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Histidine Regulatory Mutants in *Salmonella typhimurium*

III. A Class of Regulatory Mutants Deficient in tRNA for Histidine

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The *hisR* regulatory mutants in *Salmonella typhimurium* have high levels of the histidine biosynthetic enzymes even when grown in the presence of histidine. Each of the *hisR* mutants examined has approximately 55% of the amount of histidine-tRNA that is present in the wild type. These data suggest that histidine-tRNA has a role in the repression of the histidine operon.

1. Introduction

Studies on the repression mechanism of the histidine operon of *Salmonella typhimurium* have focused on a group of mutants which are de-repressed for the histidine biosynthetic enzymes. These strains were isolated as mutants resistant to triazolealanine, a histidine analogue which represses the formation of the histidine biosynthetic enzymes (Roth, Anton & Hartman, 1966). The mutants have been shown to belong to four genetically distinct classes. One of these classes, the *hisR*, maps near *metE*, far from the histidine operon (Roth *et al.*, 1966). It is the purpose of this communication to describe some properties of the regulatory mutations in the *hisR* gene and to show the *hisR* mutants have less histidine-transfer RNA than the wild-type strain. The finding of decreased tRNA(his)† in the *hisR* class strengthens the evidence in the accompanying papers (Roth *et al.*, 1966; Roth & Ames, 1966) on the role of histidyl-tRNA in repression.

2. Materials and Methods

(a) *Materials*

(i) *Chemicals*

DL-1,2,4-Triazole-3-alanine was a gift of R. G. Jones and C. Ainsworth, Eli Lilly and Co. Crystalline bovine serum albumin and all the [^{12}C]amino acids were products of the California Corp. for Biochemical research. Amino acids uniformly labeled with ^{14}C, or specifically with ^{3}H, were obtained from the New England Nuclear Corp. and included L-proline (185 μc/μmole), L-serine (120 μc/μmole), L-histidine (220 μc/μmole), and L-[3,5-^{3}H]tyrosine (5580 μc/μmole). The following amino acids uniformly labeled with ^{14}C, or labeled as indicated with ^{3}H, were purchased from the Nuclear Chicago Corp.:

† Abbreviations used: tRNA, transfer ribonucleic acid; tRNA(his), transfer ribonucleic acid that can accept L-histidine; L-histidyl-tRNA, transfer ribonucleic acid aminoacylated with L-histidine; TRA, DL-1,2,4-triazole-3-alanine; DEAE-cellulose, diethylaminoethyl cellulose; MAK, methylated albumin kieselguhr; O.D., absorbancy at 260 mμ for a 1-cm light path at 25°C.

L-lysine (190 μc/μmole), L-glutamine (32 μc/μmole), L-aspartic acid (106 μc/μmole), L-isoleucine (174 μc/μmole), L-tyrosine (234 μc/μmole), L-methionine (29 μc/μmole), L-leucine (170 μc/μmole), L-asparagine (30 μc/μmole) and L-[2,5-^{3}H]histidine (38·1 mc/μmole). L-[^{14}C]Glutamic acid (147 μc/μmole) was a product of Schwarz BioResearch, Inc. Sephadex G25, G50, G100, all in medium-sized beads, and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals, Inc. Celite 545 (kieselguhr) was a product of Johns–Manville. Hydroxylapatite (Biogel HT) was purchased through Calbiochem. and DEAE-cellulose, standard type, from Schleicher & Schuell Co. Streptomycin sulfate, U.S.P., was obtained from E. R. Squibb and Sons; ammonium sulfate, enzyme grade, from the Mann Research Laboratories, Inc.; inorganic pyrophosphatase from the Worthington Biochemical Corp., and phenol (88%), analytical reagent, from Mallinckrodt Chemical Works. Metricel, type GA-8, solvent-resistant membrane filters were a product of the Gelman Instrument Co., and Millipore filters, type HA 0·45 μ, a product of the Millipore Filter Corp.

(ii) *Bacteria*

The parental strains were *S. typhimurium* strains LT_2 and LT_7 or their derivatives. Mutant strains *hisG70*, *hisS1520*, *hisO1202hisH107*, *hisO1202*, *hisS1210* and *hisR1203-hisH107* were obtained through the courtesy of John R. Roth and Philip E. Hartman. *HisR1813* was kindly provided by Dora N. Antón and *hisR1200* by Giovanna Ames. *HisR1203* was obtained by transduction of *hisR1203hisH107* with P22 bacteriophage grown on LT_7.

(b) *Methods*

(i) *Preparation of tRNA*

The strains were grown in a 300-l. fermenter on the medium E of Vogel & Bonner (1956) with 0·5% dextrose. When L-histidine, L-histidinol, or adenine was used as growth supplements, they were present in concentrations of 0·1, 0·05 and 0·4 mM, respectively. Cells were harvested in late log phase and stored in 100g portions at −15°C. Yields varied from 0·5 to 1·0 kg wet weight per fermenter lot.

The extraction of tRNA was designed after the phenol method of Kirby (1954) and the DEAE-cellulose chromatographic technique of Holley, Apgar, Doctor, Farrow, Marini & Merrill (1961). 100 g of frozen bacterial cells were placed without thawing into 200 cc of 0·001 M-Tris buffer (pH 7·5) containing 0·01 M-magnesium acetate and 175 cc of 88% phenol, and vigorously agitated at 4°C for 1 hr. Unlike the Holley procedure, the aqueous phase from phenol extraction was introduced directly onto a DEAE-cellulose column (100 cc bed volume) where phenol and polysaccharides could be washed through with 0·02 M-LiCl. Batch elution was then conducted at room temperature with 0·1 M-LiCl and then 1·0 M-LiCl, following optical density at 260 mμ for each eluate until readings were below 0·100. RNA was recovered from the 1·0 M-LiCl eluate by precipitating with 3 vol. of cold alcohol. Yields were consistently 150 mg/100 g wet weight of cells with one exception. Auxotroph *hisG70* grown under conditions of histidine starvation in order to produce de-repression yielded 75 mg RNA/100 g wet weight. Keeping the reagents in the same proportion, the scale of preparation could be varied from a few grams to a kilogram of cells. The tRNA so isolated was free of protein and deoxyribonucleic acid as determined by the Lowry, Rosebrough, Farr & Randall (1951) and diphenylamine (Burton, 1955) procedures, respectively. A 10-mg/ml. solution had an absorbancy at 260 mμ of about 210 O.D. units. Upon examination of *S. typhimurium* wild-type tRNA in the ultracentrifuge, one large peak and a barely perceptible second peak on the faster sedimenting limb was observed. When chromatographed on G100 Sephadex, 90% of the absorbancy at 260 mμ migrated in a single peak midway between the excluded dye (blue dextran 2000) and the retained salts. 10% was present as a faster component moving just behind the indicator front.

The above method of isolation of tRNA was modified slightly to estimate the percentage present as the charged form of tRNA(his) *in vivo*. The scale was adapted to 500-ml. cultures (approx. 1 g wet weight of cells). Extraction procedures were carried out on fresh log-phase cells entirely at 0°C and pH 5·0 using 0·01 M-potassium cacodylate to

buffer both the aqueous phase in the phenol step and the eluants in the DEAE-cellulose chromatography. The 1·0 M-LiCl eluate contained approximately 1·5 mg tRNA in 40 ml. Quantitative recovery was obtained by the addition of 2 vol. of cold 95% alcohol, collection of the tRNA on Gelman GA-8 solvent-resistant filters, pore size 0·20 μ, and elution with 1·5 ml. of 0·2 M-LiCl containing 0·01 M-potassium cacodylate (pH 5·0). The entire extraction from harvest to recovery required 2 hr.

(ii) *Hydrolysis of amino acyl transfer tRNA*

The stability of amino acyl linkages varies widely among the different amino acid tRNA species (Coles, Bukenberger & Meister, 1962; Sarin & Zamecnik, 1964). Many of the amino acyl tRNA linkages are hydrolyzed in the process of isolating tRNA over DEAE-cellulose at *room temperature* and *neutral pH*. For those amino acid tRNA's where chemical stripping could be shown to enhance acceptor capacity, the tRNA was treated with 1·8 M-Tris (pH 8·0) at 37°C for 45 min (Sarin & Zamecnik, 1964) or with sodium hydroxide (pH 10·0) at 37°C for 10 min.

(iii) *Preparation of activating enzymes*

S. typhimurium histidyl-tRNA synthetase was obtained as a by-product during purification of PR–ATP pyrophosphorylase (Voll, Appella & Martin, 1967). Several amino acid synthetases were partially purified by the procedure. The histidyl-tRNA synthetase was obtained from step 4, a G200 column (Voll *et al.*, 1967), and was approximately 100-fold purified. The synthetase was stored for weeks without loss in activity in a solution of 0·1 M-Tris buffer (pH 7·5) containing 0·1 M-NaCl, 0·4 mM-L-histidine, 0·5 mM-EDTA, and 2·8 mM-β-mercaptoethanol at 4°C. The preparation was passed over Sephadex G50 just prior to use, and one μg was more than sufficient to charge fully the tRNA within 15 min in the usual transfer system. Prolonging the incubation for 60 min revealed no loss in acid-precipitable radioactive material, suggesting that the enzyme and tRNA preparations were relatively free of nuclease activity.

A fresh crude extract of *S. typhimurium* was used as the source for other amino acid synthetases. 0·5 g of frozen cells in 2·0 ml. of 0·01 M-Tris buffer (pH 7·0) were disrupted at 0°C with a Branson sonifier, model LS-75 set at position 3 for 30 sec. The supernatant portions were recovered by centrifugation in the cold at 38,000 *g* for 30 min. 20 μg of extract from the Sephadex treatment were used per assay.

Yeast histidyl-tRNA synthetase was prepared from Fleischman's baker's yeast according to the method of Peterkofsky (1964), except that the yeast cells were disrupted in a Gaulin laboratory homogenizer (Manton-Gaulin Manufacturing Corp.), a continuous-flow French pressure cell. The yeast protein was separated on a DEAE-cellulose column and the fractions with maximum activity were used. The addition of 100 μg of protein gave full tRNA(his) charging in 30 min. Incubation for 80 min did not lead to any loss in acid-precipitable radioactive material.

(iv) *Enzyme assays*

Assay for amino acid-acceptor activity. This assay was based on the method of Berg, Bergmann, Ofengand & Dieckman (1951). The reaction mixture contained the following components in a final volume of 0·25 ml. at pH 7·0: 20 μmoles potassium cacodylate; 1·0 μmole $MgCl_2$; 0·5 μmole ATP; 20 mμmoles of each of 19 [^{12}C]amino acids; 1 to 3 mμmoles of the appropriate [^{14}C]amino acid; 0·1 to 2·0 O.D. units (260 mμ) of tRNA; 25 μg crystalline bovine serum albumin; and 1, 20 or 40 μg of partially purified *Salmonella* enzyme, crude *Salmonella* extract, or partially purified yeast enzyme, respectively. One μmole of reduced glutathione was added to the mixture when yeast histidyl-tRNA synthetase was used. Controls contained no tRNA. The reaction was started by the addition of tRNA or enzyme to the incubation mixture at 37°C, and after 30 min (sufficient time to charge tRNA(his) in all systems examined) it was stopped with 2·0 ml. of cold 5% trichloroacetic acid. The trichloroacetic acid precipitates were collected on membrane filters (Millipore, 0·45 μ), dried and glued to planchets, and counted. Net counts were converted into $\mu\mu$moles of amino acid accepted per 1 O.D. tRNA.

The $\mu\mu$moles of amino acid accepted were proportional to tRNA added.† All determinations reported represent the average of at least duplicate determinations with less than 10% variation.

(v) *Chromatographic procedures*

Gel filtration was carried out using columns (2·4 cm × 100 cm) of Sephadex G100 equilibrated with 0·7 M-LiCl containing 0·02 M-acetate (pH 4·0). The Sephadex was repeatedly de-fined in 0·5 M-LiCl and was allowed to swell for 24 hr prior to pouring the column. Under 50 cm water pressure, the column had a flow rate of 0·65 ml./min. Fractions of 4·5 ml. were collected.

Chromatography on MAK is described in the legend to Fig. 2. DEAE-cellulose–Sephadex and hydroxylapatite fractionations of tRNA were performed according to the methods of Smith (1966) and Pearson & Kelmers (1966), respectively.

3. Results

(a) *Altered specific activity of histidyl-tRNA in the* hisR *class of regulatory mutants*

Triazolealanine-resistant mutants mapping at the *hisR* locus of *S. typhimurium* have approximately 55% of the tRNA(his) present in the isogenic wild-type organism. Table 1 illustrates this observation with one pair of isogenic strains. The specific

TABLE 1

Transfer RNA activity for histidine in a hisR *mutant*

Source of tRNA	Quantity assayed (O.D. unit)	Histidine accepted ($\mu\mu$moles)	Acceptance relative to wild type (%)
Wild type	1·0	23·8	100
hisR1813	1·0	12·0	51
Wild type + *hisR1813*	1·0 + 1·0	36·0	152

Activity is based on the average of duplicate determinations with less than 10% variation. 3×10^{-5} M-L-[^{14}C]histidine was used in the assay.

activities for the two preparations were constant over the range of 0·1 to 2·0 O.D. of unfractionated tRNA added per assay volume of 0·25 ml. In addition, when assayed together, the activities of the two preparations were additive. This latter finding suggests that the *hisR1813*-tRNA does not contain an inhibitor responsible for the reduced acceptor capacity.

Figure 1 shows that both wild-type and *hisR* mutant tRNA are fully charged by 15 minutes and, furthermore, that between 15 minutes and 30 minutes there is no loss of acid-precipitable radioactive materials.

† The absolute extent of charging tRNA(his) was influenced by concentration of L-[^{14}C]histidine in the assay. The effect occurred with both wild-type and mutant tRNA(his) and did not affect the ratio of their activities. Unless indicated otherwise, 4×10^{-6} M-L-histidine was used in the assay.

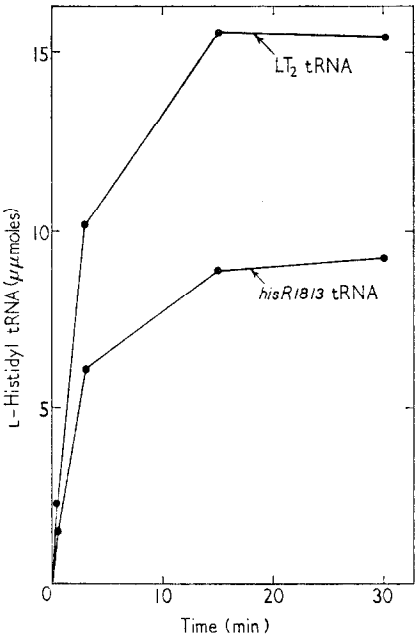

FIG. 1. Amino acylation with L-[^{14}C]histidine of equal amounts of unfractionated tRNA from wild type (LT_2) and *hisR1813* cells.

The assay system contained 1 O.D. of tRNA. Identical curves were obtained with either 20 μg of crude extract protein or 5 μg of purified enzyme. Each point represents the average value of duplicate determinations.

(b) *tRNA activity in regulatory mutants for histidine biosynthesis*

Regulatory mutants mapping in the *hisR* region of *S. typhimurium* chromosome all have reduced histidine-acceptor capacity. The *hisR* mutants also appear to have a slight increase in acceptor activity for a number of other amino acids. Regulatory mutants mapping outside of this region do not show either of these changes (Table 2). The findings indicate clearly that the *hisR* mutation simultaneously produces de-repression and lowers specifically the tRNA(his) activity. The *hisR* mutants have the wild-type doubling time, and addition of L-histidine to the growth medium does not correct either enzyme or tRNA levels. Twelve other amino acid tRNA activities have been examined, and none shows a lowering of activity. Four tRNA species studied closely, appear to have 120 to 140% of the wild-type levels (Table 2). Three other species (asparagine, arginine and lysine) also have increased acceptor capacity, three species (proline, serine and leucine) probably are unchanged, and two species (methionine and glutamic acid) require further work to establish valid assay conditions.

Regulatory mutants of the other three classes (*hisO*, *hisS* and *hisT*) showed no change in their tRNA(his) levels and, where measured, no change in the activity of other tRNA species (Table 2).

Changes in tRNA levels similar to those of the *hisR* class but less marked also were observed with *hisG70*. This strain, containing a point mutation in the first enzyme of the histidine operon, was de-repressed by growth on inadequate amounts of histidine. Although the tRNA levels in *hisG70* resemble those of the *hisR* regulatory mutants, one cannot definitely conclude that these changes are part of physiological

TABLE 2

Transfer RNA activity in regulatory mutants

Strain	Growth condition	Doubling time	Extent de-repressed	tRNA activity (% wild type grown on M)					
				His	Tyr	Gln	Asp	Ile	Pro
Wild type LT_2	M†	48	1	100	100	100	100	100	100
	H†	48	1	100					
Regulatory mutants									
hisR1813	M	48	6	54	134	128	132	115	93
				55	128				99
				57	126				
				60	125				
				51					
				50					
				48					
	H	48		58					
hisR1203	M	48	8	56					
hisR1200	M	48	8	57		124	117		
	H		7						
hisT1501	M	58	6	102	104			95	
				112					
				97					
hisO1202	M	48	6	104					
hisO1202 *hisH107*	H	48	6	98	98				
				103					
				96					
hisS1520	H	48	1	103					
hisS1210	M	48	8	101					
				107					
				93					
	H	48	2	102					
De-repressed *hisG70*	Ad,† HOL	300	17	74	115		116	118	102
				79					

The extent of de-repression was measured by the activity of histidinol dehydrogenase and/or histidinol phosphate phosphatase. These assays were performed as previously described (Ames, Hartman & Jacob, 1963). The description of the phosphatase assay has 0·22 ml. of substrate as a misprint for 0·02 ml.; currently, 0·01 ml. is used.

Strains *hisR1203*, *hisR1200*, *hisO1202* and *hisS1210* were derived from the wild-type strain LT_7; all other strains were from wild-type LT_2. *HisR1200* and *hisR1203* are lysogenic for bacteriophage P22. Wild-type strains LT_2 and LT_7 have growth rates, enzyme levels, and tRNA activities within 3% of each other and lysogenization of strains with P22 does not affect these values.

tRNA activity was determined in duplicate. The scatter of values obtained from assays on different days or with different preparations is given for tRNA(his) in the several classes of de-repressed mutants and tRNA(tyr) and tRNA(pro) in the *hisR* class.

† M, H and Ad, HOL stand for miminal E medium supplemented respectively with (i) 0·5 g glucose/100 g, (ii) 0·5 g glucose/100 g and 0·1 mM-L-histidine, or (iii) 0·5 g glucose/100 g, 0·005 mM-L-histidine, 0·05 mM-L-histidinol and 0·4 mM-adenine.

de-repression, because this auxotroph was grown at an abnormally slow rate (300 min doubling time). A better study might utilize the wild-type organism under conditions where de-repression permits the bacterium to overcome simultaneously an inhibitor of one of the biosynthetic enzymes and restore the normal growth rate.

Strain *his-1509*, derived from LT_2, is one of a few regulatory mutants that have not been successfully mapped due to technical difficulties (Roth *et al.*, 1966). It resembles the *hisR* mutants biochemically in that it has their tRNA levels (60% of the wild type for His, 136% for Asp, 124% for Ile). However, this strain is unlike the *hisR* mutants in that it is only slightly de-repressed.

(c) *Effect of triazolealanine on the formation of histidyl-tRNA*

The regulatory mutants were selected as resistant to the histidine analogue, triazolealanine, which is known to be incorporated into protein (Levin & Hartman, 1963). It was of interest, therefore, to see *in vitro* (i) what effect TRA had on the formation of histidyl-tRNA, and (ii) if mutant resistance to the analogue could be explained by a loss of sensitivity at this level. TRA was observed to affect the extent of charging tRNA(his) but not the rate. At TRA concentrations considerably in excess of the histidine concentration, the total incorporation of histidine was decreased (e.g. 50-fold excess gave 50% inhibition). The behavior of tRNA(his) from the *hisR* mutants and also the other classes of regulatory mutants was identical to that of the wild-type tRNA. Presumably TRA is incorporated into protein via tRNA(his). It appears then that TRA resistance is not due to a loss of TRA charging to tRNA. In the *hisR* class, the lowered tRNA(his) is not a consequence of the removal of a tRNA(his) which accepts TRA.

(d) *Investigations for inactive forms of histidine tRNA*

A number of types of inactivation has been examined and none of these appears to explain the decrease in tRNA(his) of the *hisR* class.

(i) It might be imagined that a fraction of the tRNA(his) was acylated with a substance other than histidine, which is difficult to hydrolyze. This possibility has not been completely ruled out, but a variety of hydrolysis conditions has shown no differences between *hisR* and wild-type.

(ii) In general, tRNA(his) in the *hisR* preparations appears to have its -CCA end intact. No difference in charging between partially purified synthetase and crude extracts has been observed. The presence of CTP as well as ATP in the assay containing crude extract did not improve histidine incorporation, although an increase would be expected if the -CCA end for some chains needed to be regenerated.

(iii) The difference in acceptor capacity of wild-type and mutant tRNA(his) might reflect modification of the tRNA by enzymic activity present in one strain but not in the other. With the possibility that such a difference might be detected in crude extracts, histidine acceptor capacity for tRNA from wild-type and mutant organisms was determined using histidyl-tRNA synthetase from wild type, regulatory mutant strains *hisR* and *hisS* (the latter have reduced histidyl-tRNA synthetase with increased K_m for histidine), and auxotrophs de-repressed by growth on limiting histidine. This study failed to show any qualitative difference in transfer activity between extracts.

(iv) Several mechanisms which partially inactivate specific tRNA species are known. As described below, none of these appears to contribute to the reduced

tRNA(his) in the *hisR* mutants. Carbon, Hung & Jones (1965) demonstrated that mild treatment with iodine led to partial inactivation of tRNA(lys), tRNA(phe), and tRNA(ser). The inactivation was associated with oxidation of thiopyrimidine bases, and the tRNA activity could be regenerated with several reducing agents. Transfer tRNA(his) activity in both wild type and *hisR* strains was not affected by oxidation with iodine nor by reduction with β-mercaptoethanol (0·3 M) or sodium thiosulfate (in 8·0 M-urea buffered at pH 8·5). Heating tRNA to 60°C in the presence of magnesium ions according to the method of Lindahl, Adams & Fresco (1966) did not produce any change in tRNA(his) activity of either wild type or *hisR* mutant. It might be noted that tRNA in this study was not exposed to chelating agents during its isolation, unlike that prepared by Lindahl *et al.* (1966).

(e) *Chromatography of transfer tRNA from wild-type and* hisR *mutants*

Using a number of the available methods for fractionating transfer RNA, it has not been possible to demonstrate a qualitiative difference between the wild type and *hisR1813* tRNA(his). However, the quantitative differences between crude tRNA from wild type and the *hisR* mutants have been borne out in all the chromatographic procedures. This finding, in addition to the evidence presented in Table 2, rules out the possibility that the reduced specific activity of the *hisR* mutants is a consequence either of contamination with ribosomal RNA or of a general increase in other tRNA species.

Figure 2 shows a double-labeling experiment, following histidyl-tRNA from wild type and *hisR1813* on a MAK column. The profiles are nearly identical and depict one peak of tRNA activity with a slight trailing of the descending limb. The ratio of *hisR1813* to wild-type activity increases from 0·61 on the left side of the peak to 0·79 on the right. This small change in the ratio cannot be taken as strong evidence, however, of a difference in histidyl-tRNA composition between wild type and mutant. Formation of aggregates (Schleich & Goldstein, 1964,1966) could easily account for the trailing. In several runs of wild-type histidyl-tRNA on MAK columns, trailing was a variable feature and at times negligible.

That several amino acid-tRNA activities are increased in the *hisR* mutant (Table 2) might be explained by an altered specificity in the acceptor activity of part of the tRNA(his). Since tyrosyl-tRNA is well resolved from histidyl-tRNA on an MAK column (Sueoka & Yamane, 1962), a double-labeling experiment identical to that given in Fig. 2 was performed to see if the increased *hisR1813* tyrosyl-tRNA activity chromatographed in the histidyl-tRNA region. The two tyrosyl-tRNA profiles were congruent; the descending limb trailed; and the ratio of *hisR1813* to wild type was 1·4 across the peak. Thus, the enhanced activity of the mutant was confirmed, but there was no evidence that it migrated as a separate peak, for example, at the site of histidyl-tRNA.

Transfer RNA was fractionated on DEAE-cellulose–Sephadex according to the method of Smith (1966). Employing this technique with a double linear gradient of 0·53 to 0·90 M-LiCl and 0·02 M-acetate buffer, (pH 3·5 to 4·5), only one tRNA(his) peak was found. On some columns this peak was followed by a shoulder or a second low peak; however, the latter was redistributed on an identical DEAE-cellulose–Sephadex column largely with the first peak and rechromatographed on a G100 in part with the bulk of tRNA and in part ahead of it. Combining preliminary G100 chromatography followed by DEAE-cellulose–Sephadex fractionation, an eightfold

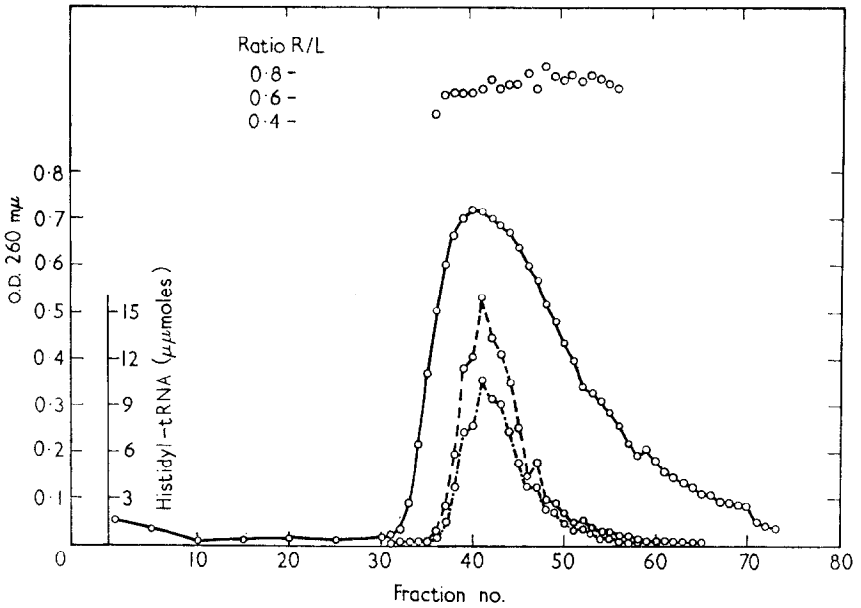

FIG. 2. MAK chromatography of histidyl-tRNA from wild type and *hisR1813* strains.

The column was prepared as described by Mandell & Hershey (1960). Dimensions were 22 mm × 200 mm and the actual bed volume was 45 ml., equilibrated in 0·2 M-NaCl containing 0·05 M-$NaHPO_4$ (pH 6·7). L-[^{14}C]histidyl-tRNA from wild-type tRNA and L-[^{3}H]histidyl-tRNA from *hisR1813*-tRNA were prepared in a transfer system containing the following components in a final volume of 1·0 ml. at pH 7·0: 80 μmoles potassium cacodylate; 4·0 μmoles $MgCl_2$; 2·0 μmoles ATP; 80 mμmoles of each of 19 [^{12}C]amino acids; 18·0 mμmoles of L-[^{14}C]histidine or 1·05 mμmoles of L-[2,5-^{3}H]histidine; 20·0 O.D. of tRNA; and 40 μg of partially purified *Salmonella* histidyl-tRNA synthetase. After 30 min incubation at 37°C, 0·1 ml. of 2% potassium acetate (pH 5·0) and 1·1 ml. of 88% Mallinckrodt phenol were added to each reaction. The two systems then were combined at room temperature for 5 min and centrifuged at 38,000 *g*; the aqueous phase was decanted by suction, filtered over Sephadex G50, and applied to the column in 30 ml. of 0·2 M-NaCl containing 0·05 M-$NaHPO_4$ (pH 6·7) under a pressure of 3 lb./in^2.

Following the method of Sueoka & Yamane (1962), tRNA was eluted at room temperature using a gradient from 0·2 M-NaCl (110 ml.) containing 0·05 M-$NaHPO_4$ (pH 6·7) to 1·2 M-NaCl (110 ml.) containing 0·05 M-$NaHPO_4$ (pH 6·7). A flow rate of 1 ml. per min was maintained with a Durrum pump. 2-ml. fractions were collected and O.D. at 260 mμ was read concurrently. Radioactive material was recovered from each fraction as follows: 100 μg of crystalline bovine serum albumin and 2 ml. of 10% perchloric acid were added to produce a precipitate which was collected on membrane filters (Millipore type HA). The filters were washed with 1 N-HCl, dried thoroughly, and counted in a Nuclear-Chicago dual-channel scintillation counter. Counts were corrected for efficiency, quenching and overlap, and converted to μμmoles L-histidine per fraction.

—○—○—, O.D. 260 mμ; --○--○--, LT_2 [^{14}C]histidyl-tRNA; —·○—·—○—, *hisR1813*-[^{3}H]histidyl-tRNA.

enrichment in the peak fractions of tRNA(his) was obtained with negligible loss in acceptor activity. Both the wild-type and *hisR* mutant tRNA yielded one symmetrical peak eluting in identical fractions. The *hisR1813* to wild-type ratio of 0·60 was preserved.

Hydroxylapatite chromatography also has confirmed the quantitative difference, but has not demonstrated any qualitative change between the mutant and wild type.

We are indebted to B. P. Doctor for counter-current chromatographic fractions of *Escherichia coli* B tRNA. These fractions were obtained after two serial 225 transfer distributions in different solvent systems. There was evidence of only one tRNA(his).

(f) *Charging of* S. typhimurium *tRNA(his) by a synthetase from yeast*

Structural differences in tRNA preparations can sometimes be recognized through the use of a heterologous source for the tRNA synthetase (Berg *et al.*, 1961). This discrimination is most convincing when the heterologous enzyme compared to the homologous one fails to charge one of multiple tRNA sub-species for a given amino acid (Bennett, Goldstein & Lipmann, 1963,1965; Yamane & Sueoka, 1963). The existence of subspecies which have not been fractionated may be suspected if heterologous enzyme only partially charges a given tRNA species and provided that premature termination of aminoacyl-tRNA formation due to enzyme inactivation or to nuclease activity can be excluded.

Using histidyl-tRNA synthetase from baker's yeast and from *S. typhimurium*, respectively, *S. typhimurium* wild-type tRNA accepts 7·7 $\pm$ 0·4 (standard error of mean) and 8·8 $\pm$ 0·6 $\mu\mu$moles of L-histidine per 0·5 O.D. unit; by a similar comparison *hisR1813*-tRNA accepts 4·5 $\pm$ 0·3 and 4·4 $\pm$ 0·5 $\mu\mu$moles of L-histidine per 0·5 O.D. unit. Thus, both enzymes charge *S. typhimurium* tRNA(his) from either source to the same extent, and thus this method provides no indication of more than one tRNA(his) species.

(g) *Percentage of charged tRNA(his)* in vivo

Table 3 demonstrates that the lowered total acceptor capacity of *hisR1813* probably reflects *in vivo* a proportional decrease of charged as well as total tRNA(his). The percentage charging of tRNA(his) is not substantially altered relative to wild type, but there is considerably less acylated tRNA(his) in the mutant.

TABLE 3

Estimation of acylated transfer RNA(his) in vivo

	tRNA source		
	Wild type		*hisR1813*
	Exp. 1	Exp. 2	Exp. 1
Total	32·0†	27·3	12·9 (40)‡
Unacylated	6·9	8·6	2·7 (39)
Acylated	25·1	18·7	10·2 (41)
% Acylated	79	69	79

Data are based on average values of duplicate determinations with less than 5% variation. 1×10^{-5} M-L-[^{14}C]histidine was used in the assay.

† Numbers to one decimal place represent $\mu\mu$moles L-histidine accepted per O.D. unit of tRNA.

‡ Numbers in parentheses are *hisR1813* values expressed as percentage of wild-type values for the same experiment.

As described in Methods, the tRNA was isolated rapidly from cells in log phase under conditions stabilizing preformed aminoacyl linkages. The amount of uncharged tRNA(his) was estimated in the following fashion immediately after isolation of the tRNA and prior to chemical stripping. The kinetics of charging were plotted for 30 minutes to show that there was a phase of rapid charging followed by a slow but further increase in [^{14}C]histidine incorporation. Since enzymic exchange due to the

generation of AMP and pyrophosphate was prohibited by the presence of pyrophosphatase in the assay, this second phase probably represented gradual chemical hydrolysis of [^{12}C]histidyl-acyl linkages. The slope of this slow charging was extrapolated to zero time and the value of the ordinate at this point taken as the best approximation of the uncharged fraction. Total acceptor capacity was determined following chemical stripping of the preparation, and the amino acylated fraction was calculated as the difference between total and uncharged values.

The specific activity of *hisR1813*-tRNA(his) isolated under these modified conditions is in good agreement with that obtained using frozen cells, more prolonged extraction, and chromatography at room temperature and neutral pH (Table 1). The wild-type histidine-acceptor capacity for tRNA isolated from fresh cells (32·0 $\mu\mu$moles per O.D.) is greater than that from frozen cells (23·8 $\mu\mu$moles per O.D.). This observation has been found repeatedly and might reflect a relatively labile component in the wild-type tRNA(his); however, there is no direct evidence that wild-type tRNA(his) is more readily inactivated than *hisR*-tRNA(his) by exposure to alkaline pH or preincubation with crude extract.

The percentage acylation of total tRNA(his) reported here for log-phase *S. typhimurium* is very similar to the value reported by Yegian, Stent & Martin (1966) for *E. coli* cells, although the methodology of the two estimations is considerably different. A number of important controls are needed to show that neither additional charging nor deacylation occurs during tRNA isolation and, therefore, that the present study constitutes a valid measurement of the *in vivo* value for charged histidine tRNA.

4. Discussion

(a) *Evidence that the* hisR *mutation results in a loss of tRNA(his) activity*

The present investigation demonstrates that the *hisR* class of regulatory mutants for the histidine operon contains only 55% of the wild-type amount of tRNA(his). The other classes of regulatory mutants (O, S and T) have normal levels of histidine tRNA. This decrease in tRNA(his) in the *hisR* mutants seems to be due to an actual decrease of the tRNA(his) species relative to the total tRNA, rather than some decreased specific activity due to contaminating nucleic acid that is not tRNA. This conclusion is based on chromatography of tRNA from wild type and from the *hisR* mutant on many different columns. Chromatography showed that the acceptor capacity of the *hisR*-tRNA(his) is only 55% of that of the wild type, although the bulk tRNA O.D. profiles are the same. The chromatography of equal amounts of tRNA from a *hisR* mutant and from wild type on a G100 Sephadex column, and then on a DEAE-cellulose–Sephadex column yielding an eightfold purification of the tRNA(his), preserved the *hisR* to wild type ratio of about 0·55 in all the fractions. A double-labeling experiment on a MAK column showed that this ratio was preserved in the region where only tRNA is eluted.

In addition to the decrease in tRNA(his), the *hisR* mutants have somewhat increased activity of tRNA for several other amino acids. The small increase of tRNA species other than histidine in the *hisR* cannot account for the marked decrease in the specific activity of tRNA(his). A doubling of all the other tRNA species would be necessary to lower the specific activity of tRNA(his) by half. In addition, as noted in the Methods section, the yield of tRNA isolated from the *hisR* mutants was identical to that from wild type, indicating no general increase in cellular tRNA.

(b) *Number of transfer RNA(his) species*

Chromatographic fractionation of tRNA on MAK, DEAE-cellulose–Sephadex, and hydroxylapatite and gel filtration on G100 Sephadex did not reveal two tRNA(his) species or show a qualitative difference between mutant and wild-type tRNA(his) accounting for the difference in specific activity. There is evidence of only one tRNA(his) in *E. coli* by countercurrent fractionation. This conclusion is based on an examination of fractions obtained from B. P. Doctor and on published results of Goldstein, Bennett & Craig (1964). Assay with a heterologous enzyme also failed to resolve tRNA(his) into two or more components. In contrast to an earlier report, in *E. coli* (Doctor & Mudd, 1963), there was no difference between yeast and *S. typhimurium* histidyl-tRNA synthetases in the extent to which they charged *S. typhimurium* tRNA(his). The failure to demonstrate more than one histidyl-tRNA by these several procedures, nevertheless, does not eliminate the possibility that there are two very similar species, not easily separable by available methods, and that one of these is inactivated by the *hisR* mutation.

Another approach to the problem of whether there are two histidine-tRNA species is the comparison of the tRNA(his) from *hisR* and wild type by a codon-binding analysis. This has been done by M. Nirenberg (unpublished observations) on preparations furnished by this laboratory. He has found no differences between *hisR* and wild type. Trinucleotides CAU and CAC stimulated ribosome binding of histidyl-tRNA from unfractionated tRNA, and the extent of binding was the same for the tRNA from mutant and wild type. In addition, histidyl-tRNA(his) from both sources responded with moderate ambiguity to the polynucleotide UC. Thus, if there are two species, they probably have similar if not the same anti-codons, and similar structures.

(c) *Nature of the product of the* hisR *gene*

(i) *Is the* hisR *gene a structural gene for an enzyme involved in the biosynthesis of several transfer RNA's?*

To convert nascent tRNA to its final mature structure involves many enzymes (Hurwitz, Gold & Anders, 1964; Srinivasan & Borek, 1964; Comb & Katz, 1964). If some of these enzymes work on a number of different tRNA species, it is possible that an altered or missing enzyme could affect several different types of tRNA. Such a change might explain the finding in the *hisR* class of regulatory mutants that the level of tRNA(his) is reduced and tRNA activity for several amino acids (though not all) is simultaneously increased.

However, there appears to be no striking difference between unfractionated preparations of tRNA from wild type and *hisR* strains in their content of minor nucleotides (Robert Holley, unpublished observations). Thus, if the *hisR* mutation does affect one of the tRNA biosynthetic enzymes, it is not reflected in a gross change in minor nucleotides.

(ii) *Is the* hisR *gene the structural gene for tRNA(his)?*

If there were two very similar species of tRNA(his), one of them (a 45% component) could be coded for by the *hisR* gene and be missing in the *hisR* mutants. However, if there were only one species, then it would also be necessary to say that the altered tRNA(his) was inactivated partially so that only 55% remained in the

several mutants. Neither of these alternatives would easily explain the increases in the other tRNA species occurring along with the decrease in tRNA(his).

The isolation and characterization of tRNA(his) should help to decide between hypotheses (i) and (ii).

(d) *Role of tRNA(his) in regulation of the histidine operon*

The present finding that the amount of tRNA(his) is lowered in the *hisR* class of regulatory mutants again focuses attention on the tRNA(his) as being important in repression. This work supports the conclusion reached in the accompanying papers (Roth *et al.*, 1966; Roth & Ames, 1966) that the involvement of histidyl-tRNA in represssion is more direct than that of free histidine.

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