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Metabolic Interlock

THE INFLUENCE OF HISTIDINE ON TRYPTOPHAN BIOSYNTHESIS IN *BACILLUS SUBTILIS**

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SUMMARY

A regulatory relationship in *Bacillus subtilis* between histidine and the synthesis of tryptophan exemplifies "metabolic interlock," a phenomenon of regulatory interactions exerted between biochemical pathways. The enzymatic basis for the histidine-tryptophan relationship was examined. Histidine activates anthranilate synthetase by increasing the apparent value of V_{max}. Hence, histidine antagonizes growth inhibition caused by 5-methyltryptophan, a false feedback inhibitor of anthranilate synthetase, by increasing the velocity of the reaction. Mutant strain F-2, which synthesizes the tryptophan enzymes constitutively, excretes 4 times more tryptophan in minimal medium containing histidine than in unsupplemented minimal medium. This reflects the stimulation of anthranilate synthetase by histidine.

In contrast to the situation in F-2, growth of wild type in the presence of histidine does not affect tryptophan excretion. Histidine supplementation indirectly leads to repression of the specific activity of at least one tryptophan enzyme, anthranilate synthetase, presumably as a consequence of the stimulation of tryptophan production. A histidine-excreting mutant, NP 187, exhibits the repressed level of anthranilate synthetase that is characteristic of wild type grown in the presence of histidine. This compensatory mechanism in which histidine plays a dual role, activating anthranilate synthetase activity but also indirectly repressing its biosynthesis, does not operate in F-2. The tryptophan enzymes of F-2 are not repressible and therefore lack the compensatory adjusting mechanism of wild type.

Another mutant strain, NP 100, which is derepressed in the synthesis of tryptophan enzymes and which accumulates chorismate because of a block in chorismate mutase, excretes significantly more tryptophan than does F-2. This results from an increased level of chorismate available to anthranilate synthetase. Tryptophan excretion is not further elevated in this strain by histidine. To explain this, we propose that anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase (PR transferase), not anthranilate synthetase, is the rate-limiting reaction in the tryptophan pathway in *B. subtilis*. Although tryptophan and histidine do not allosterically influence PR transferase, both amino acids could effectively regulate PR transferase activity by controlling the availability of one of its substrates, anthranilate.

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The occurrence of regulatory interactions exerted between different metabolic pathways has been termed metabolic interlock (1). That a general, possibly ubiquitous relationship between the histidine and the aromatic amino acid pathways may exist in microorganisms is implicated by a variety of observations. Ames (2) has reported that an aromatic amino acid permease also transports histidine in *Salmonella typhimurium*. In *Neurospora crassa*, Carsiotis and Lacy (3) have demonstrated an increase in the specific activity of some of the tryptophan biosynthetic enzymes when histidine-requiring mutants were grown on limiting amounts of histidine. Nester (4) has shown that histidine affects tyrosine biosynthesis by influencing the synthesis of prephenate dehydrogenase in *Bacillus subtilis*. Furthermore, the biosynthetic enzymes of both the histidine pathway and the aromatic amino acid pathway in *B. subtilis* may share a common repressing element (5). Although the precise details of the relationship between histidine and the aromatic amino acids vary considerably from one microorganism to another, independent observations of such regulatory interactions could reflect an ancient ancestral relationship between the two pathways. In this context, it is intriguing that, in *B. subtilis*, some histidine loci are located within the cluster of aromatic genes (6, 7).

We have pursued the details of the histidine-tryptophan relationship in *B. subtilis* as an example of metabolic interlock (1). The following observations formed the basis for the initial recognition of this relationship in *B. subtilis*. The histidine markers within the aromatic cluster on the *B. subtilis* chromosome are adjacent to the tryptophan loci (7). Also, reversion of certain histidine-requiring mutants results in prototrophic strains which are strong excretors of tryptophan (8). We demonstrated that histidine reverses the growth-inhibiting effects of 5-methyltryptophan, a structural analogue of L-tryptophan (9). We proposed a hypothetical regulatory relationship between histidine and tryptophan synthesis which focuses upon PP-ribose-P, a metabolite common to the synthesis of both histidine and tryptophan (1). The enzymatic basis for the influence of histidine upon tryptophan biosynthesis is the subject of this report.

MATERIALS AND METHODS

Organisms—All strains used in this study were derivatives of *B. subtilis* 168 (10). Details are given in Table I.

Growth of Cells and Extract Preparation—All strains were grown in 200 ml of minimal medium supplemented with 0.5% glucose and a trace element mixture (8). Histidine when added was at a final concentration of 50 μg per ml.

TABLE I

Strain designation	Description	Reference
NP 40	Prototrophic derivative of strain 168	8
F-2	5-Methyltryptophan-resistant prototroph; excretes tryptophan; synonymous with SB 455	7
NP 100	5-Methyltryptophan-resistant auxotroph blocked in chorismate mutase; excretes tryptophan	20
NP 187	1,2,4-Triazole-3-alanine-resistant prototroph; excretes histidine	1

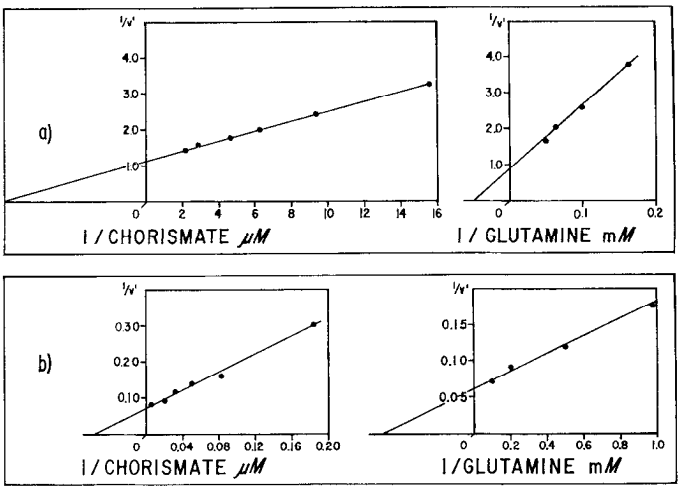

FIG. 1. Secondary plots of anthranilate synthetase activity. Assay conditions are described under "Materials and Methods." The *ordinate*, $1/v'$, represents the intercept of $1/v$ from the Lineweaver-Burk plots plotted as a function of the fixed substrate. The kinetic data for the enzyme from crude extracts of NP 40 are illustrated in *Part a*. The data from the crude extracts of F-2 are illustrated in *Part b*.

Cells were harvested in late logarithmic phase of growth unless otherwise indicated. The cells were suspended in a 10^{-1} M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 1 mM KCl, 6 mM β-mercaptoethanol, and 30% glycerol. Glycerol stabilized anthranilate synthetase and PR transferase[1] in crude extracts. Lysozyme (100 μg per ml) was added to the cell suspension and incubated at 37° for 15 min. At this time, 20 μg per ml of DNase in 0.5% $MgSO_4$ were added and the extract was incubated at 37° for an additional 15 min. The extract was centrifuged at 8000 × *g* for 20 min at 4°. The supernatant fraction was passed through Sephadex G-25 previously equilibrated with cold buffer. Protein was determined by the method of Lowry *et al.* (11), with bovine serum albumin as a standard.

Anthranilate Synthetase Assay—The reaction mixture contained 50 μmoles of Tris buffer, pH 7.5, 10 μmoles of $MgSO_4$, 20 μmoles of L-glutamine, and 0.2 μmole of potassium chorismate in a final volume of 1 ml. The reaction was initiated by the addition of enzyme (the concentration of protein used varied depending on the strain; the range being 0.15 to 2.0 mg per ml) to a reaction vessel previously incubated at 37°. The rate of reaction was

[1] The abbreviations used are: PR transferase, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

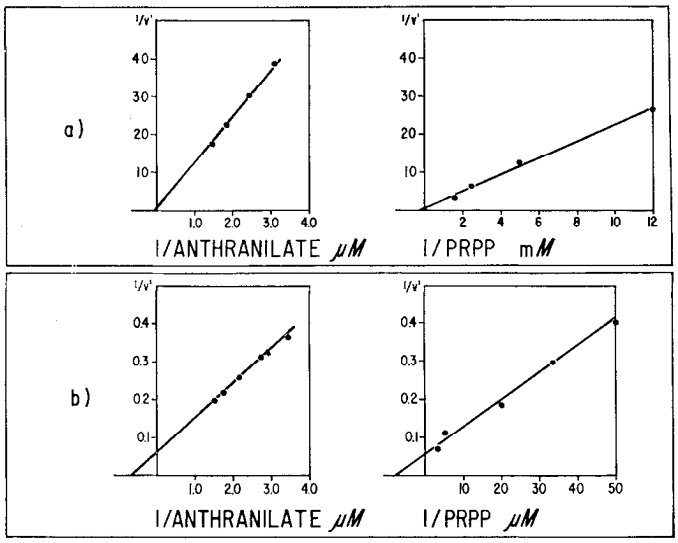

FIG. 2. Secondary plots of PR transferase activity. Assay conditions are described under "Materials and Methods." The *ordinate*, $1/v'$, represents the intercept of $1/v$ from the Lineweaver-Burk plots plotted as a function of the fixed substrate. The kinetic data for the enzyme from crude extracts of NP 40 are illustrated in *Part a*. The data from the crude extracts of F-2 are illustrated in *Part b*. *PRPP*, 5-phosphoribosyl 1-pyrophosphate.

followed continuously in an Aminco-Bowman spectrophotofluorometer for 5 min by observing the increase in fluorescence of anthranilate at an excitation wave length of 313 nm and an emission wave length of 393 nm, and comparing the fluorescence to a standard curve. Authentic anthranilate showed peak fluorescence at these wave lengths. The velocity was a linear function of time in this interval.

PR Transferase Assay—The reaction mixture contained 50 μmoles of Tris buffer, pH 7.5, 10 μmoles of $MgSO_4$, 300 nmoles of PP-ribose-P, and 2.0 nmoles of anthranilate in a final volume of 1 ml. The reaction was started by the addition of enzyme (0.15 to 2.0 mg per ml) to a reaction mixture previously warmed at 37°. The rate was followed continuously as decrease in fluorescence of anthranilate at the wave lengths indicated above. The rate was linear for at least 1½ min at the concentration of protein used.

Determination of Tryptophan Excretion—Wild type *B. subtilis* and two mutants derepressed for tryptophan biosynthetic enzymes were grown in the presence and absence of histidine. Aliquots of 2 ml each were taken at various sampling times throughout the growth phase. After determination of the optical density at 600 nm, the cell suspension was passed through a 0.45-μ Millipore filter. The filter was examined fluorometrically at an excitation wave length of 282 nm and an emission wave length of 355 nm. The excreted tryptophan was quantitatively determined by comparing the observed fluorescence to a standard curve which was determined with authentic L-tryptophan.

Chemicals—Barium chorismate (80% pure) was prepared by the method of Gibson (12). The potassium salt was made by dissolving barium chorismate in water and adding twice the calculated equivalent of K_2SO_4; the $BaSO_4$ precipitate was removed by filtration. PP-ribose-P, L-tryptophan, DL-5-methyltryptophan, and anthranilate were obtained from Calbiochem; bovine serum albumin was purchased from Sigma. All other chemicals were reagent grade.

TABLE II

Comparison of activities of anthranilate synthetase and PR transferase in crude extracts of F-2 and NP 40

Strain	Anthranilate synthetase			PR transferase		
	V_{max}[a]	K_m		V_{max}[b]	K_m	
		Glutamine	Chorismate		Anthranilate	PP-ribose-P
		mM	mM		μM	μM
NP 40.....	1.00	20	0.16	1.00	12.5	2500
F-2.....	14.70	2	0.02	18.00	1.25	150

[a] V_{max} is expressed as nanomoles of anthranilate produced per min per mg of protein.

[b] V_{max} is expressed as nanomoles of anthranilate utilized per min per mg of protein.

TABLE III

Effect of histidine (6 mM) on inhibition of anthranilate synthetase by 5 μM tryptophan

Strain	Inhibition	
	− Histidine	+ Histidine
	%	%
NP 40.....	72	72
F-2.....	40	50
NP 100.....	35	39

RESULTS

Kinetic Parameters of Anthranilate Synthetase and PR Transferase—The first two enzymes specific to tryptophan synthesis, namely, anthranilate synthetase and PR transferase, were examined in extracts of strains NP 40 and F-2. Fig. 1 shows the intercepts of the primary Lineweaver-Burk graphs as a function of the reciprocal of the concentration of the fixed substrate (secondary plots) obtained with anthranilate synthetase from NP 40 (Fig. 1*a*) and F-2 (Fig. 1*b*). The kinetic properties of PR transferase are illustrated in Fig. 2 for NP 40 (*a*) and F-2 (*b*). The kinetic parameters of both enzymes from NP 40 and F-2 are compared in Table II. The mutations resulting in derepressed levels of anthranilate synthetase and PR transferase appear to influence the K_m values of both enzymes (Table II). This is consistent with the observation that tryptophan does not inhibit the anthranilate synthetase of F-2 as well as the anthranilate synthetase from NP 40 (Table III). Since the methyltryptophan resistance mutation in F-2 maps away from the tryptophan cluster (7), these results are not comparable with the observations in *Escherichia coli* (13) where a mutation in the structural gene for anthranilate synthetase leads to both a loss of feedback inhibition and derepression. Our kinetic data indicate that either the gene product of the 5-methyltryptophan locus codes for some protein that complexes with anthranilate synthetase thus altering its kinetic parameters or that the locus is a regulatory gene and the number of enzyme molecules per mg of protein determines the kinetic constants. This latter possibility could reflect some equilibrium between aggregational states of the enzyme. These two possibilities are being investigated.

Both tryptophan and 5-methyltryptophan strongly inhibit anthranilate synthetase; the inhibition is competitive with respect to chorismate (Fig. 3). Since histidine reverses the inhibiting effect of 5-methyltryptophan on growth (See Reference 9 and this paper), we examined the possibility that the inhibition of anthranilate synthetase by tryptophan (or 5-methyltryptophan) might be antagonized in the presence of histidine. In the presence of 20 mM glutamine and 0.25 mM chorismate, 5 μM tryptophan potently inhibits anthranilate synthetase from NP 40, F-2, and NP 100 (Table III). Histidine, 6 mM, did not alter the magnitude of this inhibition. Similar results were observed when the same concentration of 5-methyltryptophan was used as the inhibitor.

The effect of histidine on the initial velocity of anthranilate synthetase is shown in Fig. 4. With the use of a saturating concentration of glutamine and a variable concentration of chorismate, the presence of 6 mM histidine increased the velocity of anthranilate synthetase by 50%. Histidine alters the apparent value of V_{max} without changing the kinetic parameter of

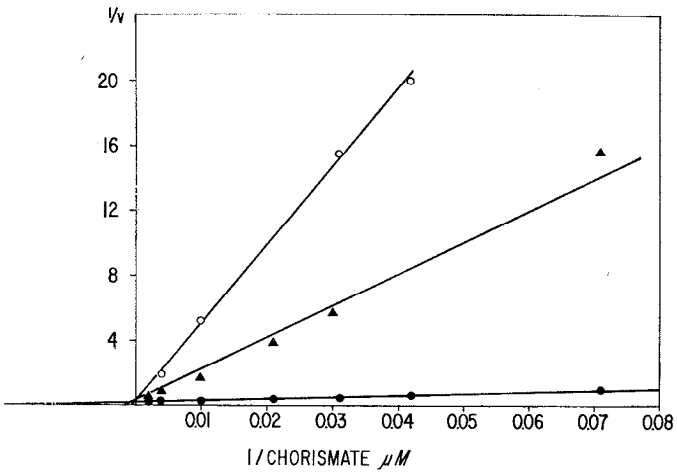

FIG. 3. Inhibition of anthranilate synthetase by L-tryptophan and DL-5-methyltryptophan. Assay conditions are described under "Materials and Methods." The data were obtained with an extract of F-2. In this Lineweaver-Burk plot the *ordinate* is $1/v$ (velocity expressed as nanomoles of anthranilate formed per min per mg of protein) and the *abscissa* is the reciprocal of the chorismate concentration. Glutamine was maintained at a constant concentration of 20 mM. The concentration of inhibitor was: ●——●, none; ▲——▲, 50 μM L-tryptophan; ○——○, 50 μM DL-5-methyltryptophan.

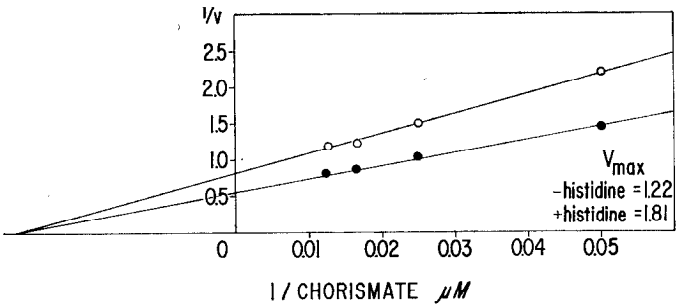

FIG. 4. Histidine as an allosteric activator of anthranilate synthetase in F-2. The concentration of L-glutamine was fixed at 20 mM. Histidine (●——●) was added at a final concentration of 6 mM. The reaction mixture contained 150 μg of protein from extracts of F-2.

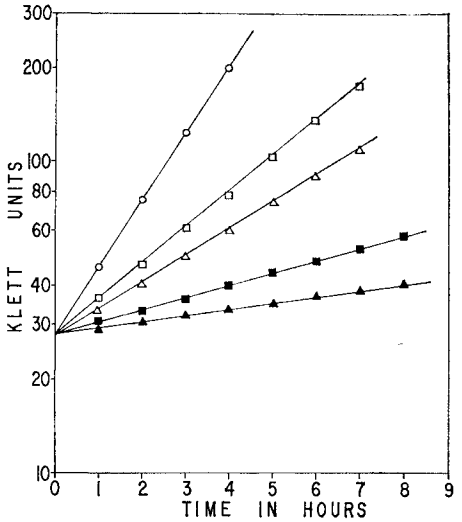

FIG. 5. Influence of histidine on the inhibition of growth of *B. subtilis* NP 40 by 5-methyltryptophan. Minimal glucose cultures of NP 40 were grown with the following supplements: none, ○——○; 50 μg per ml of 5-methyltryptophan, ■——■; 75 μg per ml of 5-methyltryptophan, ▲——▲; 50 μg per ml of 5-methyltryptophan + 50 μg per ml of histidine, □——□; 75 μg per ml of 5-methyltryptophan + 50 μg per ml of histidine, △——△. Cultures of 10 ml were grown in 125-ml side arm flasks in a shaking water bath at 37°. Turbidity was measured in a Klett colorimeter.

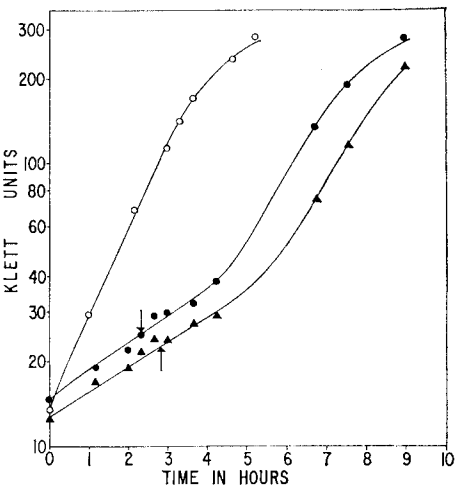

FIG. 6. The influence of tryptophan on the growth of NP 40 previously incubated with 5-methyltryptophan. Cultures of NP 40 were grown in minimal glucose media with the following supplements: none, ○——○; + 50 μg per ml of 5-methyltryptophan, ●——●; ▲——▲. At the times indicated on the curves 50 μg per ml of tryptophan (●——●) or 100 μg per ml of tryptophan (▲——▲) were added to the cultures containing the analogue. Other growth conditions are described in the legend of Fig. 5. Similar curves were found when 50 μg per ml of histidine were added instead of tryptophan. Turbidity was measured in a Klett colorimeter.

K_m for chorismate. The unaltered K_m is consistent with the observation that histidine does not affect the fractional inhibition of enzyme activity found in the presence of tryptophan. The data in Fig. 4 were obtained with extracts of F-2. Anthranilate synthetase prepared from the other strains used in these experiments yielded similar results.

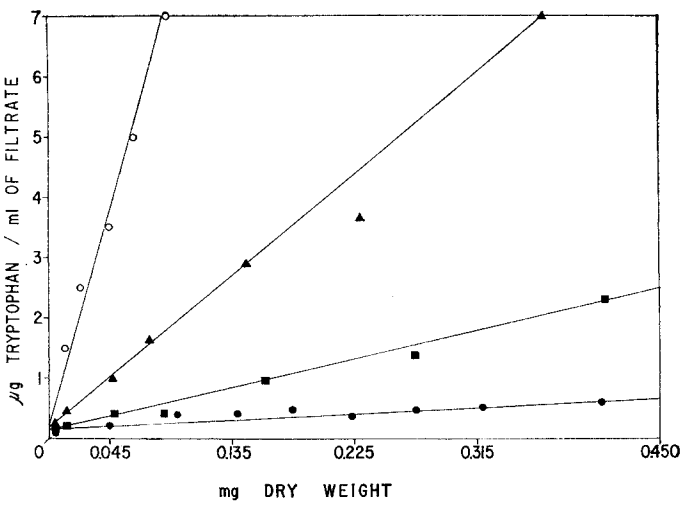

FIG. 7. The excretion of tryptophan by NP 40, F-2, and NP 100. The excretion of tryptophan is plotted as a function of dry weight. Cultures of NP 40, F-2, and NP 100 were grown in minimal media in the presence and absence of histidine (50 μg per ml); NP 40 ± histidine, ●——●; F-2 −histidine, ■——■, + histidine, ▲——▲; NP 100 ± histidine, ○——○. Other growth conditions are indicated in Table I.

Observations in Vivo of Histidine Interaction with Tryptophan Synthesis—The results shown in Fig. 5 show that the stimulative potential of histidine on the biosynthesis of tryptophan can be expressed *in vivo* in *B. subtilis*. At concentrations of 50 and 75 μg per ml, 5-methyltryptophan inhibited the growth rate of NP 40 80% and 85%, respectively. The presence of 50 μg per ml of histidine decreased the magnitude of these inhibitions to 48 and 57%, respectively. When tryptophan was added to a culture of NP 40 after growth in the presence of inhibiting concentrations of 5-methyltryptophan for 2 to 3 hours, the wild type growth rate was not restored immediately (Fig. 6). Instead, a lag of 3 to 4 hours preceded the resumption of the rate of growth found in the absence of the analogue. Since the reversal of 5-methyltryptophan inhibition of growth by tryptophan is not immediate, the inhibitory effect of the analogue must involve more than false feedback inhibition of anthranilate synthetase. The time course of the reversal by histidine of growth inhibition caused by 5-methyltryptophan was characterized by the same lag observed with tryptophan. These data implicate analogue incorporation into protein during tryptophan starvation. The lag period would be the time required to replace the false proteins. The incorporation of 5-methyltryptophan into proteins under conditions of tryptophan starvation has been indicated in *E. coli* K-12 (14).

Since histidine is an allosteric activator *in vitro* of anthranilate synthetase, we examined the effect of histidine on the excretion of tryptophan. Wild type *B. subtilis* (NP 40) and two tryptophan excretors (F-2 and NP 100) were grown in minimal media in the presence and absence of histidine. Exogenous histidine did not influence the growth rates of any of the three strains. In NP 40 and NP 100 the excretion of tryptophan was not influenced by histidine (Fig. 7). Likewise, a histidine-excreting mutant, NP 187, grown in minimal medium, excreted the same amount of tryptophan as NP 40.[2] On the other hand, when

[2] J. F. Kane, unpublished observations.

F-2 was grown in the presence of histidine there was a 4-fold increase in the tryptophan excreted compared to growth in the absence of histidine (Fig. 7).

Although in NP 40 histidine antagonized the inhibitory effect of 5-methyltryptophan upon growth and proved to be an allosteric activator of anthranilate synthetase, it, nevertheless, did not influence the excretion of tryptophan in the absence of the analogue. Insight into this seeming paradox was gained by the following enzyme repression studies. Extracts were made from cultures of NP 40, F-2, and NP 100 grown in the presence of histidine and from a culture of NP 187 grown in minimal medium. These results are presented in Table IV. Exogenous histidine consistently repressed anthranilate synthetase activity 40% in NP 40. The specific activity of anthranilate synthetase from NP 187 was at the repressed level of NP 40 grown in the presence of histidine. The specific activities of the enzyme from the derepressed mutants were not influenced by growth in a histidine-supplemented medium. For convenience the specific excretion of tryptophan in these strains is included in Table IV.

Phosphoribosyltransferase, Regulatory Role?—PR transferase is the second enzyme in the tryptophan biosynthetic pathway. Since we postulated that PR transferase might be an allosteric enzyme functioning in the regulation of tryptophan biosynthesis (1), this enzyme was examined in extracts of NP 40 and F-2. PR transferase is labile and retained activity only in extracts prepared in the presence of 30% glycerol. Anthranilate synthetase was inhibited more than 90% by 50 μM tryptophan or 5-methyltryptophan, whereas PR transferase was unaffected (Table V). Further studies on the enzyme from F-2 did not reveal any inhibition of PR transferase when the tryptophan concentration was increased to 100 μM or when substrate concentrations were decreased in the presence of inhibitor. There was no evidence for either histidine stimulation or end product-mediated substrate inhibition of the type observed with PR transferase of *S. typhimurium* (15). Hence, although we still favor an important role for PR transferase in the histidine-tryptophan interaction (see "Discussion"), there is no direct allosteric effect of tryptophan upon PR transferase activity.

TABLE IV

Repression of anthranilate synthetase by histidine

Strain	Supplement	Specific tryptophan excretion[a]	Specific activity[b]
NP 40	Minimal	0.69	0.035
	Histidine	0.69	0.021
NP 187	Minimal	0.69	0.023
F-2	Minimal	2.90	12.00
	Histidine	11.50	11.00
NP 100	Minimal	39.30	11.40
	Histidine	39.30	11.60

[a] These data were calculated from Fig. 3 and are expressed as micrograms of tryptophan per mg dry weight per hour.

[b] Specific activity is expressed as the nanomoles of anthranilate formed per min per mg of protein.

TABLE V

Effect of L-tryptophan and DL-5-methyltryptophan on anthranilate synthetase and PR transferase of NP 40 (930 μg of protein)

Enzyme	Effector concentration	Relative activity
	μM	
Anthranilate synthetase	None	1.00
	50 L-Tryptophan	0.09
	50 DL-5-Methyltryptophan	0.07
PR transferase	None	1.00
	50 L-Tryptophan	1.19
	50 DL-5-Methyltryptophan	1.00

DISCUSSION

We previously described an interaction between histidine and tryptophan synthesis (1). At that time we proposed a hypothetical regulatory relationship which was dependent upon the identity of tryptophan as a feedback inhibitor of PR transferase. This model was based on the following considerations. (*a*) The biosynthetic pathways of histidine and tryptophan share PP-ribose-P as a common intermediate. Hence, PP-ribose-P can be considered to be a branch point metabolite with respect to the synthesis of tryptophan and histidine. Enzymes at metabolic branch points are apt to be allosteric proteins. (*b*) A number of nutritional observations were consistent with the idea that tryptophan regulated PR transferase. For example, we observed the accumulation of anthranilate under certain conditions (1). (*c*) Both *E. coli* (16) and *S. typhimurium* (15), were known to have PR transferases inhibited by tryptophan. (*d*) Histidine, by feedback inhibiting the first enzyme specific to its own synthesis (5), would spare PP-ribose-P. The increased concentration of PP-ribose-P would overcome the tryptophan inhibition of PR transferase, especially if the inhibition were competitive with respect to PP-ribose-P.

We found, however, that tryptophan does not inhibit PR transferase, even at low concentrations of PP-ribose-P or anthranilate. Our data substantiate a modified scheme of the regulatory interactions between tryptophan and histidine that is diagrammatically shown in Fig. 8. In this model, histidine directly influences tryptophan biosynthesis by stimulating anthranilate synthetase activity. This activating effect of histidine allows *B. subtilis* to resist the inhibition of growth by 5-methyltryptophan, a potent growth inhibitor of *B. subtilis*.

Since the immediate effect of 5-methyltryptophan is false feedback inhibition of anthranilate synthetase, histidine must antagonize this effect in one of two ways. First, histidine could

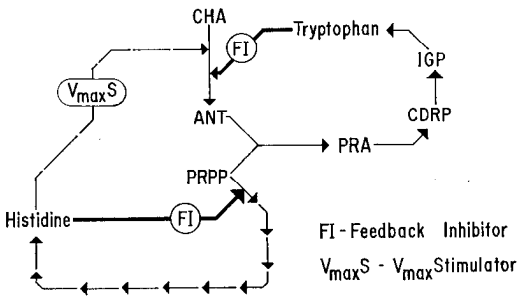

FIG. 8. Regulatory interaction between histidine and tryptophan synthesis. Feedback inhibition (*FI*) is indicated by *dark line*; V_{max} stimulator ($V_{max}S$) is indicated by *broken line*. *CHA*, chorismate; *ANT*, anthranilate; *PRPP*, 5-phosphoribosyl 1-pyrophosphate; *PRA*, *N*-(5′-phosphoribosyl)anthranilate; *CDRP*, 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate; *IGP*, indole glycerol 3-phosphate.

interfere with the inhibition of anthranilate synthetase caused by 5-methyltryptophan. This could occur by an allosteric-mediated change in conformation. Since the inhibition is competitive with respect to chorismate, a histidine-promoted decrease in the value of the apparent K_m could also produce this result. Second, histidine, could act as a V_{max} effector; that is, it would increase the velocity of the reaction without influencing the fractional sensitivity of the enzyme to feedback inhibition. The first possibility can be eliminated by the observation that both tryptophan and 5-methyltryptophan inhibit anthranilate synthetase equally well in the presence and absence of histidine. Our data show that velocity is the kinetic parameter of anthranilate synthetase activity which is altered by histidine. Although this reaction in the presence of 6 mM histidine is still normally susceptible to tryptophan inhibition, the velocity of the reaction is increased 50%. A further probable stimulative effect on tryptophan biosynthesis is that histidine, by feedback inhibiting the first enzyme of its own synthesis, would spare PP-ribose-P, a substrate for the second enzyme in the tryptophan pathway.

Although histidine overcomes 5-methyltryptophan inhibition by stimulating the production of tryptophan, histidine does not influence the quantity of tryptophan excreted by NP 40 in the absence of the analogue. The increased velocity of anthranilate synthetase caused by histidine is compensated for by an increased repression of enzyme synthesis. Thus, the quantity of tryptophan produced remains constant. This dual effect of histidine is documented by the following facts. First, the specific activity of anthranilate synthetase is lower when NP 40 is grown in histidine-supplemented media than when it is grown in minimal media. Likewise, NP 187, which excretes histidine, had a specific activity for anthranilate synthetase similar to that found in NP 40 cells grown in the presence of exogenous histidine. Since NP 187 excretes histidine one would expect this mutant to be more resistant to 5-methyltryptophan. This has, in fact, been reported (1). Second the derepressed mutant F-2, excretes 4 times as much tryptophan in histidine-supplemented media as in minimal media. Hence, the potential of histidine to stimulate tryptophan formation by causing an increased velocity of anthranilate synthetase is unmasked in F-2 which lacks the ability to repress the level of the tryptophan enzymes.

On the other hand, the excretion of tryptophan by the derepressed mutant NP 100, is not altered by histidine. Like F-2, NP 100 is derepressed for tryptophan enzymes and is known to have a feedback-sensitive anthranilate synthetase (Table III); however, NP 100 synthesizes 13 times as much tryptophan as does F-2. NP 100 also has a negligible level of chorismate mutase.[2] In *B. subtilis* prephenate is the major allosteric inhibitor of DAHP synthetase (17). The absence of appreciable chorismate mutase activity means that the level of its product, prephenate, would be low. Hence, there would be no effective feedback control or repression control of DAHP synthetase, and a large internal pool of chorismate would accumulate in NP 100. Since tryptophan is a competitive inhibitor of anthranilate synthetase with respect to chorismate, saturation of the enzyme with chorismate would effectively reverse the feedback control of this enzyme. In F-2, which has chorismate mutase activity, feedback inhibition of anthranilate synthetase by tryptophan still exerts some controlling influence on tryptophan production as seen by the fact that histidine stimulated tryptophan production 4-fold. It is probable that enzymes, in general, work under nonsaturating conditions *in vivo*. In NP 100 where the data indicate that saturating conditions exist for anthranilate synthetase with respect to chorismate, it appears that an enzyme or enzymes other than anthranilate synthetase is the rate-limiting step in tryptophan production.

Although one ordinarily expects the initial regulatory enzyme of a pathway to be the rate-limiting enzyme of that pathway, the composite of our results points to PR transferase as the rate-limiting reaction. (*a*) Histidine stimulates anthranilate synthetase activity 1½-fold but results in a 4-fold increase in tryptophan excretion by F-2. This difference could be explained by the fact that histidine increases the concentration of anthranilate and, perhaps, PP-ribose-P (by sparing action), both substrates for the rate-limiting PR transferase. The 2½-fold discrepancy could be accounted for in this way because of the complexities of a two substrate reaction. (*b*) If anthranilate synthetase were rate-limiting, it is difficult to understand the inability of histidine to stimulate tryptophan excretion in NP 100. Alternatively, if PR transferase were limiting the absence of a histidine effect on tryptophan excretion is understandable. In NP 100, which lacks chorismate mutase, the high internal pool of chorismate would result in a high concentration of anthranilate. Under these conditions, PR transferase is probably saturated, with respect to anthranilate, and the histidine stimulation of anthranilate synthetase would not be reflected in tryptophan excretion. We suggest that tryptophan controls its synthesis by allosterically inhibiting anthranilate synthetase thus regulating the flow of anthranilate to PR transferase, the rate-limiting enzyme in the pathway.

A relationship between histidine and tryptophan biosynthesis has been observed in a number of organisms. This is consistent with the speculation that at some point in evolution the enzymes of the two pathways may have had a common genetic origin. The enzymes of the histidine pathway have some rather striking similarities to the enzymes of the tryptophan pathway. Anthranilate synthetase catalyzes the formation of anthranilate from chorismate and glutamine and is postulated to catalyze two discrete steps (18) leading to the production of anthranilate. The amidotransferase in the histidine pathway requires glutamine and *N*-(5′-phospho-D-1′-ribulosylformimino-5-amino-1-5′-phosphoribosyl)-4-imidazole carboxamide to form an as yet unidentified product which is converted by the cyclase enzyme to imidazole glycerol phosphate. The cyclase maps within the aromatic cluster (19). In the tryptophan pathway there is a cyclization of enol-1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate to form indole glycerol phosphate, which is catalyzed by indole glycerol-phosphate synthetase. The condensation of anthranilate and PP-ribose-P to give phosphoribosyl anthranilate is very similar to the first reaction in the histidine pathway where ATP and PP-ribose-P are condensed to give phosphoribosyl ATP.

If the enzymes of the two pathways reflect such an evolutionary relationship, the observed differences in the specific details of the tryptophan-histidine relationships in different microorganisms probably reflect refinements brought about by the independent evolution of the two pathways. Contemporary regulatory interactions may constitute remnants of the ancestral relationships which have been preserved by natural selection. If such interpathway regulation is important in the biochemical integration of the various segments of metabolism, then mutations influencing these interactions probably would have such

far reaching effects as to be detrimental to the organism. Hence, we suggest that subtle regulatory relationships which exemplify metabolic interlock are sophisticated and highly evolved control systems which, once established, would tend to be strongly conserved as a consequence of the level of complexity.[3]

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[3] *Note Added in Proof*—It has been demonstrated that 5-methyltryptophan is incorporated into the protein of *B. subtilis* (21).