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Isolation of the Self-Regulated Repressor Protein of the *Hut* Operons of *Salmonella typhimurium*

(histidine utilization/DNA-binding/urocanate/λ*phut*)

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ABSTRACT In *Salmonella typhimurium* the structural genes of the enzymes responsible for histidine utilization (*hut*) are clustered in two adjacent operons. A single repressor regulates both operons. The repressor itself is a member of one of the *hut* operons and, thus, regulates its own synthesis. We have assayed the *hut* repressor by its ability to bind radioactive DNA to nitrocellulose filters. The binding is specific for DNA bearing the *hut* operons, and the binding is abolished by the inducer, urocanate. As a member of one of the *hut* operons, the repressor is inducible, subject to catabolite repression, and affected by a promoter mutation.

Earlier work from this laboratory has shown that the genes coding for the enzymes of histidine utilization (*hut*) in *Salmonella typhimurium* are clustered in two adjacent operons: *hutMIGC hutPUH* (1–5) (Fig. 1). A single repressor, the *hutC* gene product, regulates both operons. Urocanate, the first degradation product of histidine, is the inducer and presumably acts by inactivating the repressor.

A unique feature of the *hut* repressor is that it regulates its own synthesis, i.e., it is a member of one of the operons that it regulates (6). The evidence for this hypothesis is twofold: strains in which *M*, the left-hand operon promoter, has been deleted simultaneously lack I and G, the left-hand operon enzymes, and are constitutive for U and H, the right-hand operon enzymes. "Super-promoter" mutations of *M* with 3- to 5-fold increased amounts of I and G enzymes have depressed amounts of U and H enzymes. The depression of U and H is a result of the increase in the amount of repressor and can be relieved by point mutations in the *C* gene.

A method developed by Riggs *et al.* (7, 8) has made it possible to assay repressors in cell extracts. The principle of this method is retention by repressor of labeled DNA containing the appropriate operator region on a nitrocellulose filter. Smith (5) had previously isolated λ*phut* transducing phages, and we were thus in a position to prepare the labeled DNA required for the assay of the *hut* repressor. The results reported in this paper show that a repressor protein with the properties indicated by our earlier studies is present in extracts of cells carrying the *hutC*$^+$ allele.

MATERIALS AND METHODS

Chemicals. The following chemicals were purchased from Calbiochem: urocanic acid·H_2O, dihydrourocanic acid (imidazole propionic acid), L-histidine·HCl·H_2O, chicken-blood DNA, bovine-pancreatic DNase, bovine-pancreatic RNase, Pronase, and Cleland's reagent (dithiothreitol). The L-histidine·HCl·H_2O used in growth media and Tris were products of Sigma Chemical Co. Whatman P11 cellulose phosphate was purchased from Reeve Angel; fine particles were removed by settling and decantation, and the ion-exchanger was washed with acid and base and equilibrated with buffer before use. 3',5'-Cyclic adenosine monophosphate (P. L. Biochemicals), Bacto casamino acids, vitamin-free (Difco), bovine-serum albumin (Miles Laboratory), and carrier-free $H_3{}^{32}PO_4$ (New England Nuclear) were also commercial preparations. All other chemicals used were of reagent grade.

Strains of Bacteria and Bacteriophage. Escherichia coli strain GS156, Gal$^-$Str^{R}T1^{R}λ^R [λ*b2cI857Sam7*, λ*phut*36 (*hut*$^+$)*cI857Sam7*], was isolated in our laboratory. This

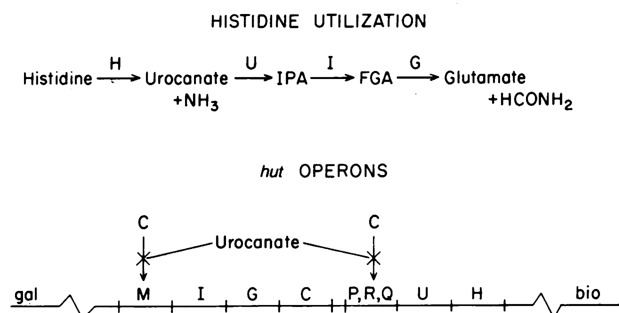

FIG. 1. Histidine utilization and the *hut* operons of *Salmonella typhimurium*. Histidine is degraded by *S. typhimurium* in four enzymatic steps to glutamate, ammonia, and formamide. The *hut* genes of this biochemical pathway are linked and lie between *gal* and *bio* on the *Salmonella* map. *HutMIGC* constitutes the "left-hand" operon and codes for the third enzyme, 4-imidazolone-5-propionate amidohydrolase ("I") (EC 3.5.2.7), the fourth enzyme, *N*-formimino-L-glutamate formiminohydrolase ("G"), and the repressor protein, the *C*-gene product. *Hut(P, R, Q)UH* constitutes the "right-hand" operon and codes for the second enzyme, urocanase ("U") and the first enzyme, L-histidine ammonia-lyase (histidase, "H") (EC 4.3.1.3). *M* is the promoter of the left-hand operon; M^{++} mutants have 3- to 5-fold increased amounts of I and G enzymes. P^-, R^-, and Q^- are mutations of the promoter–operator region of the right-hand operon. P^- mutants lack U and H enzymes; R^- mutations render U and H partially insensitive to catabolite repression; and Q^- mutations render U and H partially constitutive and partially insensitive to catabolite repression. Urocanate, the first decomposition product of histidine, is the inducer and, presumably, inactivates the repressor.

Abbreviations: Symbols in roman type indicate enzymes or phenotype, while symbols in italic type indicate genes or genotype.

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TABLE 1. *Comparison of repressor specific activity to G, H, and U enzyme specific activities*

Exp.	Strain	Relevant genotype		Growth medium		Specific activities			
		Chromosome	Episome	Inducer	Glucose	Repressor	G Enz.	H Enz.	U Enz.
1	NE449	*hutM* ++	*hutM* ++	−	−	73*	830	22	
	NE449	*hutM* ++	*hutM* ++	+	−	220*	4900	480	
	NE449	*hutM* ++	*hutM* ++	+	+	110*	1800	52	
2	NE413	*hut* del.	*hut* +	+	−	66	390	730	
	NE606	*hut* del.	*hutC* −	+	−	0	790	970	
	NE547	*hut* del.	*hut* del.	+	−	0	10	2	
	NE446	*hut* del.	*hutM* ++	+	−	140	2500	420	
	NE449†	*hutM* ++	*hutM* ++	+	−	220*	4900	480	
3	NE413	*hut* del.	*hut* +	−	−	24	280	34	5
	NE413‡	*hut* del.	*hut* +	+	−	66	390	730	150
	NE606	*hut* del.	*hutC* −	−	−	0	630	1130	156
	NE606§	*hut* del.	*hutC* −	+	−	0	790	970	177
	NE607	*hut* del.	*hutU* −	−	−	93	650	870	0
	NE607	*hut* del.	*hutU* −	+	−	92	500	1000	0

The cells were grown on succinate–ammonia minimal medium (SN) to which 0.2% histidine was added as inducer, where indicated, and 0.4% glucose was added for catabolite repression, where indicated. Extracts were prepared as described in *Methods*. G, H, and U enzymes were assayed at the "crude extract" stage of purification; specific activities are given as nmol of substrate consumed or product formed per min per mg of protein. Repressor was assayed at the "cellulose phosphate" stage; specific activity was determined from the slope of the initial, linear portion of the protein saturation curves (curves in which the background binding—binding in the presence of urocanate—had been subtracted) and is expressed as mol of λ*phut* DNA bound $\times 10^{16}$/mg of protein. Strain NE449, a strain diploid for the *hut* region, has the following genotype: *hutM145, hutR9, bio-25* (F′ *gal*+, *hutM145, hutR9, bio*+). Strains NE413, NE606, NE547, NE446, and NE607 constitute an isogenic series; they are strains derived from the same F− parent, NE264, into which episomes of the indicated *hut* genotype have been mated. NE264 carries the deletion *hut**162*, which includes *chl, hut, bio,* and *uvrB*. The episomes of strains NE413, NE606, NE446, and NE607 carry *gal*+ and *bio*+ in addition to the *hut* region. The episome of strain NE547 carries *gal*+ and *bio*+, but lacks all *hut* genes. The right-hand operon promoter allele, *hutR9*, is present here in all *hut* genomes and is considered as "wild-type" in this paper. All of the strains are lysogenic for phage P376.

* These values of repressor specific activity have been obtained from saturation curves corrected for *uvrB*-nuclease activity (see text).

† Relisted from Exp. 1, line 2.

‡ Relisted from Exp. 2, line 1, with U enzyme specific activity added.

§ Relisted from Exp. 2, line 2, with U enzyme specific activity added.

strain, a derivative of the nonlysogen M65 obtained from E. Signer, is simultaneously lysogenic for bacteriophages λ*b2cI857Sam7*, obtained from E. Signer, and λ*phut36*(*hut*+)-*cI857Sam7*, from our laboratory (5). Both phages carry *cI857*, a thermoinducible λ allele, and *Sam7*, a lysis-defective λ allele. *E. coli* strain M5107, Gal−Str^R^T1^R^λ^R^ (λ*cI857Sam7*), was also a gift of E. Signer. The *S. typhimurium* strains NE413, NE446, NE449, NE547, NE606, and NE607 are described in Table 1. These *Salmonella* strains were constructed in this laboratory by techniques reported elsewhere (1, 2, 4).

Media and Solutions. LPC broth contains 1.0% Bacto vitamin-free Casamino acids, 0.25% NaCl, 1 mM $MgSO_4$, and 0.5 μg of vitamin B1 per ml. The phosphorus content was reduced to 4 μg/ml by precipitation of phosphate as NH_4MgPO_4, and the pH was adjusted to 7.0.

Succinate–ammonium minimal medium, SN, has been described (3). SNH medium is SN plus 0.2% histidine, and GSNH is SN plus 0.4% glucose and 0.2% histidine.

Extract buffer, PM, is 10 mM potassium phosphate–10 mM 2-mercaptoethanol (pH 7.5); PMY is PM plus 5% glycerol; and PMSY is PM plus 5% glycerol and 0.4 M KCl. TDY contains 10 mM Tris·HCl–0.1 mM dithiothreitol–5% glycerol (pH 7.6). SMO is 10 mM Tris·HCl–86 mM NaCl–1 mM $MgSO_4$ (pH 7.4).

The DNA binding buffer, BBD, is identical in composition to the BB buffer developed by Riggs *et al.* (8), except that 200 μg of chicken-blood DNA per ml was added and the pH is 7.6. FB buffer is BBD minus-chicken blood DNA, bovine-serum albumin, and dithiothreitol. Both buffers were filtered through a 0.45-μm Millipore filter before use.

Bacteriophage [^{32}P]DNA. GS156 and M5107 served, respectively, as sources of λ*phut* [^{32}P]DNA and λ [^{32}P]DNA. Cells growing exponentially at 32° in LPC broth were centrifuged and resuspended at a density of 5×10^8 cells per ml in 100 ml of LPC broth plus 0.4% glucose and 5 mCi of carrier-free [^{32}P]phosphate. The cells were incubated at 43° for 20 min for thermal induction of phage production, and then grown for 4 hr at 38°. Then, after centrifugation and resuspension in 5 ml of SMO buffer, the cells were lysed with chloroform at 37° and treated with bovine-pancreatic DNase (0.05 μg/ml) for 15 min at 37°; the cellular debris was removed by centrifugation. The phage were purified by block and equilibrium CsCl centrifugation, and the DNA was extracted by a modification of the procedure of Adesnik and Levinthal (9). DNA concentrations were determined from A_{260} using a conversion factor of 54 μg/ml per A_{260} unit determined for chicken-blood DNA. The experiments reported here were performed with [^{32}P]DNA that had been stored at 4° for 2–3 weeks after extraction.

Cell Extracts. 2-l Flasks containing 500 ml of SN, SNH, or

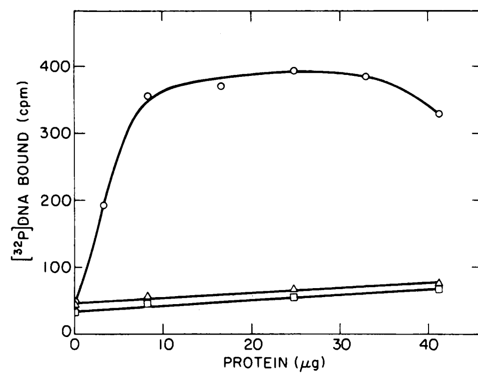

FIG. 2. DNA-binding assay of the *hut* repressor: ○——○, λp*hut* [^{32}P]DNA bound; □——□, λp*hut* [^{32}P]DNA bound in the presence of urocanate; and △——△, λ [^{32}P]DNA bound. Increasing amounts of cellulose phosphate-purified extracts of strain NE449 (described in Table 1) grown on SNH medium were added to 1.00 ml of BBD buffer or to 1.00 ml of BBD buffer plus 2.0 μmol of urocanate. The reactions were started with addition of either 14 ng of λp*hut* [^{32}P]DNA or 13 ng of λ [^{32}P]DNA. The mixtures were incubated for 30 min at room temperature (22°). Triplicate portions of 0.30 ml were filtered through nitrocellulose filters and washed with 0.30 ml of FB buffer. The radioactivity was determined by liquid scintillation counting. Each point represents the average of the three values obtained. The input was 3310 cpm for λp*hut* [^{32}P]DNA and 3180 cpm for λ [^{32}P]DNA or 993 cpm and 954 cpm, respectively, per aliquot. No background radioactivity was subtracted.

GSNH medium were inoculated with 5-ml cultures of cells grown overnight in the same medium. The flasks were shaken at 37° until the cells reached a density of about 6 × 10^{8} cells per ml and then chilled on ice. The cells were collected by centrifugation, washed with PM buffer, and resuspended in 5 ml of PMY buffer. The cells were disrupted with five 30-sec sonic treatments with an MSE Ultrasonic Power Unit. Cellular debris and ribosomes were removed by centrifugation for 20 min at 35,000 × *g* and then for 90 min at 100,000 × *g*. The final supernatant, about 5 ml, was taken as "crude extract." The crude extracts contained an average of 14 mg of protein per ml, as determined by the method of Lowry *et al.* (10), with bovine-serum albumin for standardization.

Repressor Purification. The crude extracts were applied directly to PMY-equilibrated, 1.0 × 3.0-cm columns of cellulose phosphate at 4°. Fractions were eluted from the columns first with a total of 4 ml of PMY buffer and then with 0.5-ml portions of PMSY. Most of the protein eluted by PMSY was contained in the fourth and fifth PMSY fractions. These fractions were pooled and dialyzed overnight at 4° against 400 ml of TDY buffer. The dialyzed fractions were termed "cellulose phosphate-purified extract" and contained an average of 1.5 mg of protein per ml. The degree of purification was about 50-fold.

Repressor Assay. The *hut* repressor was assayed by the DNA-binding technique of Riggs *et al.* (8). Chicken-blood DNA was added to the binding buffer to reduce backgrounds due to nonspecific DNA-binding. Cellulose phosphate-purified extracts (0–25 μl) were mixed with 1.00 ml of BBD buffer, then prefiltered λp*hut* [^{32}P]DNA at 14 ng/ml (10 μl of a 1.4 μg/ml stock solution) was added, and the mixtures were incubated at room temperature (22°) for 30 min. Triplicate portions of 0.30 ml were filtered through FB-soaked, 25-mm Schleicher and Schuell, B6 nitrocellulose filters and then washed with 0.30 ml of FB. Filters were immersed in 15 ml of water in scintillation vials and the Cherenkov radiation was counted directly on a Nuclear Chicago liquid scintillation counter.

Enzyme Assays. Histidase ("H enzyme"), urocanase ("U enzyme"), and formiminoglutamate hydrolase ("G enzyme") were assayed in crude extracts by reported procedures (2, 3).

RESULTS

Assay of the *hut* repressor and the effect of inducers

For isolation of the repressor we used cells of strain NE449, a strain diploid for *hutM145*, a 5-fold super-promoter mutation in the *hutMIGC* operon. The cells were grown in SNH medium to obtain full induction of the *hut* operons. Attempts at assaying the *hut* repressor in crude extracts of this strain proved fruitless. No binding of λp*hut* [^{32}P]DNA could be detected over the relatively high background of nonspecific DNA binding, i.e., over binding in the presence of 2 mM urocanate or binding of λ[^{32}P]DNA in place of λp*hut* [^{32}P]DNA. Purification on cellulose phosphate greatly lowered the nonspecific binding and revealed the *hut* repressor activity illustrated in Fig. 2. With increasing amounts of purified extract, as much as 40% of the input λp*hut* [^{32}P]DNA could be bound. Essentially no binding was detected in the presence of 2 mM urocanate or if λ [^{32}P]DNA was substituted for λp*hut* [^{32}P]DNA.

With the repressor concentration adjusted such that the amount of *hut* DNA bound was one-half that at saturation, urocanate at 0.07 mM reduced the standard level of binding to 50%. Imidazolepropionate, a urocanate analogue and a gratuitous inducer of the *hut* enzymes (1, 2), was needed at 2 mM to reduce the binding to the same extent. Histidine (0.5–10 mM), glucose (50–500 mM), or cyclic AMP (0.002–0.2 mM) had no effect on the binding reaction (data not presented).

Incubation of the repressor extract with Pronase (5 μg/ml for 15 min at room temperature) completely destroyed the DNA-binding activity (results not presented). A comparable

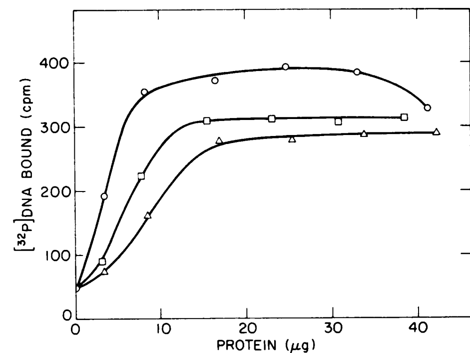

FIG. 3. Repressor activity from cells in different physiological states of growth: △——△, uninduced; ○——○, induced; and □——□, induced-catabolite repressed. Increasing amounts of cellulose phosphate-purified extracts of strain NE449 (described in Table 1) grown on SN medium (uninduced), SNH medium (induced), or GSNH medium (induced-catabolite repressed), were added to 1.00 ml of BBD buffer. 14 ng of λp*hut* [^{32}P]DNA was added to start the reaction. Incubation time, input cpm, filtering, and averaging were the same as in Fig. 2. The curve for induced cells has been repeated from Fig. 2.

incubation with pancreatic RNase (5 μg/ml) had no effect. By these criteria, the repressor is a protein.

Induction and catabolite repression of the *hut* repressor

Strain NE449 was grown in three different conditions: uninduced (SN medium), induced (SNH medium), and induced-catabolite repressed (GSNH medium). The DNA-binding capacities of the cellulose phosphate-purified extracts are illustrated in Fig. 3. The level of repressor is seen to be highest in induced cells. Cells grown either uninduced or catabolite repressed have lower repressor levels.

The G and H enzyme concentrations of these cells, assayed at the crude extract stage, and their repressor levels, assayed after cellulose phosphate purification, are presented in Table 1, Exp. 1. It can be seen that the repressor is inducible about to the same degree as the G enzyme, but to a lesser degree than the H enzyme. Similarly, the repressor is repressed by glucose in parallel with the G enzyme and not with the H enzyme.

Genotype variation and the *hut* repressor

Cells with different *hut* genotypes grown under identical conditions on SNH medium yielded different levels of repressor (Fig. 4 and Table 1, Exp. 2). Strains lacking the *hutC* gene by point mutation (NE606) or by deletion of the entire *hut* region (NE547) are devoid of *hut* DNA-binding activity. A strain (NE446) with a super-promoter mutation of *M* has increased levels of repressor as well as of G enzyme compared to wild type (NE413). The *hut* diploid strain, NE449, has about twice the repressor content as the corresponding haploid strain (NE446).

Although strain NE449 has greater repressor specific activity (a steeper slope in the initial portion of the binding curve) than strain NE446, the plateau of the binding curve is lower and degenerates at high protein concentrations (see Fig. 4). Extracts of strain NE449 were found to have greater DNase contamination than those of all other strains. This additional nuclease is possibly the *uvrB* gene product, for NE449 is $uvrB^+$ while all other strains are $uvrB^-$ by deletion. The *uvrB* gene product is known to be involved in the excision of pyrimidine dimers (see ref. 11).

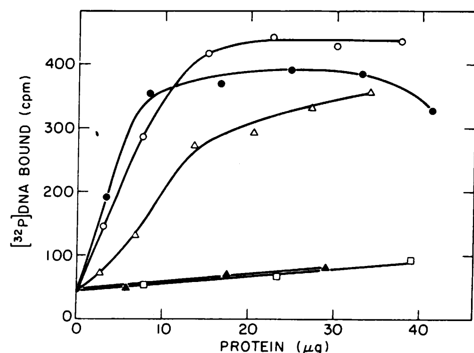

FIG. 4. Repressor activity from cells of different genotype: △——△, wild type (NE413); □——□, $hutC^-$, repressor negative (NE606); ▲——▲, *hut* deletion (NE547); ○——○, $hutM^{++}$, super-promoter (NE446); and ●——●, $hutM^{++}/hutM^{++}$, super-promoter diploid (NE449). Increasing amounts of cellulose phosphate-purified extracts of cells grown on SNH medium were added to 1.00 ml of BBD buffer, and 14 mg of λ*phut* [^{32}P]-DNA was added to start the reaction. Incubation time, input cpm, filtering, and averaging were the same as in Fig. 2. The strains are described in greater detail in Table 1. The curve for the super-promoter diploid strain has been repeated from Fig. 2.

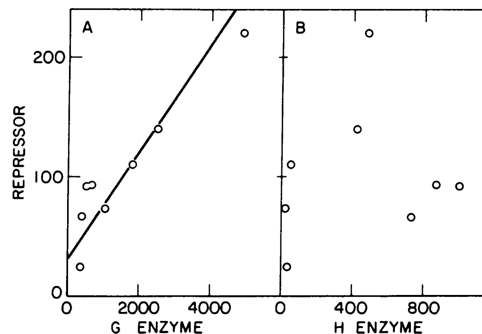

FIG. 5. (*A*) Coordinacy of repressor and G enzyme activities. (*B*) Noncoordinacy of repressor and H enzyme activities. Repressor, G enzyme, and H enzyme specific activities have been plotted from the data given in Table 1. Strains NE606, carrying a point mutation in *hutC*, and NE547, lacking all *hut* genes, have been omitted.

Constitutive mutants of *hut*

Two classes of mutations in the *hut* operons have been found to bring about full constitutive expression of both operons: repressor-negative ($hutC^-$) and urocanase-negative ($hutU^-$) mutations (1, 2). Urocanase-negative cells are constitutive for the remaining *hut* enzymes (H, I, and G) due to an accumulation of endogenously produced urocanate. Repressor and enzyme levels from induced and uninduced wild-type (NE-413), repressor-negative (NE606), and urocanase-negative (NE607) cells are compared in Table 1, Exp. 3. It can be seen that, whereas wild-type cells have an inducible repressor and $hutC^-$ cells lack repressor altogether, $hutU^-$ cells have a repressor that is synthesized constitutively.

Coordinacy of the repressor and G enzyme activities

In Fig. 5 the data of Table 1 are presented graphically to show the coordinacy of repressor with G enzyme and the lack of coordinacy of repressor with H enzyme. The ratio of repressor specific activity to G enzyme specific activity is about constant for several growth conditions and for several genotypes.

DISCUSSION

In this paper we have reported the partial purification of the *hut* repressor protein and have demonstrated that the repressor binds specifically to *hut* DNA and that this binding is abolished by the presence of the inducer, urocanate. In keeping with our earlier evidence that the repressor is a member of one of the operons that it regulates, we have found that the repressor is inducible, subject to catabolite repression, and affected by a promoter mutation.

Purification by chromatography on cellulose phosphate is a convenient and rapid means of eliminating 98% of the protein present in the crude extracts. We have made the assumption that the amount of repressor eluted from the columns is proportional to the amount of repressor applied. The procedure can be performed without bias to repressor-negative extracts. The preparations, however, remain quite heterogeneous, and quantitations of repressor activity are necessarily approximate. The incubation period for the repressor assays was arbitrarily set at 30 min. At high protein concentrations of cellulose phosphate-purified repressor extracts, 30 min is

ample time for repressor–DNA binding to reach equilibrium; however, contaminant DNases reduce the quantity of DNA retained on the filter. At low extract concentrations, on the other hand, 30 min is not long enough for the repressor–DNA binding reaction to equilibrate. The sigmoidal shape of the saturation curves (Figs. 3 and 4) is probably a result of sub-equilibria at low repressor concentrations. Other possible explanations of the sigmoidicity (see ref. 12), however, cannot be ruled out at this time. Fitting a straight line to the initial portion of the binding curves and taking the slope as an estimate of repressor specific activity proved to be sufficiently accurate for us to demonstrate the coordinacy of repressor to G enzyme specific activity (Fig. 5).

The *hut* system has a complex regulatory mechanism; the operons are strongly buffered against premature induction. The repressor is inducible; hence, as induction is initiated, further induction becomes more difficult. The inducer-forming enzyme (H) is in balance with the inducer-destroying enzyme (U), and these enzymes, because they belong to the same operon, are synthesized coordinately. In the induced state, the intracellular concentration of urocanate is presumably high enough to inactivate the existing repressor completely. Once histidine is exhausted from the medium, the concentration of urocanate drops, and the high level of repressor rapidly shuts off *hut* enzyme synthesis. The *hut* operons are thus very cautious in their expression.

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