

# Metabolic Interlock

## THE MULTI-METABOLITE CONTROL OF PREPHENATE DEHYDRATASE ACTIVITY IN *BACILLUS SUBTILIS*\*

(Received for publication, March 2, 1970)

JOHN L. REBELLO AND ROY A. JENSEN

From the Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025

### SUMMARY

Regulatory interactions which relate small molecules and enzymes that are located in different sequences of biochemical reactions have been termed "metabolic interlock." One of the simplest examples of the phenomenon occurs in the branching biochemical pathway for the synthesis of aromatic amino acids in *Bacillus subtilis*. Although prephenate dehydratase is not a member enzyme of the tryptophan-specific biosynthetic pathway, tryptophan inhibits the activity of prephenate dehydratase as effectively as the feedback inhibition exerted by phenylalanine. The inhibitions by tryptophan and phenylalanine are competitive with respect to prephenic acid, the substrate for the enzyme. Tyrosine, the end product of the third branch of the pathway, reverses the inhibition of prephenate dehydratase by tryptophan although it does not exhibit a strong allosteric effect alone.

Several experiments showed that the allosteric specificities of prephenate dehydratase for tryptophan and tyrosine function *in vivo*. It was possible to show physiological conditions under which wild type cells were susceptible to growth inhibition by tryptophan. These conditions were equated with lower levels of intracellular tyrosine. More striking effects were noted by using mutant strains having a partial requirement for tyrosine. Hence, a mechanism appears to operate which decreases intracellular phenylalanine levels whenever intracellular levels of tyrosine are limiting or depleted. Methionine and leucine, allosteric activators of prephenate dehydratase, were found to reverse growth inhibition caused by tryptophan. The enzyme properties of several classes of mutants selected for resistance to growth inhibition by tryptophan are described.

In biosynthetic pathways, enzymes located at branch points are usually controlled by end products of that specific pathway. Supplementing such primary allosteric controls by end products, several metabolic interrelations have been found involving biosynthetic enzymes regulated by metabolites which are not produced in that pathway. We refer to regulatory interactions between small molecules and enzymes that are located in dif-

ferent, apparently remote, metabolic pathways as "metabolic interlock" (1).

Recently, we provided several examples of metabolic interlock in *Bacillus subtilis*: (a) prephenate dehydratase, a regulatory enzyme of phenylalanine biosynthesis, is activated by either leucine or methionine (1), and (b) Kane and Jensen (2) found that histidine stimulates anthranilate synthetase, the first enzyme in the tryptophan pathway.

A scan of the literature produces many suggestive observations which may prove to exemplify further the phenomenon of metabolic interlock. For example, in *Salmonella typhimurium*, Kredich, Becker, and Tomkins (3) discovered that histidine auxotrophs grown on histidins synthesized levels of serine transacetylase 3 to 6 times higher than those obtained from prototrophs grown on minimal medium. In *Pseudomonas putida* (4) histidine deaminase is inhibited by L-tyrosine and *p*-hydroxyphenylpyruvate. In *Neurospora crassa* an intermediary metabolite of the tryptophan biosynthetic pathway, anthranilic acid, inhibits the activity of glutamine synthetase (5).

Perhaps the simplest example of the phenomenon occurs in branching biochemical pathways in which a metabolite formed in one branch allosterically influences the activity of an enzyme in one of the other branches. For example, in *N. crassa* chorismate mutase is activated by tryptophan (6), and prephenate dehydrogenase is activated by phenylalanine (7); in *Escherichia coli* aspartate transcarbamylase is activated by ATP (8), and threonine deaminase is activated by valine (9). All of these can be treated as control devices which act to reinforce the primary feedback controls. This communication deals with a somewhat more complex relationship in the multibranching pathway used for the synthesis of aromatic amino acids in *B. subtilis*.

### MATERIALS AND METHODS

**Bacterial Strains**—The *B. subtilis* strains used in this investigation are described in Table I. All of these were derived from the prototroph, NP 40, a derivative of strain 168 (10). Isolates H-38 through H-46 were selected at 50° as spontaneous variants from a lawn of NP 40 spread on a minimal salts agar medium (11) lacking glucose but containing 200 µg per ml of tryptophan and 0.4% citrate. The use of citrate as the sole source of carbon appears to accentuate the sensitivity of the strain to growth inhibition by tryptophan. Isolate H-47 was selected on minimal glucose agar medium at 50° as a spontaneous mutant resistant to 1 mg per ml of tryptophan. Strains with I prefixes are slow growing prototrophs which were obtained spontaneously

\* This investigation was supported by Research Grants AM 13105 and GRS P-69-50 from the United States Public Health Service.

TABLE I  
*Bacillus subtilis* strains

Collection No.	Description
NP 40	Prototroph; spontaneous full revertant of 168 (10)
NP 100	Partial growth requirement for phenylalanine; tryptophan excretor; low level of chorismate mutase (17)
NP 164	Partial growth requirement for phenylalanine; excretes tyrosine; prephenate dehydrogenase is feedback-resistant (19)
H-38 through H-46	Spontaneous derivatives of NP 40 which are resistant to inhibition by tryptophan at 50° on citrate agar
H-47	Spontaneous derivative of NP 40 which is resistant to tryptophan at 50° on glucose agar
F-3	Phenylalanine excretor; $\beta$ -2-thienylalanine-resistant (WB 740; Reference 20)
F-4	Phenylalanine excretor; <i>p</i> -fluorophenylalanine-resistant (WB 2136; Reference 20)
I-6, I-8	Suppressed revertants of NP 100

as extracistronic suppressor mutations of NP 100. These strains have partial blocks in the tryptophan pathway. The consequent decreased metabolism of chorismate allows increased synthesis of prephenate, which then satisfies the phenylalanine requirement of NP 100.

**Growth Conditions**—Cells for physiological experiments were grown in minimal glucose medium at 37° or at 50° in a shaking water bath. Cultures in the exponential phase of growth served as the inoculum for 10-ml cultures in 125-ml side arm flasks. Growth was followed by monitoring the increase in culture turbidity in a Klett-Summerson colorimeter. The specific growth rate,  $k$  (12), is expressed in reciprocal hours.

**Chemicals**—The preparation of barium prephenate was described (13). Its purity was estimated as 95% (uncorrected for solvation). Lysozyme (crystallized three times) and THA<sup>1</sup> were obtained from Sigma. Deoxyribonuclease (B grade) and amino acids were purchased from Calbiochem. All other chemicals used were of reagent grade. All amino acids were L isomers.

**Enzyme Assay**—Cells for the preparation of crude extracts were grown in minimal medium at 37° with vigorous shaking. They were harvested in the late logarithmic phase of growth at an optical density of about 1.2 at 600 nm. After centrifuging at  $10,000 \times g$ , the pellet was suspended in 75 mM Tris·HCl buffer, pH 8.1, containing 10 mM  $\beta$ -mercaptoethanol, 0.1 mM EDTA, 1 mM MgCl<sub>2</sub>, 50 mM KCl, and 30% (v/v) glycerol. Cells were lysed as previously described (1). Extracts were clarified by spinning the cell lysate at  $12,000 \times g$  for 20 min. Small molecules were removed from all extracts by passage through a column of Sephadex G-25. Protein concentrations were assayed by the procedure of Lowry *et al.*, with bovine serum albumin as standard (14). Solutions of allosteric effectors were made in the same buffer as that used for suspending cells except that glycerol was omitted.

The assay procedure for prephenate dehydratase was described previously (1). Barium prephenate was converted to potassium prephenate and this was ordinarily used at final

<sup>1</sup> The abbreviation used is: THA,  $\beta$ -2-thienylalanine.

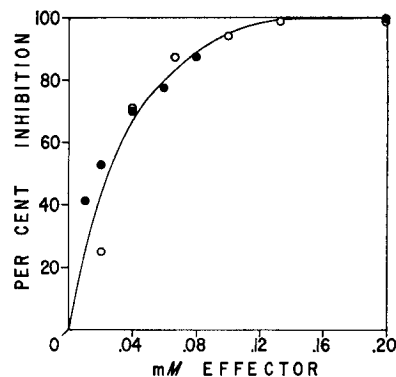


FIG. 1. Inhibition curve of prephenate dehydratase by phenylalanine (○—○) and by tryptophan (●—●). Percentage of inhibition on the ordinate was calculated by relating the activity observed in the presence of effector to that of the control vessel, lacking effector. The reaction mixture contained 1.04 mg per ml of protein from an extract prepared from NP 40. The activity of the control equaled an absorbance of 0.085 at 320 nm after 30 min at 32°.

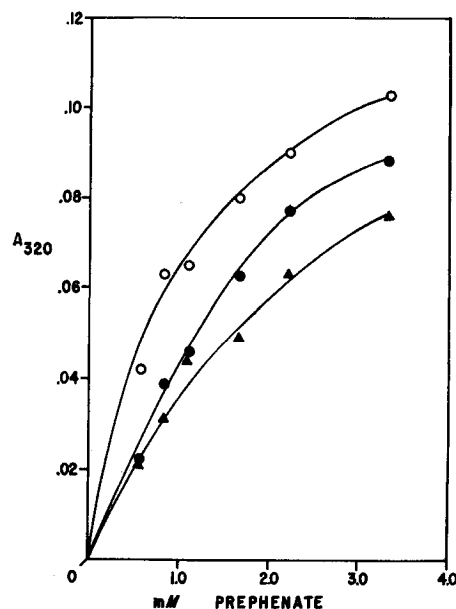


FIG. 2. Substrate saturation curves for prephenate dehydratase. Reaction velocity is expressed on the ordinate as absorbance at 320 nm/30 min. Control vessel contained no inhibitor (○—○), test vessels contained 25  $\mu$ M phenylalanine (●—●), and 50  $\mu$ M tryptophan (▲—▲). The reaction mixture contained 2.4 mg per ml of protein of an extract prepared from NP 40.

substrate concentrations of 3.33 mM. The reaction volume was scaled down to a volume of 0.3 ml, and the reaction was started by adding 0.1 ml of substrate. The reaction mixture was incubated at 32° for 30 min and terminated by adding 1.2 ml of 1 N NaOH. The amount of product, phenylpyruvate, formed was measured as the change in optical density at 320 nm. When allosteric effectors were used, equal volumes of effector solution and extract protein were mixed together and placed in an ice bath for 20 min. After allowing 5 min for equilibration, the usual assay procedure was then followed.

**Gradient Plates**—Gradient plates were prepared by pouring 15 ml of minimal glucose agar containing 10  $\mu$ g per ml of THA into a Petri dish and allowing the medium to solidify in a slanted

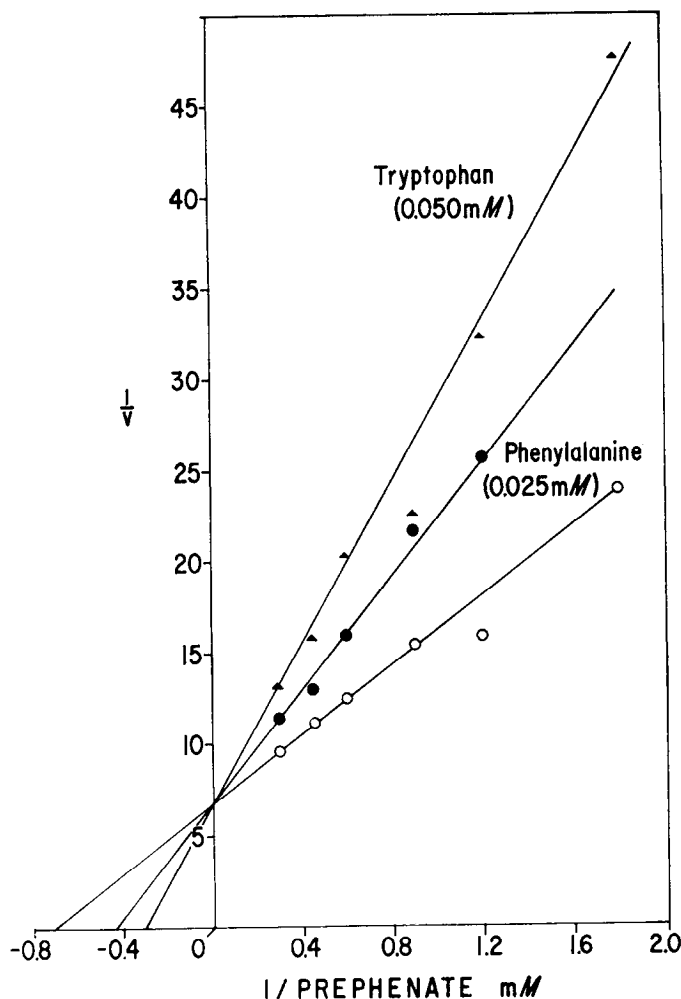


FIG. 3. Kinetic analysis of the influence of phenylalanine and tryptophan on prephenate dehydratase activity, double reciprocal plot. The data were calculated from those presented in Fig. 2.  $\circ$ — $\circ$ , control;  $\bullet$ — $\bullet$ , phenylalanine at 25  $\mu$ M;  $\blacktriangle$ — $\blacktriangle$ , tryptophan, 50  $\mu$ M.

position. The plates were then placed on a level surface and 15 ml of unsupplemented minimal glucose agar were added. The top layer was allowed to solidify in this position.

#### RESULTS

**Allosteric Effect of Modulators on Prephenate Dehydratase**—L-Phenylalanine is a potent and physiologically expected inhibitor of the first enzyme of the pathway leading to its own biosynthesis. Surprisingly, L-tryptophan is nearly as effective as phenylalanine in inhibiting the activity of prephenate dehydratase (15). Under usual assay conditions concentrations of about 20  $\mu$ M inhibitor will inhibit catalytic activity about 50% (Fig. 1). Substrate saturation curves for prephenate dehydratase in the absence of inhibitor, in the presence of 25  $\mu$ M L-phenylalanine, and in the presence of 50  $\mu$ M L-tryptophan are shown in Fig. 2. Neither phenylalanine nor tryptophan promoted a change in the shape of the substrate saturation curve. It is clear from the double reciprocal plots of these data (Fig. 3) that both inhibitors increase the value of the apparent  $K_m$ , indicating that the inhibition is competitive with respect to prephenate.

L-Tyrosine by itself was not found to affect the activity of prephenate dehydratase significantly (Fig. 4). However, when

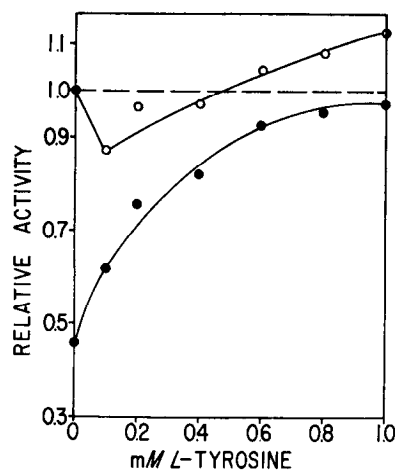


FIG. 4 (left). Reversal of tryptophan inhibition of prephenate dehydratase by L-tyrosine. Relative activity on the ordinate was calculated by relating the activity observed in the presence of effector to that of the control, observed in the absence of effector. Control activity is arbitrarily taken as 1.0 (dashed line) which equaled an absorbance of 0.147 at 320 nm after 30 min at 32°.  $\circ$ — $\circ$ , tyrosine alone;  $\bullet$ — $\bullet$ , tyrosine plus 40  $\mu$ M tryptophan.

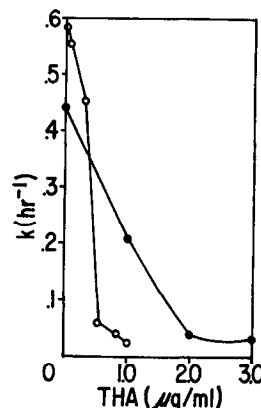


FIG. 5 (right). Growth inhibition by  $\beta$ -2-thienylalanine in *B. subtilis* NP 40. The specific growth rate,  $k$ , plotted as a function of variable concentrations of THA, is compared for cells grown at 37° ( $\bullet$ — $\bullet$ ) and at 50° ( $\circ$ — $\circ$ ). The specific growth rate,  $k$ , is expressed in reciprocal hours.

it was added to the enzyme simultaneously with tryptophan, 1.0 mM tyrosine reversed the inhibition caused by 40  $\mu$ M tryptophan, thus increasing the relative activity of the enzyme from 46% to 97% of the control activity (Fig. 4).

**$\beta$ -2-Thienylalanine Studies**—In spite of the demonstrated sensitivity *in vitro* of prephenate dehydratase to inhibition by tryptophan, we initially could not show a corresponding inhibition *in vivo* of phenylalanine synthesis by tryptophan (15). Attempts were subsequently made to see whether growth inhibition by tryptophan could be shown under conditions in which the internal concentration of phenylalanine was decreased.

Preliminary investigations with the gradient plate technique for antimetabolite sensitivity indicated that the synthesis of phenylalanine decreased at higher temperatures. A lawn of wild type *B. subtilis*, NP 40, was spread on minimal medium plates containing a gradient of THA, 0 to 10  $\mu$ g per ml. Duplicate plates were incubated at both 37° and 50°. Plates incubated

TABLE II

Effect of *L*-tryptophan on growth<sup>a</sup> of *Bacillus subtilis*, NP 40

Concentration of tryptophan <i>mM</i>	Specific growth rate, <i>k</i> <sup>b</sup>	
	At 37°	At 50°
0	0.41	0.58
1	0.37	0.21
2	0.35	0.11
3	0.32	0.08

<sup>a</sup> The cells used as inocula in this experiment were grown in minimal glucose medium. Ten milliliters of cells were grown in 125-ml side arm flasks in 37° and 50° shaking water baths. Growth was followed as the increase in turbidity in a Klett-Summerson colorimeter.

<sup>b</sup> The specific growth rate, *k*, is expressed in reciprocal hours.

at 50° developed a very small zone of growth in the lowest concentration range of THA. Plates incubated at 37° showed a much larger area of growth. Growth on control plates lacking the analogue was slightly heavier at 50° than at 37°. THA, as a phenylalanine analogue, inhibits growth by competing with phenylalanine. Inhibition by THA is readily reversed by THA. We assume that the greater resistance to the analogue at the lower temperature reflects a higher level of endogenous phenylalanine available to antagonize the antimetabolite action of THA.

The results of quantitative experiments in liquid cultures are shown in Fig. 5. At 37°, 2 μg per ml of THA were required to cause almost complete inhibition of the specific growth rate. However, at 50°, a 4-fold lower concentration, 0.5 μg per ml, was sufficient to cause the same inhibition. In another experiment, 250 μg per ml of tryptophan completely inhibited the growth of wild type cells at 50°, whereas 1 mg per ml was required to promote the same inhibition at 37°. Quantitative results relating the tryptophan concentration to growth rate at 37° and at 50° are shown in Table II. Tryptophan, at 2 mM, caused 81% inhibition of growth rate at 50°, whereas the same concentration caused a 15% inhibition of growth rate at 37°.

**Physiological Experiments**—Since tyrosine reverses the inhibition of prephenate dehydratase by tryptophan *in vitro*, it seemed likely that the elevated concentrations of tyrosine at lower temperatures (16) explained, in whole or in part, the finding that more tryptophan was required to cause inhibition of growth as the growth temperature was decreased. We, therefore, tested the effect *in vivo* of tryptophan upon phenylalanine synthesis at high growth temperatures predicted on the basis of experiments *in vitro*.

In Fig. 6 the optical density of a culture of wild type NP 40 growing at 50° is plotted against incubation time. A concentration of 2 mM tryptophan caused a significant inhibition of the growth rate. Addition of 50 μg per ml of phenylalanine completely reversed the inhibition, restoring the growth rate to that of the control (not shown in figure). Leucine and methionine are known to stimulate the activity of prephenate dehydratase (1). Methionine also reverses the inhibition of prephenate dehydratase caused by tryptophan.<sup>2</sup> Consistent with these characteristics *in vitro*, methionine and, to a lesser extent,

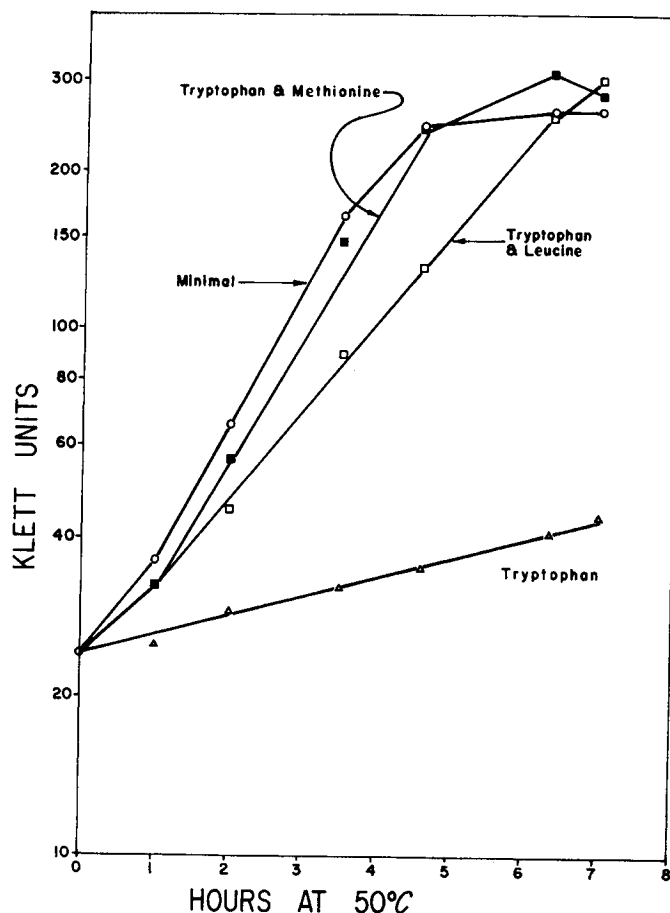


Fig. 6. Antagonism of tryptophan inhibition of growth in *B. subtilis* NP 40. Cells grown in minimal glucose medium at 50° were used as inoculum. The growth of 10-ml cultures was monitored in 125-ml side arm flasks, incubated in a shaking water bath at 50°. The growth rate in tryptophan plus phenylalanine (not shown) was the same as in minimal glucose (○—○). Tryptophan (△—△) inhibited growth, whereas methionine (■—■) and leucine (□—□) partially reversed this inhibition. Tryptophan was added at a concentration of 2 mM; other amino acids were added at final concentrations of 50 μg per ml.

leucine, caused significant increases in the growth rate when these were added to the tryptophan-containing medium (Fig. 6).

The experiments described in Fig. 6 involved the use of rather high concentrations of tryptophan (2 mM). Presumably, even at 50° in NP 40 sufficient tyrosine was present to reverse partially the potential inhibitory effects of tryptophan. Therefore, similar experiments were performed with the mutant, NP 100. This mutant has a low level of chorismate mutase and has derepressed tryptophan enzymes. As a result, it excretes large quantities of tryptophan, 57 μg per mg, dry weight, as compared to 1 μg per mg in the wild type (2). Therefore, NP 100 has high internal levels of tryptophan and low levels of phenylalanine and tyrosine. Presumably, the limiting amounts of prephenate formed owing to the block in chorismate mutase are not sufficient to provide enough tyrosine to negate a strong inhibition of prephenate dehydratase by tryptophan. In minimal medium at 37°, the growth rate of NP 100 was very poor,  $k = 0.09$  hour<sup>-1</sup> (Fig. 7). The addition of 50 μg per ml of *L*-phenylalanine increased the rate of growth ( $k = 0.47$  hour<sup>-1</sup>) to almost that of the wild type in minimal medium. Again, addition of the two

<sup>2</sup> J. Rebello, unpublished experiments.

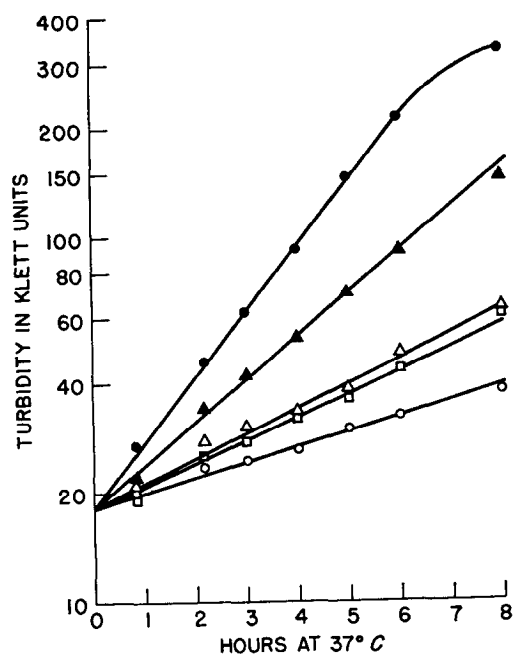


Fig. 7. Antagonism of tryptophan inhibition of growth in *B. subtilis* NP 100. Ten-milliliter cultures were grown in 125-ml side arm flasks in a shaking water bath at 37°. ○—○, minimal glucose; □—□, L-tyrosine; △—△, L-leucine; ▲—▲, L-methionine; ●—●, L-phenylalanine. Each amino acid was added at a final concentration of 50  $\mu$ g per ml.

activators of prephenate dehydratase, L-methionine and L-leucine, stimulated the growth rate.

That the level of L-tyrosine was low in NP 100 was shown in three ways: (a) 50  $\mu$ g per ml of D-tyrosine (an antimetabolite of L-tyrosine) inhibited the growth rate of NP 100 55% as compared to NP 40, which was inhibited 23%. (b) Cultures of NP 100 and NP 40 were each plated out (200 to 300 cells per plate) on solid phenylalanine-containing medium which had been surface-spread with serial dilutions of D-tyrosine. Colony size, under conditions of partial inhibition by D-tyrosine, constitutes a sensitive assay for intracellular levels of L-tyrosine (16). Higher concentrations of D-tyrosine by a factor of 6 were required in NP 40 to achieve the same decrement in colony size observed in NP 100. (c) The specific activity of prephenate dehydrogenase in NP 100 was derepressed when the strain was grown in minimal medium (17). Tyrosine is known to repress the synthesis of this enzyme in *B. subtilis* (18).

**Reversal of Tryptophan Inhibition of Growth by Tyrosine**—Data of experiments *in vitro* show that tyrosine does not significantly alter the activity of prephenate dehydratase by itself; however, it reverses the inhibition caused by tryptophan. This potential *in vitro* can be realized *in vivo*. The growth rate of NP 100 is presumably diminished by the effect of a high endogenous rate of tryptophan synthesis. The high levels of internal tryptophan then inhibit phenylalanine synthesis. When tyrosine was added to a culture of NP 100, an increase in the growth rate was observed (Fig. 7). The importance of tyrosine in the over-all pattern of regulation of aromatic amino acid synthesis can be further appreciated from some experiments performed with NP 164. This strain of *B. subtilis* has a feedback-resistant prephenate dehydrogenase and consequently excretes large amounts of tyrosine into the medium. It grows

TABLE III  
Mutational alteration of prephenate dehydratase to inhibition by phenylalanine and tryptophan

Collection No.	Relative activity <sup>a</sup> in the presence of				Allosteric type
	Phenylalanine		Tryptophan		
	0.1 mM	1.0 mM	0.1 mM	1.0 mM	
NP 40	10	0	10	0	S(trp), S(phe)
H-38	57	38	4	0	S(trp), R(phe)
H-39	97	72	14	19	
H-40	120	100	52	0	
H-41	87	41	41	0	
H-42	127	80	20	0	
H-43	98	72	25	16	
H-44	96	76	4	0	
H-45	113	60	0	0	
H-46	38	31	0	0	
H-47	115	257	0	0	S(trp), A(phe)
I-6	105	168	30	20	
I-8	90	142	36	7	
F-3	107	106	93	89	R(trp), R(phe)
F-4	254	1210	99	97	R(trp), A(phe)

<sup>a</sup> Control activity is arbitrarily taken as 100. Enzyme reaction was carried out at 32° for 30 min. When effectors were tested, equal volumes of enzyme and effector were mixed and placed in an ice bath for 20 min. The reaction was initiated by the addition of the substrate, prephenate, at a final concentration of 3.33 mM.

<sup>b</sup> Allosteric type: S(trp), S(phe): sensitive to inhibition by tryptophan and phenylalanine, respectively. R(trp), R(phe): resistant to inhibition by tryptophan and phenylalanine, respectively. A(phe): activated by phenylalanine.

poorly in minimal medium ( $k = 0.34 \text{ hour}^{-1}$ ) but grows at a normal rate ( $k = 0.41 \text{ hour}^{-1}$ ) when the medium is supplemented with phenylalanine. Presumably, prephenic acid is shunted preferentially into the tyrosine pathway, resulting in limiting phenylalanine for protein synthesis (19). Nevertheless, exogenous tryptophan does not inhibit growth of NP 164. Hence, low levels of intracellular phenylalanine are not sufficient to cause growth inhibition in the presence of tryptophan. Rather, there is an inverse correlation between tyrosine concentration and the ability of tryptophan to inhibit phenylalanine synthesis. Accordingly, exogenous tryptophan strongly inhibits the growth of NP 100 (low levels of tyrosine) and weakly inhibits the growth of NP 40 (normal levels of tyrosine), while the growth rate of NP 164 (high levels of tyrosine) is unaffected by tryptophan.

**Analysis of Prephenate Dehydratase Mutants**—The use of chemical techniques to desensitize prephenate dehydratase to various allosteric effectors has so far met with only limited success. Therefore, genetic methodology was used to obtain mutants with altered prephenate dehydratases. The prephenate dehydratases of these mutants were analyzed for sensitivity to inhibition by phenylalanine and tryptophan (Table III). Several classes of mutants with overlapping allosteric characteristics were isolated. Even though high concentrations of tryptophan

were used as the selective agent, the enzymes of the mutants obtained were modified mostly in their allosteric sensitivity to phenylalanine, rather than to tryptophan. Isolate H-40, although more extreme, is generally representative of the mutant group H-38 through H-46. The excretion of phenylalanine by H-40 was verified by its growth on THA plates and by syntrophic cross-feeding with phenylalanine auxotrophs. Although prephenate dehydratase from H-40 was less sensitive to inhibition by tryptophan, it was totally insensitive to concentrations of phenylalanine as high as 1.0 mM. The other mutants in this group had enzymes which were less sensitive to inhibition by phenylalanine than in NP 40. Feedback sensitivity to tryptophan was only slightly altered. Except for H-40 none of these mutants excreted significant amounts of phenylalanine. Mutant type H-47 had a prephenate dehydratase activity which was activated by phenylalanine but was completely inhibited by 0.1 mM tryptophan. Isolates I-6 and I-8 were similar except for slight quantitative differences. We confirmed the findings of Coats and Nester (20) that the prephenate dehydratase of isolate F-3 was insensitive to feedback inhibition by phenylalanine, and that the enzyme of isolate F-4 was activated by phenylalanine. In addition, we found the enzymes of F-3 and F-4 to be relatively insensitive to inhibition by tryptophan (Table III).

Apparently, there are numerous alternatives for mutational changes which confer resistance to growth inhibition by tryptophan. Although it is known that methionine, leucine, or tyrosine excretors are resistant to tryptophan inhibition, none of these excretor types were obtained when tryptophan was used as the selective agent. Examination of a greater number of mutants probably will yield these types as well as mutants with enzymes that are inhibited normally by phenylalanine but which are totally resistant to inhibition by tryptophan.

#### DISCUSSION

The prephenate dehydratase of *B. subtilis* is a remarkable allosteric protein. It has the expected specificity for the end product, phenylalanine, which regulates the enzyme activity by feedback inhibition. In addition, prephenate dehydratase bears allosteric specificities for methionine (activator), leucine (activator), tryptophan (inhibitor), and tyrosine (reversal of inhibition by tryptophan). The totally unexpected identity of the latter metabolites as allosteric effectors constitutes further documentation of the phenomenon of metabolic interlock (1, 2). For convenience, we refer to metabolites which possess an obvious control relationship to the activity of a regulatory enzyme as "primary effectors"; metabolites which are synthesized in one pathway but influence the activity of an enzyme in another pathway are "remote effectors." Thus, phenylalanine is the primary effector for prephenate dehydratase, and methionine, leucine, tryptophan, and tyrosine are remote effectors. Similarly, in the case of anthranilate synthetase of *B. subtilis* (2), tryptophan is the primary effector and histidine is a remote effector.

Remote effectors ordinarily exert subtle, secondary, and mutually reinforcing allosteric modifications of enzyme activities. Such modifying influences would allow the cell to approach ideal metabolite balances because of the response of allosteric proteins to small molecules other than end products and substrates. For example, under conditions of substantial feedback inhibition of prephenate dehydratase, methionine and

leucine would not elevate enzyme activity very much since they only activate the uninhibited fraction of activity (1). However, methionine and leucine would elevate enzyme activity appreciably when intracellular levels of phenylalanine are low. In like vein, although the great sensitivity of prephenate dehydratase to inhibition by tryptophan *in vitro* leads one to anticipate that exogenous tryptophan would inhibit the synthesis of phenylalanine *in vivo*, this prediction is not fulfilled under ordinary conditions of growth (15). This communication shows some physiological and nutritional conditions which unmask this potential for inhibition of phenylalanine synthesis by tryptophan in wild type *B. subtilis*. The demonstration *in vivo* of this relationship becomes even more apparent when further biosynthetic restraints are introduced by mutation.

It seems clear that the crucial determinant of whether tryptophan will inhibit growth depends upon the level of tyrosine in the cell. Although tyrosine does not appear to exert a significant allosteric effect upon the activity of prephenate dehydratase *per se*, it reverses the inhibitory effects of tryptophan. It therefore seems likely that the intracellular levels of tyrosine in wild type at 37° are sufficient to antagonize the inhibition of phenylalanine synthesis by tryptophan. This theory is consistent with recent results which reveal that excess tyrosine is synthesized and excreted into the medium by wild type cells (16). It was also shown that tyrosine overproduction is inversely correlated to growth temperature (16). Therefore, it seemed possible that growth at higher temperatures might decrease the intracellular levels of tyrosine sufficiently to reveal growth inhibition in the presence of tryptophan. This, in fact, proved to be the case when cultures were grown at 50°.

Even at 50° wild type cells produced an excess of tyrosine beyond the cellular requirement for protein synthesis (16). Therefore, a number of mutants were used in order to manipulate the balance of aromatic metabolites. Mutant NP 100 has a lowered level of chorismate mutase and is therefore limited in its ability to synthesize both tyrosine and phenylalanine; it is also derepressed for the enzymes of the tryptophan pathway, and excretes high concentrations of tryptophan (2, 17). This strain is very sensitive to inhibition by exogenous tryptophan at 37°. It grows poorly in minimal medium, but its growth rate approaches that of wild type in the presence of phenylalanine. It is inferred that the limiting levels of prephenate are mainly diverted to tyrosine because of the inhibitory influence of tryptophan upon the activity of prephenate dehydratase.

Mutant NP 164 also has a partial nutritional requirement for phenylalanine (19). It has a prephenate dehydrogenase that

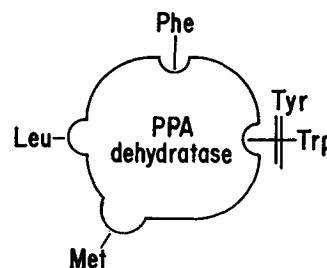


FIG. 8. Schematic model of the prephenate (PPA) dehydratase molecule of *B. subtilis*. Separate sites exist for the different allosteric effectors: *Leu*, leucine; *Met*, methionine; *Phe*, phenylalanine; *Trp*, tryptophan. Tyrosine (*Tyr*) antagonizes the inhibition by tryptophan; this could involve another allosteric site or tyrosine may compete with tryptophan for the same site.

is resistant to feedback inhibition by tyrosine, and it excretes large quantities of tyrosine. Presumably, the partial phenylalanine requirement reflects the excess drain of prephenate into the tyrosine pathway. The growth of this strain is not inhibited by tryptophan, even at 50°. Mutants NP 164 and NP 100 are similar in their partial growth requirements for phenylalanine; however, NP 164 maintains a large excess of tyrosine while NP 100 produces near limiting amounts of tyrosine. Therefore, limitation of prephenate for phenylalanine synthesis is not sufficient to lead to growth inhibition by tryptophan, even though the inhibition of prephenate dehydratase by tryptophan is kinetically competitive with respect to prephenate. A suitably low level of tyrosine is also necessary in order for tryptophan inhibition to be expressed. Further confirmation that growth inhibition by tryptophan is caused by its inhibitory effect upon prephenate dehydratase was provided by the observations that phenylalanine (the end product) and leucine or methionine (activators of prephenate dehydratase) reversed the growth inhibition caused by tryptophan in wild type and in NP 100.

Another approach involved the examination of mutants derived from NP 40 and NP 100 which are resistant to growth inhibition by tryptophan. These mutants characteristically were altered in the allosteric properties of prephenate dehydratase. The fact that tryptophan can be used as a selective agent to obtain mutational changes in an enzyme of phenylalanine synthesis confirms the relationship *in vivo* of these metabolites to prephenate dehydratase that was implicated by enzyme studies *in vitro*. The major change in our mutants was an alteration in the sensitivity of the enzyme to phenylalanine. Presumably, the normal level of enzyme sensitivity to tryptophan inhibition is permissive if the regulatory restraint usually imposed by phenylalanine is removed by mutation. Selection of more mutants would be expected to produce variants with altered allosteric sites for tryptophan, defective transport systems for tryptophan, and methionine, leucine, or tyrosine excretors.

Genetic and biochemical evidence presented here and elsewhere<sup>3</sup> supports the theory that separate sites on the enzyme exist for the various effectors tested. There are probably separate activator sites for leucine and methionine, and distinct inhibitor sites for phenylalanine and tryptophan. Mutants have been obtained which no longer respond to methionine but which retain the normal response to leucine<sup>4</sup>; also, variants exist in which the phenylalanine site has been affected, leaving the tryptophan site intact, or others in which both phenylalanine and tryptophan sites have been altered.

A schematic representation of the allosteric control of prephenate dehydratase by metabolic interlock is shown in Fig. 8. The metabolite specificities indicated by *indentations* represent inhibitors (tryptophan and phenylalanine) while those indicated

as *outward bulges* represent activators (methionine and leucine). Tyrosine antagonizes the inhibitory action of tryptophan. A possible explanation for these regulatory interactions between aromatic amino acids is that they may function to maintain a favorable balance between the levels of tyrosine and phenylalanine. The latter amino acids are natural structural analogues and are potentially capable of being mistaken for one another. For example, high concentrations of phenylalanine will inhibit prephenate dehydrogenase. It is also possible that permease and protein synthesis specificities could fail if the imbalance were sufficient. Thus, whenever intracellular levels of tyrosine diminish for any reason, tryptophan would tend to cause a parallel decrease in the internal levels of phenylalanine by inhibiting prephenate dehydratase. Because aromatic metabolites can be monitored with a high degree of sensitivity by spectrofluorometric techniques, we are hopeful that the quantitative determination of metabolite concentrations in wild type and mutant cells under various appropriate nutritional conditions will provide further insight into the physiological significance of these regulatory interactions.

#### REFERENCES

- JENSEN, R. A., *J. Biol. Chem.*, **244**, 2816 (1969).
- KANE, J. F., AND JENSEN, R. A., *J. Biol. Chem.*, **245**, 2384 (1970).
- KREDICH, N. M., BECKER, M. A., AND TOMKINS, G. M., *J. Biol. Chem.*, **244**, 2428 (1969).
- HUG, D. H., AND ROTH, D., *Biochem. Biophys. Res. Commun.*, **30**, 248 (1968).
- KAPOOR, M., BRAY, D. F., AND WARD, G. W., *Arch. Biochem. Biophys.*, **128**, 810 (1968).
- BAKER, T. I., *Biochemistry*, **5**, 2654 (1966).
- CATCHESIDE, D. E. A., *Biochem. Biophys. Res. Commun.*, **36**, 651 (1969).
- GERHART, J. C., AND PARDEE, A. B., *J. Biol. Chem.*, **237**, 891 (1962).
- CHANGEUX, J. P., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 497 (1963).
- BURKHOLDER, P. R., AND GILES, N. H., *Amer. J. Botany*, **34**, 345 (1947).
- JENSEN, R. A., *Genetics*, **60**, 707 (1968).
- HERBERT, D., ELSWORTH, R., AND TELLING, R. C., *J. Gen. Microbiol.*, **14**, 601 (1956).
- JENSEN, R. A., AND NESTER, E. W., *J. Mol. Biol.*, **12**, 468 (1965).
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. I., AND RANDALL, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
- NESTER, E. W., AND JENSEN, R. A., *J. Bacteriol.*, **91**, 1594 (1966).
- CHAMPNEY, W. S., AND JENSEN, R. A., *J. Bacteriol.*, in press.
- KANE, J. F., AND JENSEN, R. A., *Biochem. Biophys. Res. Commun.*, **38**, 1161 (1970).
- NESTER, E. W., JENSEN, R. A., AND NASSER, D. S., *J. Bacteriol.*, **97**, 83 (1969).
- CHAMPNEY, W. S., AND JENSEN, R. A., *J. Bacteriol.*, **98**, 205 (1969).
- COATS, J. H., AND NESTER, E. W., *J. Biol. Chem.*, **242**, 4948 (1967).

<sup>3</sup> J. Rebello and R. A. Jensen, manuscript in preparation.

<sup>4</sup> J. Rebello, unpublished observations.