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Histidine Regulatory Mutants in *Salmonella typhimurium*

I. Isolation and General Properties

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At least four genetically distinct classes of mutations give rise to triazolealanine resistance in *Salmonella typhimurium*. Each class studied affects regulation of the histidine operon.

Mutants of one class, *hisO*, are located at one end of the histidine operon. This class may be similar to operator constitutive mutants studied in other systems. At least two genetic sites are present in this region. A second class, *hisS*, is linked in transduction tests to the *guaA* and *strB* loci. This class contains a low specific activity of histidyl-tRNA synthetase and may represent mutations in the structural gene for this enzyme (Roth & Ames, 1966). Mutations of a third class, *hisR*, are linked in transduction tests to the *ilv* and *metE* loci (Roth & Hartman, 1965) and affect the levels of effective transfer RNA(his) (Silbert, Fink & Ames, 1966). A fourth class of mutants, *hisT*, is linked to the *purF* and *aroD* loci.

1. Introduction

The biosynthesis of histidine in *Salmonella typhimurium* involves ten enzymes. These are determined by a cluster of genes, the histidine operon, which is regulated as a unit (Ames & Garry, 1959; Ames, Garry & Herzenberg, 1960; Ames & Hartman, 1962). The level of each of the ten enzymes can vary from one, the repressed level, to about 25, under conditions of histidine starvation.

Regulatory mutants were obtained which are unable to repress fully the enzymes of the histidine operon. These mutants were obtained by selection for resistance to the histidine analogue, 1,2,4 triazole-3-alanine. This analogue has been shown to be incorporated into protein and to cause repression of the histidine operon (Levin & Hartman, 1963). Among the TRA‡-resistant mutants are regulatory mutants having a defective control mechanism for the histidine operon. Such mutants are not subject to repression by the analogue and thus escape growth inhibition. The regulatory mutants have been classified into at least four genetic groups. This report describes

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‡ Abbreviations used: TRA, 1,2,4 triazole-3-alanine; tRNA, transfer RNA; AMT, 3-amino-1,2,4-triazole.

the location of the regulatory mutations of each type on the *Salmonella* chromosome and some properties of the mutants. These results are discussed in terms of tentative models for regulation of the histidine operon.

2. Materials and Methods

(a) *Bacterial strains*

All strains used are derivatives of *S. typhimurium* LT-2, except *hisH107* and its derivatives, which are derived from strain LT-7. The following strains were kindly donated by M. Demerec: *aroD5*, *guaA1*, *guaA9*, *purF145*, and *strB57*. Strain SL751 (*purC7 proA46 ile-405 rha-461 str-r iM-10 fla-56 fim*$^-$) was received through the courtesy of B. A. D. Stocker. Strain *metE338 ara-9 ilvC401* has been described by Roth & Hartman (1965). Descriptions of the above genetic symbols and properties of the mutants have been summarized by Sanderson & Demerec (1965).

Histidine regulatory mutants and their means of selection are presented in Table 1. Procedures used in their isolation are described below in section 3(a). Mutants *hisT1222*, *hisR1223*, *hisT1224*, *hisT1227* and *hisT1230* were selected by B. N. Ames in partial revertants of frameshift mutation *hisC202*. Mutants *hisR1300*, *hisT1501*, *hisT1503*, *his-1502*, and *his-1509* were selected by B. N. Ames and G. FerroLuzzi-Ames. Histidine-independent derivatives of some of the regulatory mutants isolated in *hisH107* were constructed by transduction. The double mutants, *hisO1202 hisG46*, *hisO1242 hisG46*, and *hisO1812 hisG46*, were constructed as outlined below. Mutation *hisO1242*, originally obtained in strain SL751, was re-isolated in a prototroph by transduction into deletion *his-63* and selection of a P22-sensitive, prototrophic recombinant clone (G. R. Fink, personal communication).

TABLE 1

Sources of regulatory mutants

Regulatory mutation	Parent strain	Mutagen	Selection method†
hisR1200	*hisH107*	spontaneous	TRA†
hisO1202, *hisR1203* *hisR1204*, *hisR1205* *his-1206*, *hisT1207* *hisR1208*, *hisS1209* *hisS1210*, *hisS1211* *hisS1213*, *hisS1219*	*hisH107*	ultraviolet	TRA
hisT1814, *hisT1815* *hisS1816*, *hisR1826*	*hisH107*	diethyl sulfate	TRA
hisR1223	*hisC202*, *PR-2*	spontaneous	2-methyl histidine
hisT1222, *hisT1224*	*hisC202*, *PR-2*	spontaneous	TRA
hisT1227, *hisT1230*	*hisC202*, *PR-8*	spontaneous	TRA
hisT1501, *hisT1503* *his-1502*, *his-1509*	*LT-2* wild type	spontaneous	TRA + AMT
hisO1242, *hisS1259*	SL751	spontaneous	TRA + AMT
hisO1812, *hisR1813* *his-1817*, *his-1818* *his-1820*, *his-1821* *his-1822*	*ara-9*	diethyl sulfate	TRA + AMT
his-1819, *his-1823* *his-1824*, *his-1825*	*ara-9*	spontaneous	TRA + AMT

† Described in section 3(a).

(b) *Media*

Difco nutrient broth was used as maximally supplemented liquid medium with 1·5% Difco agar as solid medium. The E medium of Vogel & Bonner (1956) was used, with 0·2% dextrose added, as minimal salts medium. Solid media used for scoring the wrinkled colony phenotype of the TRA-resistant mutants contained 2% dextrose. Amino acid supplements, when used, were added at a concentration of 50 μg/ml. unless otherwise noted. Histidinol was added at a concentration of 125 μg/ml. Triazolealanine was obtained from R. G. Jones, Eli Lilly Co., Indianapolis, Indiana. Aminotriazole was obtained from the Aldrich Chemical Co.

(c) *Transduction tests*

Phage was prepared and stored as reported by Hartman (1956). Transduction tests were performed directly on solid medium with no prior adsorption. Phage and cells were spread together on selection medium at a multiplicity of infection dependent on the chromosome region for which recombinants were selected; this adjustment compensated for different transduction frequencies found for various chromosomal regions.

In crosses for which a TRA-resistant mutation served as an unselected marker, the transduction was performed on solid medium containing 2% glucose. On this medium, TRA-resistant clones form characteristic wrinkled colonies which can be scored with the aid of a dissection microscope. TRA-sensitive clones form normal smooth-appearing colonies (Roth & Hartman, 1965).

P22-sensitive (non-lysogenic) recombinants from phage-mediated crosses were recovered using the magnesium-hypersensitive phage of N. D. Zinder (personal communication). The procedure was modified from that of Frank D. Vasington (personal communication). E medium was made up with one-tenth of the normal K_2HPO_4 concentration and the pH was adjusted to 7·0. Just prior to the pouring of plates, the medium was made 0·05 M in $MgCl_2$. Mg-hypersensitive phage was grown on the donor strain in broth and irradiated with ultraviolet light to approximately 10% survival. The transduction tests were performed and recombinants were picked after only 24 to 36 hr incubation. The tiny recombinant colonies were restreaked several times on high-magnesium medium. In each case, colonies were picked while still small. Finally, clones were tested for sensitivity to P22 phage.

(d) *Construction of* hisG46 hisO *double mutants*

HisG46 hisO double mutants were used in the mapping of the *hisO* mutations. The strains were constructed by transducing the recipient *hisD1 hisG46* to growth on histidinol (*hisD*$^+$) with phage grown on a *hisO* mutant. The recombinants were tested for growth on histidine and for colony morphology. Recombinants which required histidine and formed wrinkled colonies on medium containing 2% glucose proved to be the *hisG46 hisO* double mutants.

(e) *Growth and preparation of cells for enzyme assays*

Cells were grown in 100 ml. of minimal medium on a rotating shaker bath at 37°C. At intervals, 1·0-ml. samples were removed and the optical density determined at 650 mμ in a Beckman DU spectrophotometer. When the optical density of the culture reached 0·5 to 0·6, the cells were harvested by centrifugation for 20 min at 10,000 *g* in a Servall refrigerated centrifuge. The cells were resuspended in 30 ml. of 0·05 M-Tris–HCl buffer (pH 7·5) containing 0·1 mM-ATP and 2 mM-$MgCl_2$. In the initial experiments, 1·4 mM-mercaptoethanol was also included. Since this had no noticeable effect on the activities measured, it was omitted in later experiments. The cell suspension was then centrifuged as before; the cells were resuspended in the same buffer and sonicated for 2 min in a sonic oscillator (Measuring & Scientific Equipment, Ltd; model 160W). The sonicate was then centrifuged 1 hr at 30,000 *g* to remove cell debris. The supernatant fraction was passed through small columns of Sephadex gel (G25 or G50) to remove any remaining small molecules, and the resulting crude extract was used directly in the enzyme assays. Protein content of the extracts was estimated by the biuret method.

(f) *Assay of enzymes*

The histidyl tRNA synthetase was assayed by following the attachment of [^{14}C]histidine to tRNA. The reaction mixture of 0·25 ml. contained the following: *E. coli* tRNA, 5 O.D.$_{260}$ units or 0·2 mg (General Biochemicals Corp.); cacodylic acid (pH 7) 20 μmoles; ATP, 0·5 μmole; $MgCl_2$, 0·5 μmole; mercaptoethanol, 1·5 μmoles; L-[^{14}C]histidine (35 mC/mM; Nuclear Chicago), 0·01 μmole; gelatin, 0·1 mg. For the experiments presented in Table 10, L-[^{14}C]leucine (22 mC/mM) replaced histidine. The reaction was initiated by the addition of crude extract and was stopped by the addition of 1·0 ml. of 5% trichloroacetic acid. The reaction tubes were chilled in an ice-bath and the precipitate removed by filtration of the mixture with suction through a Millipore filter (pore size 0·45μ; 25 mm diameter). The filter disc carrying the precipitate was washed repeatedly with 5% trichloroacetic acid, dried, fastened to an aluminium planchet with rubber cement and assayed for radioactivity in a gas-flow counter (Nuclear Chicago).

Histidinol phosphate phosphatase was assayed (as an index of the degree of de-repression of the histidine operon) using the method of Ames, Garry & Herzenberg (1960). One unit of activity is the amount of enzyme which frees 1 μmole of PO_4 from histidinol phosphate in 1 hr under the conditions used. No $MgCl_2$ was added to the reaction mixture. Duplicates were run and the results averaged for each determination. In some experiments the toluenized cell assay and specific activity calculations of Ames, Hartman & Jacob (1963) were used.

3. Results

(a) *Isolation of TRA-resistant mutants*

Histidine regulatory mutants were selected for resistance to the histidine analogue, 1,2,4-triazole-3-alanine (TRA), which acts as a false co-repressor of the histidine operon (Levin & Hartman, 1963). Since wild-type *Salmonella* growing on minimal medium have almost fully repressed levels of the histidine biosynthetic enzymes (Ames & Garry, 1959), addition of a false co-repressor molecule, such as TRA, does not severely restrict growth. *Salmonella* having a partial block in the histidine pathway are able to grow on minimal medium only when they produce high levels of the histidine biosynthetic enzymes. Such cells require de-repression for growth under these conditions, and thus show sensitivity to the presence of the false repressor.

The partial block in the biosynthetic pathway, required for TRA sensitivity, can be introduced genetically by a "leaky" histidine mutation or physiologically by addition of the histidine analogue, 3-amino-1,2,4-triazole, to wild-type cells. This analogue is an inhibitor of imidazole glycerol phosphate dehydrase (Hilton, Kearney & Ames, 1965). Wild-type cells can overcome inhibition by AMT by increasing their levels of histidine biosynthetic enzymes. In the presence of AMT, wild-type cells grow with obligately de-repressed enzyme levels and are sensitive to inhibition by TRA. Either of these partial blocks allowed direct selection of TRA-resistant mutants. 2-Methyl histidine-resistant mutants may be selected by analogous techniques.

(b) *Detection and properties of de-repressed mutants*

Several classes of mutations could be responsible for resistance to TRA. One such class would be represented by mutants, the histidine operon of which is no longer repressed by TRA. Some of these regulatory mutants might be expected to be insensitive also to repression by histidine and thus be unable to repress fully the levels of histidine biosynthetic enzymes. Another class would be represented by mutants in which the partial block in the pathway has been removed, either by reversion of the leaky mutation, or by mutation of the dehydrase gene to AMT resistance. A third class would be represented by permease mutants that are unable to take up TRA or AMT.

Colonies formed by TRA-resistant regulatory mutants were distinguished from other TRA-resistant mutants by their characteristic morphology. Cells growing with de-repressed histidine enzyme levels form wrinkled-appearing colonies (B. N. Ames, personal communication). This characteristic morphology was found to depend on

TABLE 2

Effect of glucose concentration on enzyme level

Strain	Histidinol phosphate phosphatase (specific activity) 0·2% glucose	2·0% glucose
hisR1203 hisH107	2·04	2·70
hisO1202 hisH107	3·32	2·75
hisH107	0·51	0·30

All strains grown on minimal salts medium supplemented with 40 μg L-histidine per ml. Carbon sources are as indicated above. The histidinol phosphate phosphatase assay of Ames, Garry & Herzenberg (1960) was used.

TABLE 3

Doubling times and repressed enzyme levels in some TRA-resistant mutants

Strain	Doubling time (min)	Histidinol phosphate phosphatase (specific activity)
hisH107 (Control)	47(9)†	0·48(12)†
hisR1201 hisH107	63(1)	4·44(1)
hisO1202 hisH107	48(4)	3·36(5)[a]
hisR1203 hisH107	50(4)	3·28(1)
hisR1204 hisH107	55(1)	2·88(1)
hisR1205 hisH107	50(3)	2·25(1)
his-1206 hisH107	89(2)	3·3(2)
hisT1207 hisH107	61(3)	3·4(1)
hisR1208 hisH107	56(7)	4·63(8)
hisS1209 hisH107 ile-155	46(3)	0·36(7)[a,b,c]
hisS1210 hisH107	54(3)	0·86(5)[a,c]
hisS1211 hisH107	55(8)	3·91(7) [a,c]
hisS1213 hisH107	56(2)	3·43(1)
hisS1219 hisH107	52(2)	1·79(1)
hisO1242		8·2(6)[d]
hisT1501		5·93(1)
his-1509		2·6(1)
hisO1812 ara-9		4·54(1)[d]
hisR1813 ara-9		3·65(1)[d]
hisT1814 hisH107		6·11(1)[d]
hisT1815 hisH107		5·94(1)[d]
hisS1816 hisH107		5·83(1)[d]

† Numbers in parentheses indicate how many values have been averaged.

[a] Includes some values determined for *hisH107*$^+$ derivative grown on minimal medium plus 50 μg histidine/ml.

[b] Isoleucine added to medium for growth.

[c] All *hisS* mutants tested de-repress further when grown on nutrient broth. See Table 9.

[d] Phosphatase activities for these mutants were determined using the toluenized cell assay, and the values converted to the comparable degree of de-repression in terms of mμmoles phosphate per mg protein per hour of experimental and control cultures. All other assays were performed according to Ames, Garry & Herzenberg (1960).

Specific activities were assayed by the method of Ames, Garry & Herzenberg (1960). All cells were grown in the presence of excess histidine.

elevated concentrations (2%) of glucose or other fermentable carbon sources in the medium (Fig. 1 in Roth & Hartman, 1965). The wrinkled phenotype is not expressed on 0·2% glucose, although the concentration of glucose has no significant effect on the levels of histidine biosynthetic enzymes (Table 2). Mutants selected for resistance to TRA were examined for colony morphology on medium containing 2% glucose. Only those mutants which formed wrinkled colonies were studied further. In every case tested, wrinkled colony formers had high enzyme levels for the histidine biosynthetic enzymes and presumably carried an altered regulatory element for the histidine operon.

Repressed enzyme levels of histidinol phosphate phosphatase and doubling times for some TRA-resistant, de-repressed mutants are presented in Table 3. All mutants tested were able to de-repress further when grown on limiting histidine (Table 4). This suggested that the mutants tested possessed altered regulatory mechanisms and that in none of these cases had a regulatory element been completely destroyed.

TABLE 4

Further de-repression of constitutive mutants

Experiment	Strain		De-repression of the histidine operon (relative specific activity) Excess histidine	Limiting histidine
1	*hisH107*	(control)	1·0	3·0
	hisO1202	*hisH107*	7·0	16·0
	hisR1203	*hisH107*	11·0	18·5
	hisR1208	*hisH107*	17·0	26·5
	hisS1209	*hisH107*	1·5	9·5
	hisS1211	*hisH107*	7·5	22·5
2	*hisG46*	(control)	1·0	13·9
	hisO1812	*hisG46*	10·2	20·1
3	*hisH107*	(control)	1·0	17·1
	hisT1207	*hisH107*	8·6	33·2

All strains were grown on minimal medium containing 0·2% glucose as carbon source. *Repressed* enzyme levels were determined in the presence of excess histidine, usually 50 μg/ml. Additional de-repression was measured as follows:

Experiment 1: Cultures were started with 4 μg L-histidine/ml. and cells harvested 45 min after histidine was exhausted.

Experiment 2: Cultures were grown on 150 μg histidinol/ml.

Experiment 3: Cultures were grown on 0·4 mM-adenine.

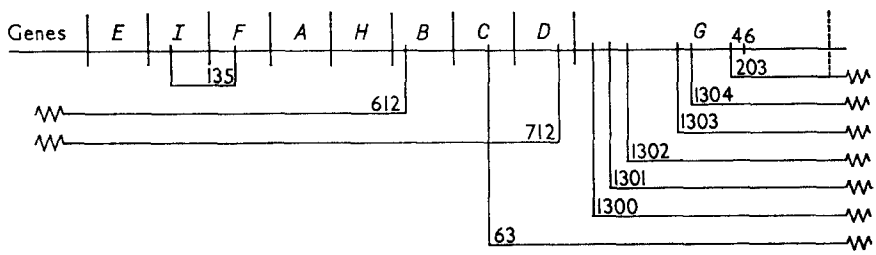
Since *hisH107* is a "leaky" histidine mutation, strains carrying this lesion can de-repress in the absence of exogenous histidine; such de-repression is enhanced by adenine. In each case activities are presented as the specific activity of histidinol phosphate phosphatase relative to the repressed level of the control culture run with each experiment. In experiment 1, the assay of Ames, Garry & Herzenberg (1960) was used; in experiments 2 and 3, the toluenized cell assay of Ames, Hartman & Jacob (1963) was used.

(c) *The* hisO *mutants*

Three of the TRA-resistant mutants have been designated *hisO* on the basis of their high levels of repressed enzyme and map position adjacent to the structural genes of the histidine operon.

(i) *Deletion mapping*

The map positions of *hisO* mutations were first determined by selecting prototrophic recombinants from transductional crosses between recipients carrying a histidine deletion mutation and donors carrying only the *hisO* mutation. Positions of the deletions tested and results of the crosses are indicated in Fig. 1 for mutant *hisO1202*. If *hisO1202* were located within the material homologous to that deleted in a recipient, all prototrophic recombinants were expected to carry the *hisO1202* mutation of the donor and, consequently, to form wrinkled colonies. If, on the other hand, *hisO1202* were located outside of the deleted region, some of the *his*$^+$ recombinants which received the deleted region from the *hisO1202* donor would fail to receive also the *hisO1202* mutation and would form smooth colonies. All of the deletion mutants numbered *1300* or larger are derived from *hisG203* and presumably represent extensions at the ends of the parental deletion (Ames, Hartman & Jacob, 1963). The single smooth colony found on the selection plates of the cross with *hisG1300* is assumed to be either a revertant of the *hisO* mutation or a modifying mutation in the recipient which prevents expression of the rough phenotype. This assumption is based on the fact that no such colonies arose from the cross with *hisG203*, a smaller deletion mapping within the deleted region of *hisG1300*. From the data presented in Fig. 1, it was inferred that *hisO1202* lies within the *hisG203* deletion and thus must be located in the right-hand end of the *hisG* gene or else outside of the structural genes of the histidine operon.



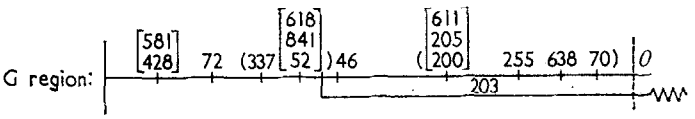
	his$^-$ deletions			*his*$^-$ deletions							*metE*‡	*ilvC*‡
Recipient strain	*135*	*612*	*712*	*63*	*1300*	*1301*	*1302*	*1303*	*1304*	*203*	*338*	*401*
Recombinant colonies scored	1087	37	765	909	2800	2000	2000	2000	3320	6792	542	2300
his01202$^+$ recombinants (smooth colonies)	300	10	55	0	1	0	0	0	0	0	542	2300
%Carrying recipient *his0*$^+$ allele	27·6	27·0	7·3	<0·1	0·04†	<0·05	<0·05	<0·05	<0·03	<0·02	100·0	100·0
Operator location	Outside deleted region but linked to it			Within deleted region							Unlinked to these loci	

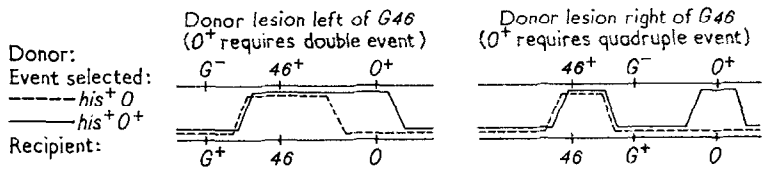
FIG. 1. Transduction tests with phage grown on *hisO1202*. Tests performed as described in section 2(c).

Presence of 100% smooth (*hisO1202*$^+$) colonies in tests with *metE* and *ilvC* recipients (‡ = controls) and presence of wrinkled colonies (*hisO1202*) in tests with *his*$^-$ deletion mutants show that *hisO1202* is unlinked to the former loci and linked with the latter. The results obtained with various *his*$^-$ deletion mutants further confine the location of *hisO1202* to the "right" end of the histidine operon. The dagger (†) indicates one probable spontaneous mutant (see text).

(ii) *Three-point crosses*

The *hisO* mutations lie to the right of all known histidine mutations, that is, very close to those sites at the extreme right end of the histidine region as the map is generally drawn. This conclusion is based on three-point transduction tests. Recipients were *hisG46 hisO* double mutants; donors were various point mutants in the *hisG* gene. Results of such crosses and the cross-over positions which best account for these results are presented in Fig. 2. With donor lesions located to the right of *hisG46*, the percentage of the recombinants which received the donor's wild-type operator allele (through quadruple cross-overs) was much lower than the percentage which received the same allele (through double cross-over) when the donor lesion was located to the left of *hisG46*. From these results, it was inferred that all three operator mutations were located either to the right of all known histidine mutations or else





		Left of *G46*							Right of *G46*					
G mutant used as donor:		[581	428]	72	(337	[618	841	52])	([611	205	200]	255	638	70)
hisG46 / *his01202* Recipient	No. of recombinants tested:	18	36	42	287	57	48	60	38	160	44	59	17	25
	% of O^+ among recombinants:	100	80	76	91	91	79	80	3	8	7	2	23	20
hisG46 / *his01242* Recipient	No. of recombinants tested:	40	79	NT	64	NT	NT	56	22	41	NT	32	15	49
	% of O^+ among recombinants: ‡	30	34	NT	55	NT	NT	50	4·8	2·4	NT	9·4	15	2
hisG46 / *his01812* Recipient	No. of recombinants tested:	31	17	NT	75	NT	NT	5	11	74	NT	36	NT	3
	% of O^+ among recombinants:	77	100	NT	84	NT	NT	100	9·1	9·5	NT	2·8	NT	0

FIG. 2. Location of *hisO* mutations through three-point transduction tests.

Phage grown in auxotrophic mutants defective in the *hisG* gene was used in transduction tests with various *hisG46 hisO* double-mutant recipients. Prototrophic recombinants were scored for the frequency of appearance of the unselected *hisO* mutation (wrinkled colony formation) as opposed to *hisO*$^+$ (smooth colony formation). The map of the *hisG* locus is that of Loper, Grabnar, Stahl, Hartman & Hartman (1964). The order of mutant sites in the "right" end of *hisG* is now known to be: (70-638) ([611-205-200] 255). Sites in brackets are not separable by transduction tests; sites in parentheses have not been ordered.

†, Includes spontaneous revertants of *hisG46* all of which carry *hisO* and form wrinkled colonies; ‡, percentage presented may be lower than true values since some spontaneous revertants are scored as wrinkled recombinants; NT, not tested.

very close to those known sites lying at the extreme right-hand end of the gene *hisG*. Thus, results are consistent with a location of all three *hisO* mutations at the extreme right-hand end of the operon either within or beyond the structural gene.

Evidence that wrinkled colony-forming recombinants scored in mapping *hisO* were in fact de-repressed is presented in Table 5. High enzyme levels accompany wrinkled colony morphology in each case.

TABLE 5

Enzyme levels in recombinants

Cross	Colony genotype selected	Colony morphology	Histidinol phosphate phosphatase (specific activity)
hisG203 × *hisO1202*	*hisG203*$^+$	W	2·8
hisG203 × *hisO1202*	*hisG*$^+$	W	2·4
hisD1 hisG46 × *hisO1202*	$\frac{hisD^+}{hisG^+}$	W	2·0
hisD1 hisG46 × *hisO1202*	$\frac{hisD^+}{hisG^-}$	W	2·2
hisD1 hisG46 × *hisO1202*	$\frac{hisD^+}{hisG^-}$	W	2·7
hisD1 hisG46 × *hisO1202*	$\frac{hisD^+}{hisG^-}$	S	0·1
hisD1 hisG46 × *hisO1202*	$\frac{hisD^+}{hisG^-}$	S	0·1

In each cross the selected recombinant received donor material for the histidine region. The recipient strains are listed first in presenting the cross. Cells for enzyme assays were grown on minimal medium containing 50 μg L-histidine/ml. The histidinol phosphate phosphatase assay of Ames, Garry & Herzenberg (1960) was used. (W, wrinkled; S, smooth.)

(iii) *Recombination between* hisO *mutations*

Since all three *hisO* mutations seemed to be located at the same place, the right-hand end of the *hisG* gene, crosses were made to determine whether recombination between *hisO* mutations could be detected. Such recombinants were first sought using the *hisO* mutations as unselected markers in transductional crosses between a double-mutant recipient, *hisG46 hisO*, and a donor strain carrying a different *hisO* lesion. The frequency of *hisO*$^+$ (smooth) recombinants (~0·01%) was too low for convenient analysis. In order to facilitate detection of recombinants, use was made of an observation by M. J. Voll (personal communication) that constitutive mutants grow poorly at 42°C whereas wild-type cells grow well at this temperature. This temperature sensitivity seems to be a consequence of de-repression of the histidine operon.

Crosses were performed using a double mutant, *hisG46 hisO*, as recipient and a second *hisO* mutant as donor. Results of these crosses are presented in Table 6. The data in Table 7 indicate that the smooth colony recombinants exhibit the *hisO*$^+$ phenotype. It is concluded that recombination can occur between *hisO1812* and the other two *hisO* mutations, but not between *hisO1202* and *hisO1242*. Thus, at least two sites are present in the *hisO* region; if the lesions in these strains are deletions, they do not overlap.

The basal (repressed) enzyme levels for *hisO1202* and *hisO1242* are quite different (Table 3). Therefore, all three *hisO* mutations can be differentiated either on genetic or on biochemical grounds.

TABLE 6

Percentage of hisO$^+$ *recombinants in transduction tests between* hisO *mutations*

Recipients	Donors		
	hisO1202	*hisO1242*	*hisO1812*
G46 O1202	<0·02% (0/5310)	<0·01% (0/7302)	0·07% (13/18813)
G46 O1242	<0·02% (0/4535)	<0·07% (0/1534)	0·3% (38/12705)
G46 O1812	0·05% (1/1983)	0·01% (1/7023)	<0·01% (0/8412)

Histidine-requiring (*hisG46*)–*hisO* double mutants were infected with phage grown on *hisO* mutants. Bacteria were plated on minimal medium containing 2% glucose. The percentage of smooth (*hisO*$^+$) recombinants among prototrophic (*hisG46*$^+$) clones is shown for each test. In parentheses the ratio *hisO*$^+$ *hisG46*$^+$/total *hisG46*$^+$ is indicated. The results are pooled for replicate experiments. In some experiments the transduction plates were incubated at 37°C. In other experiments, the plates were incubated at 20°C for 15 hr to allow expression of the *hisO*$^+$ genotype and then incubated for two more days, either at 37°C or at 42°C. The three methods produced concordant results.

The data indicate that the order of mutant sites may be *G46* (*O1202 O1242*) *O1812*.

TABLE 7

Repressed enzyme levels in smooth (hisO$^+$) *recombinants of crosses between* hisO *mutants*

Recipient	Donor	Fold de-repression (relative specific activity)
G46 O1242	*O1812*	1·1
		0·9
G46 O1202	*O1812*	0·9
		0·8
G46 O1812	*O1242*	0·7
G46 O1812	*O1202*	1·1
Controls		
G46		≡1
G46 O1812		7·1
G46 O1202		5·0
G46 O1242		20·9

Cultures of *hisG46*$^+$ *hisO*$^+$ recombinants were grown on minimal medium containing 50 μg L-histidine/ml. Cells were harvested while still in log-phase and histidinol phosphate phosphatase was assayed with the toluenized cell assay of Ames, Hartman & Jacob (1963).

Fold de-repression is the specific activity of phosphatase relative to that of *hisG46* and is indicated separately for each recombinant clone examined.

(d) *The* hisR *mutants*

A second class of TRA-resistant mutants, *hisR*, has been mapped previously (Roth & Hartman, 1965; Roth & Sanderson, 1966) and found to lie between the *metE* and the *ilvA* loci (Fig. 3). *HisR* mutants, like *hisO*, have abnormally high repressed levels of the histidine biosynthetic enzymes and are able to de-repress further these levels when grown under conditions of limiting histidine (Table 4).

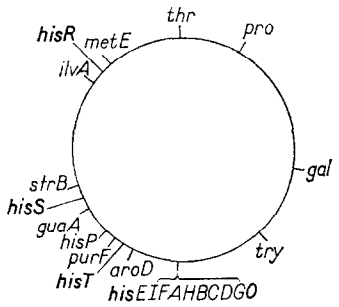


FIG. 3. Chromosome map of *Salmonella typhimurium* showing approximate locations of *his* loci. The map is based on that of Sanderson & Demerec (1965) with additions described in this report. Approximate locations of several markers are included for purposes of orientation: *thr* (threonine), *pro* (proline), *gal* (galactose) and *try* (tryptophan).

The nature of the *hisR* gene product and its role in repression have been investigated by Silbert *et al.* (1966). They find that *hisR* mutants have approximately 55% of the normal level of histidine-specific tRNA acceptor activity, whereas none of the other classes of constitutives shows such a reduction. This suggests that the *hisR* gene is involved, directly or indirectly, in the production of histidine-specific tRNA.

(e) *The* hisS *mutants*

(i) *General properties*

The class of mutants designated *hisS* was distinguished from other classes by: (1) requirement of abnormally high histidine concentrations for maximal repression; (2) possession of low specific activities of histidyl tRNA synthetase; and (3) a distinct location on the genetic map. It was first noted that several *hisS* mutants formed wrinkled colonies on nutrient agar containing 2% glucose, but normal, smooth-appearing colonies on minimal medium supplemented with histidine. After *hisH107*$^+$ derivatives were made of the original *hisH107 hisS* double mutants, it was possible to measure the enzyme levels of these strains on histidine-free medium. This study revealed that *hisS* mutants required abnormally high levels of histidine in order to repress the histidine biosynthetic enzymes (Table 8; see also Table 1 in Roth & Ames, 1966). Nutrient agar medium apparently did not contain sufficient histidine to cause repression in these mutants.

Wild-type cells grow on minimal medium with repressed levels of the histidine biosynthetic enzymes. In contrast, the above experiments show that *hisS* mutants, *hisS1210*, *hisS1211* and *hisS1520* require exogenous histidine for maximal repression, although each has an intact histidine region. Apparently the normal internal pool of histidine is not sufficient to result in repression of these mutants. In the case of *hisS1211*, even excess exogenous histidine cannot repress enzyme levels completely.

TABLE 8

Effect of histidine on enzyme levels of hisS *mutants*

Strain	L-Histidine concentration (mM)	Histidinol phosphate phosphatase (specific activity)
hisS1210 hisH107+	0	13·8
	0·01	16·4
	0·04	2·6
	0·07	1·9
	0·10	2·8
	0·15	2·9
	1·00	1·0
hisS1211 hisH107+	0	24·9
	0·3	9·9
	3·0	10·0
	10·0	9·4
LT-2 wild type	0	2·6
	0·15	1·8

Cells were grown on minimal medium with the indicated histidine concentration. Histidinol phosphate phosphatase was measured by the toluenized cell assay of Ames, Hartman & Jacob (1963).

TABLE 9

Properties of hisS *mutants*

Strain	Doubling time (min) minimal medium†	Doubling time (min) nutrient broth	Histidinol phosphate phosphatase (specific activity) minimal medium†	Histidinol phosphate phosphatase (specific activity) nutrient broth	Histidinol phosphate phosphatase (specific activity) pool medium‡	Histidyl tRNA synthetase (relative specific activity) minimal medium†	Colony morphology minimal†	Colony morphology broth
hisS1209 hisH107	46 (3)	—	0·4 (7)	3·9 (2)	0·5	0·05 (5)	S	W
hisS1210 hisH107	54 (3)	36 (1)	0·9 (5)	2·7 (3)	0·7	0·1 (3)	S	W
hisS1211 hisH107	55 (8)	50 (1)	3·9 (7)	5·4 (4)	4·2	0·15 (7)	W	W
hisS1213 hisH107	56 (2)	—	3·4 (1)	—	—	0·05 (1)	S	W
hisS1219 hisH107	52 (2)	31 (1)	1·8 (1)	2·9 (1)	—	0·08 (1)	S	W
hisR1208 hisH107 (control)	56 (7)	—	4·6 (8)	4·5 (3)	—	1·12 (4)	W	W
hisO1202 hisH107 (control)	50 (5)	—	3·3 (5)	—	—	1·07 (4)	W	W
hisH107 (control)	47 (9)	29 (1)	0·5 (12)	0·2 (3)	—	≡1·00	S	W

Numbers in parentheses are the number of times the particular assay has been made.

W, S, these letters stand for wrinkled (W) and smooth (S) colony former. The *hisS* mutants have low specific activity of the histidyl tRNA synthetase and have higher basal levels of histidinol phosphate phosphatase on nutrient broth medium than on minimal medium.

† Supplemented with 50 μg L-histidine/ml. Strains were sometimes used from which the parental *hisH107* mutation had been removed. This difference had no effect on the activities measured. The histidinol phosphate phosphatase assay of Ames, Garry & Herzenberg (1960) was used.

‡ Pool medium contains 17 amino acids plus thiamine.

(ii) *Involvement of histidyl tRNA synthetase*

Another property unique to *hisS* mutants is their low specific activity of the histidyl tRNA synthetase when measured in the transfer reaction (Table 9). In the two *hisS* mutants tested, this decrease is due to an altered synthetase having a greatly increased K_m value for histidine (Roth & Ames, 1966). Alternative explanations of the decreased activity, such as: (1) presence of an internal synthetase inhibitor, and (2) a regulatory effect on all aminoacyl tRNA synthetases, are deemed unlikely for mutant *hisS1209*. In an extract-mixing experiment, the presence of mutant extract had no effect on the activity of wild-type enzyme (Fig. 4). This argues against the

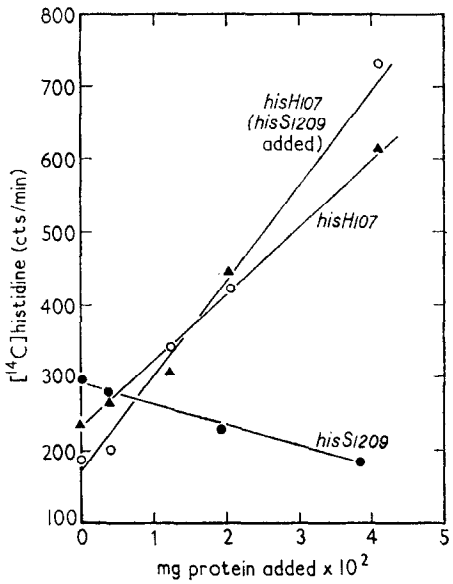


FIG. 4. Tests for the presence of an inhibitor in *hisS1209* extracts.

The transfer of [^{14}C]histidine to tRNA was examined. Each assay was incubated 7 min. Gelatin (0·04 mg) was added to increasing amounts of extract from *hisH107* in order to keep protein concentrations approximately constant.

The extract of *hisH107* catalyzed transfer of [^{14}C]histidine to tRNA, whereas the extract of *hisS1209* promoted no detectable transfer. The negative slope of the *hisS1209* curve may reflect destruction of tRNA by nucleases in the crude extract. A fixed amount (0·039 mg protein) of *hisS1209* extract was added to increasing amounts of *hisH107* extract. No inhibition of the *hisH107* enzyme activity was noted, indicating that the low specific activity of histidyl-tRNA synthetase activity in *hisS1209* extracts is not due to the presence of an internal inhibitor.

presence of an inhibitor in the mutant extract. The possibility of a regulatory effect on all synthetases is made unlikely by the observation that *hisS1209* has normal activity of the leucyl tRNA synthetase (Table 10). More information on the nature of the *hisS* mutations is presented in an accompanying paper (Roth & Ames, 1966).

(iii) *Map position*

All *hisS* mutations map near the *strB* locus and thus comprise a particular genetic class. This locus was mapped first through conjugational crosses involving Hfr donor strains (Roth, 1965). These crosses indicated that the *hisS* locus was located between *cysC* and the histidine region, and very close to the *purC* locus. It was then attempted

TABLE 10

Amino acyl tRNA synthetase activities for histidine and leucine

Strain	Histidyl tRNA synthetase (mμM/mg × min)	Leucyl tRNA synthetase (mμM/mg × min)
LT2	0·754	1·84
hisS1209, *hisH107*, *ileA155*	0·02	1·83

to identify a transducing fragment which might cotransduce both *hisS* and other genes known to be located within this region of the chromosome. For this purpose, phage grown on *hisS* mutants was used to transduce various auxotrophic mutations to prototrophy. The mutants used as recipients were indicated as located between the *cysC* locus and the histidine region (Sanderson & Demerec, 1965). Recombinant colonies were inspected for development of wrinkled colonies on minimal medium containing 2% glucose. If the *hisS* gene were contransducible with the auxotrophic mutation carried by the recipient, then some of the recombinant colonies would be expected to express the wrinkled colony phenotype of the donor *hisS* mutation. Wrinkled colonies appeared among the recombinants from crosses between *guaA* and *hisS* and crosses between *strB* and *hisS* (Table 11). Several of these recombinants were isolated, grown up on minimal medium and checked for enzyme levels. All were found to have de-repressed levels of histidinol phosphate phosphatase and abnormally low specific activities of histidyl tRNA synthetase (Table 12). Since these properties are characteristic of the donor strains in each case, these colonies were identified as true recombinants in which the mutant *hisS* allele had been co-transduced with the *guaA*$^+$ or the *strB*$^+$ allele.

TABLE 11

Transduction crosses with hisS *mutants*

Donor	Recipients: *strB57* Colonies scored	(% W)	*guaA1* Colonies scored	(% W)	*guaA9* Colonies scored	(% W)	*aroD5* (control) Colonies scored	(% W)	*purF145* (control) Colonies scored	(% W)
LT2 (control)	180	0	3625	<0·03	1720	<0·06	200	<0·5	1118	<0·09
hisS1209	855	8·3	429	<0·23	366	<0·27	30	<3·3	—	—
hisS1210	614	7·6	1220	0·98	307	0·65	200	<0·5	—	—
hisS1211	205	5·4	547	1·65	328	1·53	100	<1·0	1660	<0·06
hisS1213	273	17·5	1622	0·19	1214	0·58	—	—	—	—
hisS1219	96	9·4	1502	0·20	1353	0·66	523	<0·2	2877	<0·03
hisS1520	749	8·7	3060	0·06	—	—	—	—	—	—

Wrinkled (W) recombinant colonies result from crosses between *hisS* donors and *guaA* or *strB* recipients. All *hisS* mutants except *hisS1209* show such linkage to *guaA1*; none is linked to *aroD5* or *purF145*. *StrB* is a mutation which may be selected either for resistance to low concentrations of streptomycin or for a requirement for thiamine + nicotinic acid (Demerec & Lahr, 1960). In these experiments, *strB57* (formerly designated *nic-5*) was transduced to prototrophy.

TABLE 12

Enzyme content of recombinants of hisS *transductional crosses*

Strain	Relative specific activity: Histidinol phosphate phosphatase	Relative specific activity: Histidyl tRNA synthetase†
guaA1	1·2	≡1
guaA9	0·62	≡1
LT-2	≡1	≡1
hisS1210 hisH107$^+$	5·3	0·10
guaA1 × *hisS1210* (wrinkled recombinant)	6·7	0·06
guaA9 × *hisS1210* (wrinkled recombinant)	5·6	0·13
guaA9 × *hisS1210* (wrinkled recombinant)	5·9	0·16
hisS1211 hisH107$^+$	9·6	0·15
guaA9 × *hisS1211* (wrinkled recombinant)	12·0	0·15
hisS1213 hisH107	‡	0·05
guaA1 × *hisS1213* (wrinkled recombinant)	8·3	—
guaA9 × *hisS1213* (wrinkled recombinant)	11·7	0·21
hisS1219 hisH107	‡	0·08
guaA9 × *hisS1219* (wrinkled recombinant)	6·1	0·31

Recombinants were derived from crosses between recipient *guaA* mutants and donor *hisS* mutants. Selection was made for *gua*$^+$ and wrinkled colonies were chosen. *GuaA* mutants were grown on 50 μg guanosine/ml.; *hisS1213, hisH107* and *hisS1219, hisH107* were grown on 50 μg histidine/ml.; all other strains were grown on minimal medium. The histidinol phosphatase assay of Ames, Garry & Harzenberg (1960) was used.

†Activities are expressed relative to parental strain in each case.

‡ *hisH107*$^+$ derivatives of these strains were not available, so their enzyme levels on minimal medium could not be determined.

The transduction tests showed that the *hisS* gene is co-transducible with the *guaA* and *strB* loci and, thus, is located very close to these loci on the genetic map (Fig. 5, below). This conclusion is supported by the finding that all of the *hisS* mutants tested were found linked to *guaA* or *strB*, whereas no linkage of *hisS* to other nearby loci (Sanderson & Demerec, 1965) was detected: *try* (tyrosine), <0·045%: *phe* (phenylalanine), <0·008%; *purC* (purine), <0·06%; *purI* (purine), <0·097%; *purG* (purine), <0·050%.

(f) *The* hisT *mutants*

The *hisT* mutants have high repressed levels of the histidine biosynthetic enzymes and normal specific activity of histidyl tRNA synthetase. The *hisT* locus maps between the *aroD* and the *purF* loci on the *Salmonella* chromosome. Transduction data for these mutants are presented in Table 13. The data in Table 14 give evidence that the wrinkled colonies scored in the transduction tests are indeed histidine-regulatory mutants. Since the linkage between *aroD5* and *purF145* (Table 15, 8·1%) is weaker than the linkage of any *hisT* mutant to either of them, we infer that the

TABLE 13

Transductional crosses with hisT *mutants*

Donor	Recipient *purF145* Colonies scored	(% W)	*aroD5* Colonies scored	(% W)	*guaA1* (control) Colonies scored	(% W)	*metE338* (control) Colonies scored	(% W)
hisT1207	2225	38·9	1068	19·1	1013	<0·10	900	<0·11
hisT1222	495	38·4	—	—	395	<0·25	702	<0·14
hisT1224	348	42·8	—	—	650	<0·15	609	<0·16
hisT1227	405	38·6	—	—	352	<0·28	430	<0·23
hisT1230	917	38·6	—	—	238	<0·42	1139	<0·09
hisT1501	—	—	1252	45·0	1081	<0·09	1702	<0·06
hisT1503	36	19·4	—	—	4400	<0·03	3600	<0·03
hisS1211 (control)	1660	<0·06	100	<1·0	547	1·6	1706	<0·06
LT2 (control)	1118	<0·09	200	<0·5	3625	<0·03	1310	<0·08

W, Percentage of transductant colonies which have wrinkled morphology characteristic of de-repressed strains.

TABLE 14

Repressed enzyme levels and colony morphology of parental strains and recombinants of hisT *transductional crosses*

Strain	Histidinol phosphate phosphatase (specific activity)	Colony morphology†	Additions to medium
Parental strains:			
hisT1207 hisH107	3·4	W	50 μg L-histidine/ml.
purF145	0·5	S	20 μg adenosine/ml.
aroD5	NT	S	20 μg L-tyrosine + 20 μg L-phenylalanine/ml.
hisH107	0·5	S	50 μg L-histidine/ml.
Wrinkled recombinants:			
purF145 × *hisT1207 hisH107*	4·4	W	None
aroD5 × *hisT1207 hisH107*	5·5	W	None

Bacteria were grown on minimal medium to which the supplements listed in the last column were added. The histidinol phosphate phosphatase assay of Ames, Garry & Herzenberg (1960) was used. In the transduction tests from which wrinkled recombinants were obtained, the recipient strain is listed first and the donor strain second.

† Colony morphology: W, wrinkled, S, smooth. NT, not tested.

hisT gene is located between the *aroD* and the *purF* loci. This conclusion is substantiated by three-point tests (see also section (h) below). As yet, no biochemical alteration has been detected in *hisT* mutants. All strains tested (*hisT1207*, *1222*, *1227*, *1501*) have normal levels of histidyl tRNA synthetase. No change in the amount of histidine-specific tRNA acceptor capacity has been detected in a *hisT* mutant (Silbert, *et al.*, 1966).

TABLE 15

Co-transduction of the aroD *and* purF *loci*

Donor	Recipient			
	aroD5		*purF145*	
	No. tested	% with unselected *purF145* marker	No. tested	% with unselected *aroD5* marker
purF145 *aroD5*	586	5·6	488	10·5

(g) *Unclassified mutants*

Only four genetically distinct classes of TRA-resistant histidine regulatory mutants, *hisO*, *hisR*, *hisS* and *hisT*, have been found. However, several mutants remain which do not, at present, fit into the four categories delineated by transduction tests (i.e., show joint transduction with other appropriate markers). This may mean that additional classes remain to be found. Mutant *his-1509* may belong to the *hisR* class since, like some *hisR* mutants, it has a decreased amount of histidine-specific tRNA acceptor capacity (Silbert *et al.*, 1966). Several other possible explanations may apply to other unclassified mutants: (i) they may represent non-transducible chromosomal aberrations; (ii) they may fail to elicit de-repression in the new genetic background; (iii) they may elicit high basal enzyme levels in the new genetic background but are unable to show the wrinkled colony phenotype; and (iv) they may be lethal in the new genetic background. Material is now available to map genetically several of these mutations by conjugation tests. Location of these mutations may reveal whether or not additional genetic classes of TRA-resistant mutations exist. The currently unclassified mutants are: *his-1206*, *-1502*, *-1817*, *-1818*, *-1819*, *-1820*, *-1821*, *-1822*, *-1823*, *-1824* and *-1825*.

(h) *The* purG-metG *region of the* Salmonella *chromosome*

During studies on the map locations of *hisS* and *hisT* mutations, the genetic map of Sanderson & Demerec (1965) has been partially refined (Fig. 5). The orientation of *purG-glyA* was established by Demerec & Ohta (1964). The map also includes

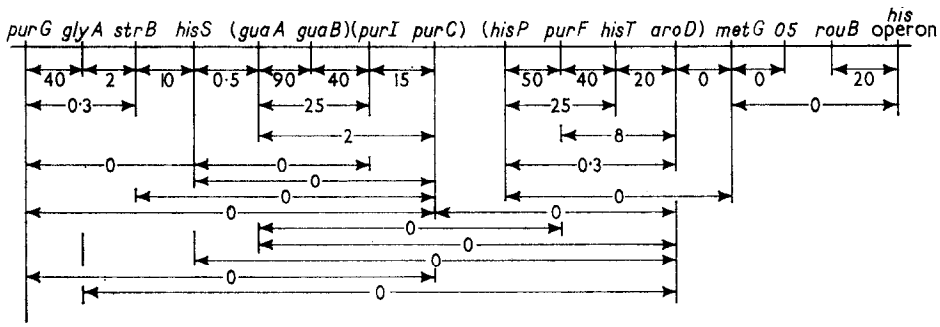


FIG. 5. Revision of a portion of the *Salmonella typhimurium* linkage map.
The map depicts the region of the chromosome designated 65 to 80 by Sanderson & Demerec (1965). Values are approximate co-transduction frequencies. 0 means less than 1% co-transduction. Parentheses indicate that the relative order of markers has not been established. Abbreviations for the markers may be found in Sanderson & Demerec (1965), Shifrin, Ames & Ferro-Luzzi-Ames (1966), or are introduced in this report.

unpublished data of M. Demerec (*purG-glyA* interval), G. FerroLuzzi-Ames (*hisP-aroD* interval) and J. S. Gots (intervals for *guaA-guaB-purI-purC* in extension of data of Ozeki (1959)). Gene locus *cysA*, formerly indicated as being located approximately in this region of the *Salmonella* chromosome (Sanderson & Demerec, 1965), is not linked by P22-mediated transduction tests (less than 1% co-transduction) with *purG*, *strB*, *guaA*, *guaB*, *purI*, *purC*, *purF*, *aroD* or *metG* (Maxine Levinthal, personal communication). The *05* and *rouB* loci, indicated in Sanderson & Demerec (1965) as in this region, are now thought to be located between *metG* and the *his* operon, closer to the *his* operon than to *metG* (Mäkelä, 1965; Johnson, Krauskopf & Baron, 1965; N. Nikaido & Mark Levinthal, unpublished work).

4. Discussion

A search for regulatory mutants for the histidine operon has yielded four genetically distinct classes. These mutants, similar to mutants in other systems, have been called regulatory mutants because of their inability to control normally the function of the related operon. Data presented in this paper and in the two accompanying papers indicate that two of the presumed regulatory genes, *hisS* and *hisR*, are involved respectively in the production of histidyl-tRNA synthetase and histidine-specific tRNA. Thus, these two genes may function solely to produce the "true" end-product of the pathway, histidyl-tRNA. They may not, therefore, have a direct role in the regulatory mechanism and not be true regulatory genes. The finding of so many regulatory genes, and the possibility that two of these may not have a singular regulatory function, emphasizes the difficulty of identifying a true regulatory gene without a knowledge of its biochemical function.

Available data on the regulatory mutants are consistent with several models of the control mechanism. If one considers histidyl-tRNA the co-repressor and *hisT* the regulator gene producing the aporepressor, then the data can be reconciled with the operon model of Jacob & Monod. It should be noted, however, that several *hisT* mutants manifest slower growth rates on excess histidine, a fact inconsistent with a purely regulatory role for the *hisT* gene. Furthermore, *hisT* mutants are not maximally de-repressed, suggesting that none of these mutations completely destroys the capacity to repress the histidine operon.

Several alternative mechanisms for the regulation of the histidine operon have been previously discussed (Roth *et al.*, 1966). We now favor a control mechanism acting at the level of messenger RNA translation in which histidyl-tRNA, or a derivative of it, acts directly to block translation of the histidine operon messenger.

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