding that another type of regulatory mutation, *hisR*, affects in some way the synthesis of active histidine tRNA (Silbert *et al.*, 1966).

One can think of several models involving histidyl-tRNA in repression (Roth *et al.*, 1966*b*). One fairly simple model would involve a translational control, with histidyl-tRNA causing a block in translation initiation, by acting at the site where translation of the large histidine operon messenger RNA starts. Evidence for the unidirectional translation of the histidine operon message has been presented (Martin, Silbert, Smith & Whitfield, Jr., 1966; Ames & Hartman, 1963). Other models could envisage that the histidyl-tRNA synthetase is the aporepressor, and histidine its co-repressor, or that histidyl-tRNA is a precursor of some other substance which is the repressor.

The fact that mutations in the *hisS* locus cause production of a histidyl-tRNA synthetase with altered kinetic properties leads us to conclude that this locus is the structural gene for histidyl-tRNA synthetase. Recently, Yaniv, Jacob & Gros (1965) have mapped the structural gene for valine-tRNA synthetase of *E. coli*. It does not map near our *hisS* gene, assuming that the genetic maps of *Salmonella* and *E. coli* are homologous. The close linkage of the *hisS* gene to a selective marker makes it feasible to carry out fine-structure genetics of the activating enzyme locus. Such an analysis is facilitated by the fact that *hisS* mutants can now be easily selected and identified.

The K_m values found for wild-type enzyme using the transfer and the exchange assays indicated a lower K_m value for the complete transfer, suggesting that the enzyme might have a greater affinity for histidine in the presence of tRNA, though the assay conditions are somewhat different. The K_m values for histidine are somewhat higher than values reported for other synthetases (Berg, Bergmann, Ofengand & Dieckmann, 1961; Stern & Mehler, 1965; Calender & Berg, 1966), but they are comparable to the size of pool of free histidine ($1{\cdot}5 \times 10^{-5}$ M) found by FerroLuzzi-Ames (1964).

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