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Metabolic Interlock

REGULATORY INTERACTIONS EXERTED BETWEEN BIOCHEMICAL PATHWAYS*

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SUMMARY

It is suggested that a regulatory superstructure of sophisticated interactions exerted *between* metabolic pathways acts to reinforce and modify the basic allosteric controls operating *within* specific pathways. Examples are cited to illustrate that small molecules may act as regulatory signals for enzymes that are located in different, apparently remote, metabolic pathways. (*a*) Regulatory interactions operating between the terminal branches of the biosynthetic pathway for aromatic amino acids are summarized as the simplest examples of the phenomenon. (*b*) Prephenate dehydratase, an enzyme of phenylalanine synthesis, was not only inhibited by phenylalanine but was also activated by either leucine or methionine. The activation was noncompetitive and independent of the competitive inhibition exerted by phenylalanine. Nutritional experiments with amino acid analogues verified the reality of these relationships *in vivo*. Data *in vitro* and *in vivo* lead to the conclusion that the activation of prephenate dehydratase by leucine or methionine is primarily significant under conditions of low phenylalanine (inhibitor) concentration. (*c*) A number of observations demonstrated regulatory influences of histidine upon the biosynthesis of tryptophan. A hypothesis is tendered which focuses upon 5-phosphoribosyl-1-pyrophosphate, a metabolite that is common to the synthesis of both tryptophan and histidine, as the key entity relating the two pathways.

An extensive comparative analysis of the regulation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase in well over 100 microbial species representing 32 genera produced the observation that the qualitative pattern of control exerted by small molecules upon this enzyme activity was strongly conserved at approximately the taxonomic level of genus (1). In fact, an analysis of a more quantitative characteristic of this allosteric protein, that of the temperature dependence of the enzyme-effector interaction, was shown to define microbial "relatedness" with even greater resolution (2). We concluded (1, 3) that various distinctive patterns of control existing in microbes for the regulation of the aromatic amino acid pathway illustrate differently evolved solutions to the dilemma posed by the complexities of multimetabolite regulation in a branched pathway. The facile mutability of allosteric proteins is well known. Mutational changes at allosteric sites are less likely to be lethal than those changes which impinge more directly upon the catalytic site. In view of this probable permissiveness for alterations of allosteric properties, the tenacious conservation of the control pattern observed for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetases of related microbial groups implies a powerful selection at work. One possible explanation advanced (1) was that different metabolic pathways might prove to be linked by subtle but nevertheless far reaching regulatory interactions. This report details several examples to show that regulatory interactions are indeed exerted between different metabolic pathways.

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MATERIALS AND METHODS

Bacterial Strains—The description of strains used in this investigation are listed in Table I. NP 187 was derived from NP 93. NP 17, NP 28, NP 108, NP 164, NP 285, and NP 297 were derived from NP 40. Analogue-resistant mutants were selected as spontaneous variants from lawns plated on analogue-containing media. Excretors were selected by picking resistant clones supporting the most prominent halos of background growth. They were verified by syntrophic cross-feeding analyses with appropriate auxotrophs. NP 285 was selected on plates containing 10 μg of L-methionine DL-sulfoximine per ml. NP 28, NP 108, NP 164, and NP 187 were originally selected on plates containing 50 μg of the appropriate amino acid analogue per ml. The requirements of NP 17 and NP 297 were induced by the mutagenic action of nitrosoguanidine. NP 67 and NP 83 were obtained from Dr. Takahashi (H12 and W3, respectively (4)). A complete discussion of parental stocks, strains 23 and 168, is given by Armstrong *et al.* (5).

Nutritional Procedures—The mixture of nonaromatic amino acids contained the following final concentrations of amino acids in micrograms per ml: alanine, 40; aspartic, 200; glutamic, 400; glycine, 80; isoleucine, 160; methionine, 40; threonine, 80; cystine, 40; proline, 120; serine, 200; valine, 160; arginine, 80; histidine, 80; lysine, 160; leucine, 200. The composition of growth media was previously described (6).

5-Methyltryptophan sensitivities of various strains were quantitated by spreading 0.1 ml of 10^{-2} dilutions of cells cultured

to a population size of 5×10^8 cells per ml in liquid minimal medium onto the surface of minimal agar plates. Sterile 11-mm paper discs were placed on the center surface of the plates and spotted with 0.02 ml of 2 mg of 5-methyltryptophan per ml. The diameter of the zone of inhibition was measured following incubation at 37° for 2 days.

Gradient plates of THA[1] were made by pouring 12 ml of minimal agar into tilted Petri dishes, allowing the agar to harden, repositioning the plates horizontally, and adding 12 ml of minimal agar containing 15 μg of THA per ml.

Chemicals—Barium prephenate was prepared as previously described (7). Its purity was estimated at 81% (uncorrected for solvation). THA and lysozyme (crystallized three times) were obtained from Sigma. D-Tyrosine, deoxyribonuclease (B grade), and amino acids were acquired from Calbiochem. 1,2,4-Triazole 3-alanine was the generous gift of Dr. Robert Guthrie. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) was prepared and used as previously described (6). All other chemicals were of reagent quality.

Analytical Procedures—Cells for the preparation of extracts were grown at 32° with vigorous aeration in 1-liter flasks containing 200 ml of medium and were harvested in the logarithmic phase of growth. Extracts for enzyme assay were prepared as described by Coats and Nester (8) except that cells were disrupted by a 15-min treatment with lysozyme, 200 μg per ml, plus deoxyribonuclease, 5 μg per ml at 34° followed by a short sonic oscillation treatment for 1 min with a Müller sonic oscillator. Small molecules were removed by gel filtration with Sephadex G-25. Protein concentrations were estimated chemically (9).

Prephenate dehydratase was assayed as previously described (8). The analysis of prephenate dehydratase activity in the presence of positive or negative effectors was routinely carried out by mixing a solution of the effector under test with an equal volume of extract protein. This mixture was then added to an appropriate volume of substrate to initiate the reaction. The reaction volume was scaled down to 0.3 ml.

The assay for PR transferase was negative in *Bacillus subtilis* when a sensitive fluorometric procedure was used to follow loss of anthranilate. Procedures which were tried in order to obtain an active enzyme preparation included variation of growth conditions; the use of derepressed tryptophan excretors; the use of added extract from a PR transferase mutant of *Salmonella typhimurium* to "pull" the reaction; the variation of buffer composition as well as pH, temperature, and ionic strength; variations in methods of cell disruption; the use of whole cell assays with permeabilized cells; the use of sulfhydryl protective reagents; fractionation of extract proteins to separate possible inhibitors; changes in concentrations of reaction components; and the addition of various metals or chelating agents.

Analogue Approach—Structural analogues of amino acids may mimic any function of the natural compound to a greater or lesser extent. Regardless of the particular site or sites of antimetabolite action, the inhibitory influence of the analogue upon the growth rate will be reversed by the natural compound. The inhibition of growth by all of the analogues used in these studies is totally reversed by the addition of relatively low concentrations of the natural metabolites. Therefore, a convenient method for observing internal changes in the regulation of the synthesis of the natural compound following the exogenous addition of a metabolite is to monitor alterations in the rate of growth in the presence of partially inhibitory concentrations of analogue.

[1] The abbreviations used are: THA, β-2-thienylalanine; PR transferase, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase.

TABLE I

Bacillus subtilis strains

Collection No.	Description
NP 17	Anthranilate synthetase-deficient, tryptophan auxotroph
NP 28	β-2-Thienylalanine-resistant, phenylalanine excretor
NP 40	Prototroph, spontaneous revertant of strain 168
NP 67	Phenylalanine auxotroph
NP 83	Leucine auxotroph
NP 93	Prototroph, spontaneous revertant of strain 23
NP 108	DL-4-Azaleucine-resistant, leucine excretor
NP 164	D-Tyrosine-resistant, tyrosine excretor
NP 187	1,2,4-Triazole 3-alanine-resistant, histidine excretor
NP 285	L-Methionine DL-sulfoximide-resistant, methionine excretor
NP 297	Methionine auxotroph

RESULTS

Regulatory Interaction between Synthesis of Phenylalanine and Tyrosine—Sometimes an allosteric agent may regulate an enzyme which does not participate in its own synthesis. For example, chorismate mutase activity is stimulated by tryptophan in *Neurospora crassa* (10). This phenomenon can be looked upon as an accessory control device acting as a secondary reinforcement which guarantees that, in the presence of excess tryptophan, chorismate will be shunted into precursors for the synthesis of phenylalanine and tyrosine. Hence, tryptophan possesses a dual signaling function as a negative effector for anthranilate synthetase and as a positive effector for chorismate mutase. Similarly, mutually reinforcing regulatory roles undoubtedly explain the "cross-inhibition" exerted by phenylalanine and tyrosine upon prephenate dehydrogenase and prephenate dehydratase, respectively, in *B. subtilis* (11, 12).

Another approach which implicates the occurrence of regulatory interactions between tyrosine and phenylalanine synthesis in *B. subtilis* is illustrated in Fig. 1. A regulatory mutant, NP 164, was isolated by selection in the presence of the D isomer of tyrosine, a growth-inhibitory compound which acts as a potent antimetabolite of L-tyrosine (13). The mutant possesses a prephenate dehydrogenase that is insensitive to inhibition by tyrosine, an effect which leads to the overproduction and excretion of L-tyrosine during growth. A consequence of this defect in tyrosine regulation was a decreased ability of the mutant to synthesize sufficient phenylalanine to maintain the wild-type growth rate. The stimulation by phenylalanine of growth rate in the mutant is shown in Fig. 1. The presence of nonaromatic amino acids in the growth medium accentuates the metabolic imbalance in the mutant, and the partial phenylalanine requirement is somewhat more apparent under these conditions. The partial requirement is nearly as distinct in minimal medium (specific growth rate, k, in minimal was 0.34; k in minimal plus phenylalanine was 0.41). Further details are cited elsewhere (14).

The results above are given as the simplest examples of inter-

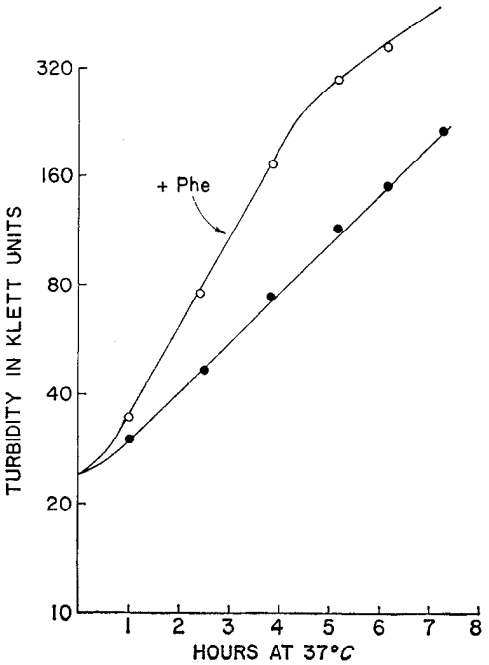


FIG. 1. Partial requirement of NP 164 for phenylalanine (*Phe*). NP 164 was grown overnight in minimal medium to the late log phase of growth at 37° and diluted into a minimal glucose medium supplemented with a mixture of the nonaromatic amino acids (●——●) or with a mixture of nonaromatic amino acids plus phenylalanine, 50 μg per ml (⊙——⊙). Growth was monitored in side arm flasks (10 ml of culture in a 125-ml flask) incubated in a shaking water bath at 37°. The Klett colorimeter was equipped with a red filter.

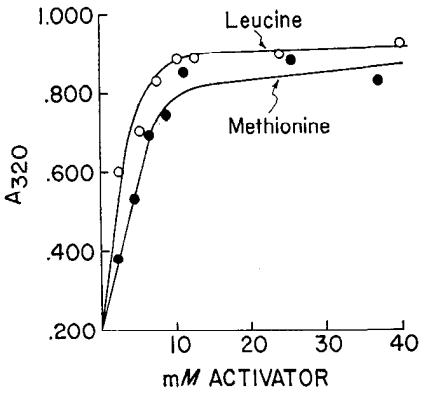


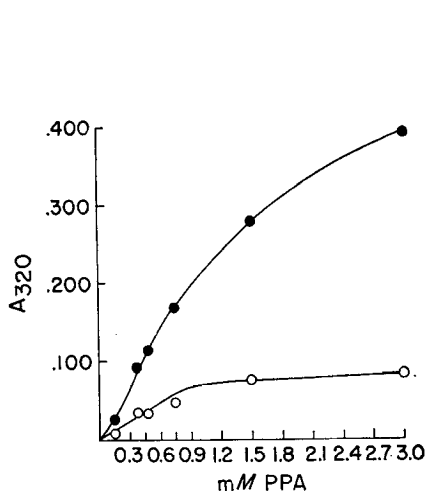
FIG. 2. Activation of prephenate dehydratase activity by leucine or methionine. Serial dilutions of leucine or methionine were added to the enzyme preparation and mixed with the prephenate substrate to initiate the reaction. The reaction velocity, v, is expressed as the absorbance of phenylpyruvate formed, A_{320}. The reaction time was 20 min at 32°. The reaction mixture contained 0.5 mg of protein. The extract was prepared from NP 40.

pathway regulation. The interacting metabolites and macromolecules are not in the same linear sequence of reactions. However, their relationships are relatively obvious since they are components of the same complex pathway.

Regulatory Interaction between Phenylalanine and Leucine-Methionine Synthesis—In contrast to the foregoing, leucine and methionine have no obvious metabolic connection with the synthesis of phenylalanine. Both methionine and leucine were allosteric activators of prephenate dehydratase as shown in Fig. 2. An activation exceeding 4-fold plateaued at 10^{-2} M concentrations of activator. Coats and Nester (8) also noted a stimulatory effect of these amino acids at 10^{-3}. Leucine was somewhat more effective than methionine on a molar basis. Appropriate controls established that the extract did not convert leucine or methionine to 320 mμ-absorbing material and that neither compound interacted nonenzymatically with prephenate or with the extract to produce absorbance at 320 mμ. Both leucine and methionine protected the enzyme from storage inactivation. Both amino acids at 10^{-2} M concentration resulted in the complete stabilization of enzyme activity after 28 hours at 4° under conditions in which the enzyme otherwise lost about 90% of the original activity. The substrate saturation curve for prephenate was slightly sigmoid in shape (Fig. 3), and therefore the double reciprocal plot of these data (Fig. 4) resulted in curves constantly bending upward in the direction away from the ordinate. In spite of this departure from Michaelian behavior, it is clear that all or most of the activation by leucine (or methionine) can be attributed to an increase in the apparent V_{max}.

The activation by leucine or methionine does not reverse or otherwise influence the inhibition of prephenate dehydratase activity by phenylalanine. The phenylalanine inhibition data shown in Fig. 5 were obtained in the presence of two fixed concentrations of leucine, following activity as a function of variable concentrations of phenylalanine. The experimental points were superimposed on the same inhibition curve. The phenylalanine inhibition curve in the absence of activator (experimental points not shown) were also superimposed with those shown in Fig. 5. Identical experiments with several fixed concentrations of methionine yielded similar data. The *inset* indicates the variation of the actual reaction velocities measured as a function of phenylalanine concentration at each of the two fixed concentrations of leucine indicated. The effect of activator molecules is obviously more significant in the low concentration range of the phenylalanine inhibition curve, becoming of little quantitative import at higher inhibitor concentrations. Thus, the activation of the enzyme is independent of the inhibition by phenylalanine and influences only the residual, noninhibited portion of the enzyme activity. It follows that the potential for activation would be greatest under conditions of minimal phenylalanine concentration in the cell.

The physiological interpretation of the results *in vitro* with prephenate dehydratase are further supported by experiments *in vivo* with the phenylalanine analogue, THA. (*a*) In a minimal medium the analogue decreased the growth rate of wild-type *B. subtilis* sharply as the concentration was increased to 3 μg per ml (Fig. 6). The addition of 5 μg of leucine per ml partially antagonized the growth inhibition in this range of analogue concentration; at higher analogue concentrations leucine did not appreciably alter the inhibition of growth by the analogue, even when leucine concentrations were increased to 50 μg per ml. Entirely comparable effects were found with the use of methionine. This is consistent with the *inset* of Fig. 5 illustrating activation of enzyme activity at low inhibitor concentrations of phenylalanine, but not at high inhibitor concentrations *in vitro*. (*b*) Secondly, a THA-resistant phenylalanine excretor which possesses a feedback-resistant prephenate dehydratase cross-feeds wild type better on analogue plates (50 μg per ml) supplemented with leucine or methionine (50 μg per ml) than on analogue plates alone. Similarly, the excretor feeds NP 67, a phenylalanine auxotroph, better on methionine or leucine plates



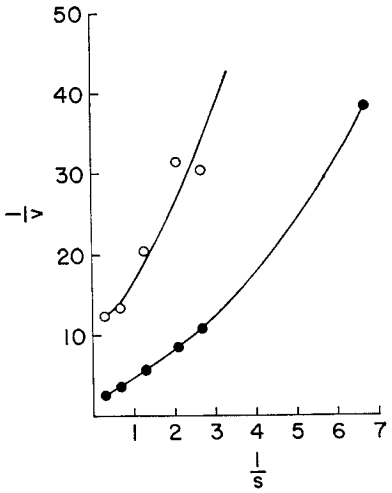


FIG. 3 (*left*). Substrate saturation curves for prephenate dehydratase. Reaction velocity is expressed on the *ordinate* as absorbance at 320 mμ. Leucine was absent in one reaction vessel (⊙——⊙) and present as a final concentration of 1.3×10^{-2} M in the other (●——●). The reaction time was 20 min at 32°. The reaction mixture contained 0.3 mg of protein of an extract prepared from NP 40. The potassium salt of the prephenate (*PPA*) substrate was used in the reaction.

FIG. 4 (*center*). Kinetic analysis of the influence of leucine upon prephenate dehydratase activity, double reciprocal plot. The data were calculated from those presented in Fig. 3. ⊙——⊙, no leucine present; ●——●, leucine present at 1.3×10^{-2} M.

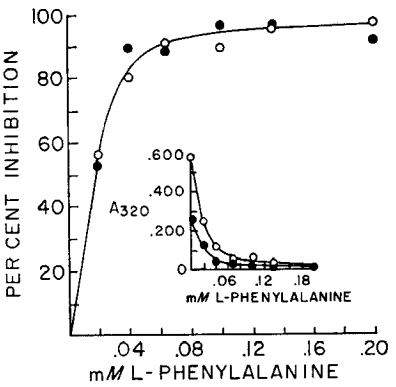


FIG. 5 (*right*). Inhibition curve of prephenate dehydratase by phenylalanine in the presence of 1.3×10^{-3} M leucine (●——●) and by 5.6×10^{-3} M leucine (⊙——⊙). Percentage of inhibition on the *ordinate* was calculated by relating the activity observed in the presence of phenylalanine to the activity measured in the control vessel lacking phenylalanine. The *inset* shows the original data in which v, expressed as A_{320}, is plotted as a function of phenylalanine concentration. Control activity determined in the absence of leucine was A_{320}, 0.143. The reaction mixture contained 0.4 mg of protein from an extract prepared from NP 40.

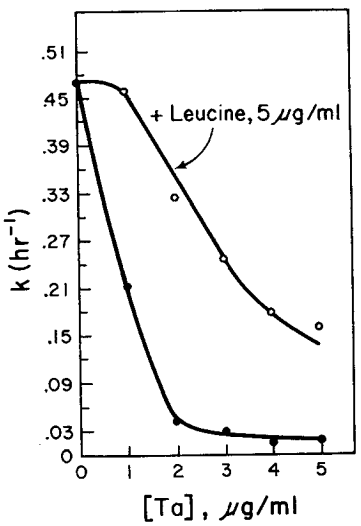


FIG. 6. Antagonism by leucine of growth inhibition by β-2-thienylalanine (*Ta*) in *B. subtilis* NP 40. The specific growth rate, k, plotted as a function of variable concentrations of THA is compared in the presence and absence of leucine. Growth rates were monitored at 37° as described for Fig. 1. The specific growth rate, k, is expressed in reciprocal hours.

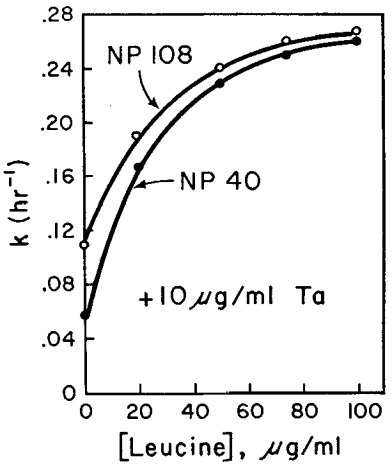


FIG. 7. Comparison of the sensitivity of a leucine excretor and wild type to growth inhibition by β-2-thienylalanine (*Ta*). With THA fixed at 10 μg per ml, k is plotted as a function of leucine concentration. The conditions of growth were as described for Fig. 1.

than on minimal plates. (*c*) Mutants which excrete either leucine or methionine ought to be more resistant to low concentrations of THA than wild type. NP 108, a leucine excretor, is indeed more resistant than the parental NP 40 (Fig. 7). In the presence of 10 μg THA per ml, NP 40 required 8 μg of leucine per ml to achieve the intrinsic resistance of NP 108 to this concentration of THA. Again there is little to distinguish the two at higher concentrations of THA. Similar results were achieved when a L-methionine sulfoximine-resistant methionine excretor was compared with wild type in the presence of THA. A summary of the analogue resistances of a leucine excretor (NP 108), a methionine excretor (NP 285), a phenylalanine excretor (NP 28), and wild type (NP 40) are given in Fig. 8. Only the leucine excretor was resistant to DL-4-azaleucine and only the methionine excretor was resistant to L-methionine sulfoximine, but both of these mutants additionally possessed a concomitant increased level of resistance to low concentrations of THA. The leucine excretor was somewhat more resistant to THA than the methionine excretor. The level of the increased resistances of NP 108

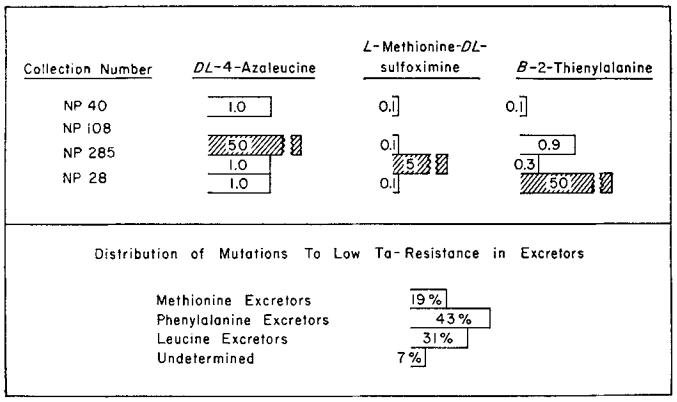


FIG. 8. Summary of analysis of phenylalanine, leucine, and methionine excretors. *Upper half*, the *numbers within the horizontal bars* indicate the concentration of analogue in micrograms per ml which prevented the visible colony formation of 100 to 500 cells after 3 days at 37° when surface-spread on minimal agar plates. The *numbers within the shaded horizontal bars* indicate the highest concentration of analogue tested. Each of the latter concentrations was not sufficient to produce detectable inhibition. *Lower half*, 100 resistant mutants of NP 40 that were judged to be excretors were selected from agar plates made up with a gradient of β-2-thienylalanine (*Ta*). They were all selected at the low concentration margin of the gradient adjacent to confluent growth. The THA-resistant isolates were cross-streaked against auxotrophs requiring phenylalanine, methionine, or leucine on minimal agar to identify the excreted metabolite. Of the seven uncharacterized isolates, two seemed to excrete more than one of the amino acids, and five were nonexcretors which apparently were erroneously picked.

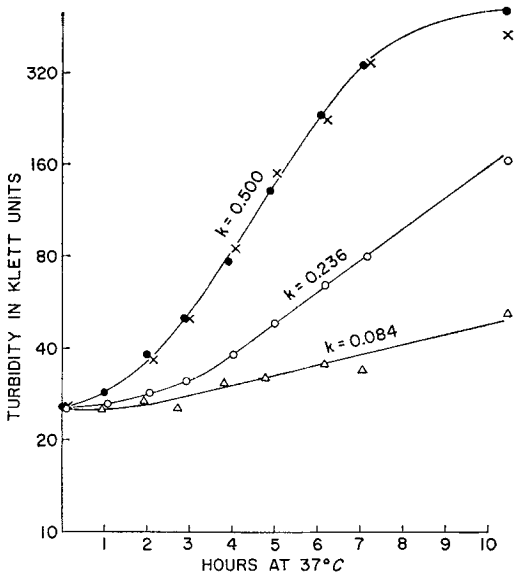


FIG. 9. Increased resistance of a histidine-excreting mutant to inhibition by 5-methyltryptophan. The growth of 10-ml cultures was monitored in 125-ml side arm flasks, incubated in a shaking water bath at 37°. The parental strain, NP 93 (×——×), and its histidine-excreting derivative, NP 187 (●——●), grew equally well in the absence of 5-methyltryptophan. 5-Methyltryptophan at a final concentration of 25 μg per ml was present in growth flasks inoculated with NP 93 (△——△) or with NP 187 (○——○). Values calculated for *k*, the specific growth rate, are indicated *along the curve*.

and NP 285 is substantially lower than that of the phenylalanine excretor, a result in agreement with conclusions drawn from Fig. 5 to the effect that the activators of prephenate dehydratase do not interfere with the feedback inhibition of the enzyme by phenylalanine. The *lower portion* of Fig. 8 indicates the distribution of various classes of THA-resistant excretors selected on gradient plates at low THA concentrations. Substantial numbers of leucine and methionine excretors were obtained in addition to phenylalanine excretors. As expected, high level THA-resistant excretors invariably proved to be phenylalanine excretors.

Regulatory Interaction between Tryptophan and Histidine Synthesis—It had been observed in the course of routine experiments that 5-methyltryptophan did not inhibit growth on casein hydrolysate media. Likewise, the presence of an amino acid solution lacking the aromatic amino acids also negated the usual inhibitory action of 5-methyltryptophan. Since acid-hydrolyzed casein lacks tryptophan, one would expect that the sparing action of other amino acids might tend to stress the pathway of tryptophan biosynthesis and, if anything, render 5-methyltryptophan more inhibitory to growth. Subsequent experiments showed that the deletion of histidine from the amino acid solution restored the potent antimetabolite action of 5-methyltryptophan. It was then possible to show that histidine alone influenced the sensitivity of *B. subtilis* to 5-methyltryptophan (15), presumably by some effect on the biosynthesis of tryptophan.

The following observations bear upon the stimulation of tryptophan synthesis by histidine. (*a*) A regulator mutant of *B. subtilis* which is derepressed for the tryptophan enzymes and excretes tryptophan feeds tryptophan-requiring auxotrophs distinctly better on histidine agar than on minimal agar. (*b*) When lawns of wild-type NP 40 were placed on histidine and minimal plates, 5-methyltryptophan-saturated discs produced smaller zones of inhibition on histidine agar than on minimal agar. The zones of inhibition following equal times of incubation at 37° were 4.1 and 2.9 cm in diameter on minimal and histidine-supplemented agar, respectively. (*c*) Wild-type NP 40 is capable of cross-feeding some tryptophan auxotrophs on minimal agar, namely those of the class which will grow on anthranilic acid and which possess a mutant deficiency in the anthranilate synthetase reaction. Other auxotrophs with later enzyme blocks in indole glycerol 3-phosphate synthetase and tryptophan synthetase are not fed by wild type appreciably. This suggests that NP 40 accumulates anthranilate, *N*-(5'-phosphoribosyl)anthranilate, or 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate. This feeding of anthranilate synthetase-less auxotrophs by wild type is increased on histidine plates. (*d*) We had previously reported a partial reversal of 5-methyltryptophan inhibition by anthranilate and showed that anthranilate and histidine in combination antagonize the analogue much more effectively than either alone (15). (*e*) If histidine does promote the increased synthesis of tryptophan, then regulatory mutants which overproduce histidine ought to be more resistant to 5-methyltryptophan than wild type. 1,2,4-Triazole 3-alanine, an antimetabolite of histidine in *B. subtilis*,[2,3] acts as a potent inhibitor of growth and was used to isolate a histidine excretor, NP 187. The data of Fig. 9 demonstrate the elevated resistance to 5-methyltryptophan of NP 187. The mutant growth rate exceeds that of the wild-type parent by a factor of nearly 3 in the pres-

[2] R. Guthrie, personal communication.
[3] R. A. Jensen, unpublished observations.

ence of 5-methyltryptophan, 25 μg per ml. Cross-streaking experiments established that NP 187 fed a mutant blocked in anthranilate synthetase (NP 17) much better than did the parental wild type, NP 93, on plates containing low concentrations (15 μg per ml) of 5-methyltryptophan. NP 187 did not cross-feed NP 17 any better on histidine-supplemented agar. On the other hand, syntrophism of NP 93 with NP 17 improved decidedly in the presence of histidine. (*f*) Various mutations of *B. subtilis* have been described in the literature in which concomitant alterations involving histidine and tryptophan were found (16, 17).

The foregoing results summarize the findings that both histidine and anthranilate act to antagonize the inhibitory effects of 5-methyltryptophan. The combination of histidine and anthranilate exerts a positive stimulus to the synthesis of tryptophan, presumably by reversing or bypassing regulated steps in the pathway.

DISCUSSION

The simplest and most direct allosteric control of a biosynthetic enzyme activity involves the binding of an end product molecule with the macromolecular enzyme that catalyzes the formation of the first unique precursor in the synthesis of the end product, *i.e.* feedback inhibition. The diverging multibranched chemical route used by microorganisms for the synthesis of aromatic amino acids potentially offers a regulatory dilemma. The essence of the dilemma centers about the ability of an enzyme at the beginning of the pathway to be suitably responsive to more than one metabolic signal. The pathway of aromatic synthesis is not so complicated as to prevent the appreciation of the individuality of the many ingenious control patterns which exist in microorganisms (1). At still one higher level of complexity are those regulatory interactions within the aromatic pathway in which end products of one terminal branch influence regulatory enzymes of other branches (11, 12). Total understanding of the subtleties of control at this level is far from complete. Meaningful physiological interactions can nevertheless be imagined, *e.g.* between tyrosine and an enzyme of phenylalanine synthesis, because both are removed from the common chain of precursor molecules by only a few unique enzyme steps. Explanations similar to those presented by Cerutti and Guroff (18) or by Baker (10) could apply.

The regulatory interactions herein noted between phenylalanine synthesis and that of methionine-leucine or the interactions between the synthesis of histidine and tryptophan depart much more abruptly from conventional expectations. A glance at any metabolic chart quickly conveys the impression of a complexly interwoven set of biochemical relationships. Hence, if all of metabolism is looked upon as one extraordinarily complex branched pathway, then it seems attractive that the action of relatively obvious metabolic signals is refined by additional influences of molecular signals, which may not be so large in magnitude. In analogy of regulatory interactions operating between the individual branches of the aromatic amino acid pathway, controlling interactions between relatively remote metabolic segments may amplify and modify in systemic fashion the more obvious and direct local controls in individual sequences of reactions.

It seems probable that the control relationships described in this paper are of such a secondary, reinforcing nature. For example, concentrations of leucine and methionine which activate

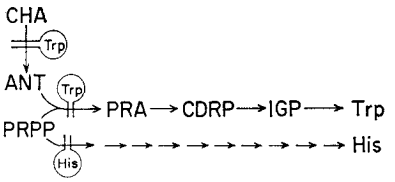


FIG. 10. Hypothetical regulatory relationship between histidine and tryptophan synthesis. The *symbols intersecting with arrows* designating enzyme reactions indicate regulatory enzymes that are feedback inhibited by the amino acid shown *within the symbol*. *CHA*, chorismate; *ANT*, anthranilate; *PRPP*, 5-phosphoribosyl-1-pyrophosphate; *PRA*, *N*-(5′-phosphoribosyl)anthranilate; *CDRP*, 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate; *IGP*, indole glycerol 3-phosphate; *Trp*, L-tryptophan; *His*, L-histidine.

prephenate dehydratase are relatively high compared to the concentrations of phenylalanine required for inhibition. Additionally, the stimulatory amino acids do not influence the ability of phenylalanine to act as a feedback inhibitor; the activators only increase the activity of the residual, uninhibited fraction of enzyme activity. Thus it would appear certain that the ability of leucine and methionine to increase enzyme activity *in vivo* would primarily occur under conditions in which the enzyme is relatively free from restraint by phenylalanine. This conclusion agrees with the observation that leucine or methionine only produces a significant reversal of growth inhibition by THA when THA concentrations are low, a condition synonymous with a fractional degree of false feedback inhibition of prephenate dehydratase by THA. The exact meaning in physiological terms of the finding that high concentrations of methionine or leucine or both exert a positive influence upon the endogenous level of phenylalanine is unknown. In *N. crassa* leucine and methionine resemble phenylalanine in their common ability to inhibit the transport system for tryptophan (19). Perhaps if leucine, methionine, and phenylalanine share the same transport system in *B. subtilis*, the stimulation of prehenate dehydratase by leucine and methionine may reflect a regulatory device to keep the internal pool balanced. This would tend to prevent an excess of one amino acid from blocking a transport system shared with other amino acids. Alternatively, the maintenance of optimal ratios of various amino acids in intracellular pools may promote protein synthesis by minimizing the probability of translational ambiguities in the process of protein synthesis. Similar explanations could apply to the meaning of the observed stimulation of tryptophan synthesis by histidine in *B. subtilis*. A striking number of other observations relating leucine, methionine, and phenylalanine with one another occur in the literature. Mutants of *N. crassa* have been described that have alternative requirement for leucine or phenylalanine (20, 21). In *Bacillus polymyxa* leucine, methionine, and phenylalanine were among a number of nonpolar amino acids which overcame the threonine, lysine inhibition of aspartokinase (22). In RC^{rel} strains of *Escherichia coli*, leucine, methionine, and phenylalanine all inhibit growth under nutritional shift-down conditions (23).

The enzymatic basis for the influence of histidine upon the synthesis of tryptophan in *B. subtilis* has not been directly established (see "Analytical Procedures"). The existing data are, however, consistent with the relationship diagrammed in Fig. 10. Here the significant entity common to both pathways is 5-phosphoribosyl-1-pyrophosphate, a substrate for the first enzyme of the histidine pathway as well as for the second enzyme of the

tryptophan pathway. The hypothesis is that tryptophan not only inhibits the first enzyme, anthranilate synthetase (11), but also the second enzyme, PR transferase. Histidine would be expected to spare the requirement for 5-phosphoribosyl-1-pyrophosphate molecules normally utilized by ATP-phosphoribosyl-pyrophosphate pyrophosphorylase as a consequence of the action of histidine as a feedback effector for the enzyme. Assuming the control of PR transferase by tryptophan (or 5-methyltryptophan) to be competitive, 5-phosphoribosyl-1-pyrophosphate would tend to reduce the inhibitory restraints exerted by tryptophan. The following data supported the existence of a tryptophan-controlled PR transferase. (*a*) Control by tryptophan upon the first enzyme, anthranilate synthetase, is incomplete based upon the observed accumulation of anthranilate under numerous conditions of growth in wild type (paper chromatography and fluorescence analysis).[3] (*b*) Anthranilate only partially reverses false feedback inhibition by 5-methyltryptophan even though anthranilate bypasses the first control site, anthranilate synthetase (11). However, the combination of histidine and anthranilate *does* overcome the inhibition almost completely (15). Indole and tryptophan also antagonize inhibition by 5-methyltryptophan as expected since they bypass both control points. (*c*) It has been shown that tryptophan does inhibit the activity of the PR transferase in *E. coli* (24) and in *S. typhimurium* (25). (*d*) If both tryptophan and 5-methyltryptophan inhibit PR transferase, then one ought to be able to select mutants having a PR transferase that is insensitive to false feedback inhibition by 5-methyltryptophan together with an anthranilate synthetase that is normally sensitive to 5-methyltryptophan. Such mutants should be conditionally 5-methyltryptophan-resistant, being 5-methyltryptophan-sensitive in minimal media but 5-methyltryptophan-resistant (50 μg per ml) in the presence of anthranilate. Such mutants are readily obtained on anthranilate-5-methyltryptophan agar. The concentration of anthranilate used (5 μg per ml) overcomes 5-methyltryptophan inhibition poorly in wild type (15).

Regulatory enzymes occur at metabolic branch points. Just as chorismate is a branch point with respect to tryptophan and to phenylalanine plus tyrosine, so 5-phosphoribosyl-1-pyrophosphate is a branch point with respect to tryptophan and histidine. Thus the regulation of PR transferase as well as anthranilate synthetase by tryptophan is consistent with physiological expectations. The molecular mechanism for inhibition of the second enzyme in *E. coli* involves its association with anthranilate synthetase within a macromolecular aggregate (26). We have not found conditions permissive for the quantitative assay of this enzyme (see Reference 27), and this may involve its aggregation with other tryptophan enzymes in *B. subtilis* (28).

From microorganisms in general, data are emerging to formulate a general relationship of histidine and aromatic amino acids. Carsiotis and Lacy (29) have described the increased synthesis of tryptophan enzymes in histidine auxotrophs of *N. crassa*. Ames (30) described an aromatic permease which mediated the common transfer of histidine and aromatic amino acids in *S. typhimurium*. Hug and Roth (31) found an allosteric control of histidine deaminase by L-tyrosine in *Pseudomonas putida*. Other observations relating to tryptophan and histidine synthesis have been made in *B. subtilis* (6, 12, 15–17). The exact details of the histidine-aromatic relationship undoubtedly vary in microorganisms differing in their recent evolutionary histories. However, the maintenance of some qualitative relationship in such different organisms probably reflects primitive ancestral relationships centering about the common derivation of histidine and aromatic amino acids from the pentose pathway.

We previously observed that patterns of control for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase are conserved sufficiently to mirror ancestral relationships of microbes (1). Such a system of communication between different metabolic pathways by the agency of regulatory molecules offers some ground for the understanding of the persistance of control patterns in related groups of microbes. A mutation leading to an alteration in the regulation of one pathway might be very likely to perturb the delicate balance of one or more other pathways. Secondary effects produced by these imbalances upon still other pathways could generate a highly unfit pleiotrophic result, undoing in one fell swoop the accrued advantages of a long evolutionary history. Therefore, once a given basic pattern of control was qualitatively established in primitive cells, there may have followed a sequence of relatively subtle mutations effecting the more intimate integration of this control with the total metabolic context of the cell as a result of selective pressures. This proposes a complex genetic background specifying the regulatory properties of allosteric enzymes. If so, a transition from one distinct pattern of metabolic control to another probably could not be accomplished through a single step mutation. Hence, the gross qualitative pattern of control for an enzyme in a sufficiently complex pathway is viewed as a primitive characteristic of the enzyme. The subsequent juxtaposition of secondary, modifying mutations in the course of evolution would tend to enhance the survival value of the basic pattern and therefore to promote its conservation.

Other observations *in vitro* suggestive of regulatory interactions mediated by small molecules of one pathway with macromolecules are known. For example, a strain of *N. crassa* was described in which threonine interfered with the metabolism of methionine, thiamine, adenine, and serine (32). Histidine or lysine will inhibit the growth of arginine bradytrophs in *E. coli* (33). An analogue of histidine was found to inhibit the growth of *E. coli* because of its interaction with an enzyme of arginine biosynthesis (34). Leucine, methionine, phenylalanine, tryptophan, and alanine were found to activate aspartokinase in *B. polymyxa* (22). The same nonpolar L-amino acids potentiate the inhibition of the lysine-sensitive aspartokinase by lysine in *E. coli* (35). Our results *in vitro* correlate with supporting observations *in vivo*. Previously there has been a general inclination to question the significance *in vivo* of such results *in vitro*. Although these specific data deal with allostery, it seems equally likely that interpathway regulatory relationships exist at the level of the regulation of enzyme synthesis (*e.g.* Reference 29).

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