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Metabolic Interlock

THE ROLE OF THE SUBORDINATE TYPE OF ENZYME IN THE REGULATION OF A COMPLEX PATHWAY*

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SUMMARY

The activity of chorismate mutase plays a strategic role in mediating the flow of chorismate between the synthesis of tyrosine and phenylalanine on the one hand, and of tryptophan and a number of aromatic vitamins on the other hand. A gene specifying chorismate mutase in strain 23 of *Bacillus subtilis* was introduced into the genetic background of strain 168 by deoxyribonucleate transformation. The newly introduced chorismate mutase has a specific activity which is 10 times greater than that of strain 168. This hybrid strain was used to study the effect of the altered activity of chorismate mutase upon the routing of common pathway precursors into the divergent terminal branches of the aromatic amino acid pathway of biosynthesis.

1. Compared to strain 168, the hybrid strain (carrying chorismate mutase-H) was more resistant to growth inhibition by low concentrations of D-tyrosine and less resistant to growth inhibition by low concentrations of 5-methyltryptophan. The hybrid strain was resistant to a weak inhibition of growth which may occur in derivatives of strain 168 in the presence of the combination of tryptophan and tyrosine. The hybrid strain also possesses an increased capacity for incorporation of exogenous shikimate.

2. Chorismate mutase was product-inhibited by prephenate, a finding which we suggest is physiologically significant—a part of the pattern of sequential feedback inhibition which regulates the aromatic pathway in *B. subtilis*.

3. The considerable ability of the hybrid strain to adjust to the unbalancing influence of chorismate mutase-H is attributed to the compensatory capabilities of allosteric and repressive controls existing in the pathway. Accordingly, when these regulatory controls in the terminal branches of the pathway were lost by mutation, the full potential of an alteration in level of chorismate mutase to unbalance the flow of common precursors to the terminal branches of the pathway was unmasked. The dependence of the aromatic pathway in *B. subtilis* upon the regulatory enzymes in its terminal branches for the appropriate function of chorismate mutase leads us to describe this chorismate mutase as an example of a subordinate enzyme.

4. Hybrid strains of *B. subtilis* are widely distributed, often inadvertently. The properties of hybrid strains explain a number of findings in the literature.

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The allosteric regulation of the biochemical pathway serving the synthesis of aromatic amino acids is shown in schematic form in Fig. 1. The diagram is intended to summarize an over-all pattern of allosteric control in *Bacillus subtilis* which we have called sequential feedback inhibition (1, 2) This pattern consists of four interacting loops of feedback inhibition, one in each of the terminal branches of the pathway and the fourth spanning the common sequence of reactions. These four regulatory loops are ingeniously integrated insofar as the substrates of the three allosteric enzymes in the terminal branches are also the feedback

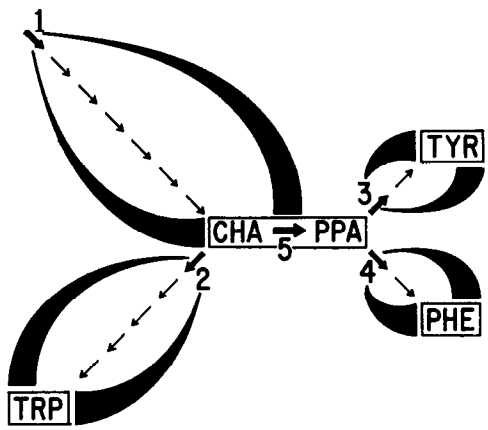

FIG. 1. Schematic representation of the biochemical pathway for the synthesis of aromatic amino acids. The allosteric pattern of regulation in *Bacillus subtilis* is known as sequential feedback inhibition (2). Feedback effectors appear in *boxes* (*TYR*, tyrosine; *PHE*, phenylalanine; *TRP*, tryptophan; *CHA*, chorismate; and *PPA*, prephenate). The *pincer-like wedges* point to the key regulatory enzymes which are inhibited by the feedback effectors. These enzymes are indicated by *heavier arrows* (*1*, DAHP synthase; *2*, anthranilate synthase; *3*, prephenate dehydrogenase; *4*, prephenate dehydratase; and *5*, chorismate mutase).

effectors for the first enzyme in the sequence, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. (In this paper we also suggest that the product inhibition of chorismate mutase by prephenate may be a fifth regulatory element in the sequential feedback inhibitory pattern of control.)

In retrospect these control relationships seem to be obvious ones compared to other unexpected, more subtle allosteric specificities which we have termed metabolic interlock (3–5). Within the branched aromatic pathway itself, various regulatory interplays have been found. For example, both tryptophan and tyrosine influence the activity of prephenate dehydratase (5). Indeed, perhaps the simplest examples of metabolic interlock are these allosteric interactions exerted between the terminal branches of the aromatic amino acid pathway in *B. subtilis*. The role of these interpathway relationships of control may be to coordinate the catalytic activity of a particular pathway with the over-all metabolic state of the cell. The general metabolic state of microbial cells is known to be subject to a great deal of flux in nature. We have suggested that the significance of interpathway relationships of regulatory interlock may be to maintain balanced ratios of metabolites, *e.g.* for optimal rates of protein synthesis (3).

Chorismate mutase is located at a metabolic branch point in the aromatic amino acid pathway of biosynthesis. Metabolic branch points usually mark the location of regulatory enzymes which serve as foci of allosteric control governing the utilization of multifunctional metabolites into competing pathways. Chorismate mutase acts to divert the common intermediate, chorismate, from pathways of tryptophan and aromatic vitamin synthesis toward those of phenylalanine and tyrosine. In contrast to the regulatory devices found for chorismate mutase in other microorganisms, the chorismate mutase of *B. subtilis* is not subject to negative allosteric control by tyrosine or phenylalanine or to positive allosteric control by tryptophan. Its activity is product-inhibited by prephenate. Only a small repression of enzyme synthesis occurs in the presence of end products (6, 7). Isoenzymes of chorismate mutase exist in *Escherichia coli* and are controlled by a channeling mechanism involving two multifunctional protein complexes. One isoenzyme is associated with prephenate dehydrogenase and the other isoenzyme purifies with prephenate dehydratase (8). In *Neurospora crassa* the balanced partitioning of chorismate into the terminal branches is controlled in part by the allosteric activation of chorismate mutase by tryptophan (9).

In view of these differences between the regulation of chorismate mutase in *B. subtilis* and that of other microorganisms, we used the following system to examine the regulatory significance of this branch point enzyme. Strains 168 and 23 of *B. subtilis* differ about 10-fold in the basal level of activity for chorismate mutase. Both strains carry a seemingly identical species of chorismate mutase which is complexed with DAHP[1] synthase (6). However, the strain difference is due to a second unique species of chorismate mutase in strain 23 which exceeds the activity of the first species by an order of magnitude. Hybrid strains carrying the gene for the higher enzyme level of strain 23 in a genetic background that is mostly that of strain 168 can be obtained as recombinants by means of deoxyribonucleate transformation. This affords the opportunity to study the effect of this enzyme alteration upon the balance of the flow of common aromatic precursors to the terminal embranchments of the pathway.

[1] The abbreviation used is: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

MATERIALS AND METHODS

Conventions—Various cultures are referred to by prefacing their acquistion number with "mutant" or "isolate" in order to reserve the word strain for the more general distinction to be made between strains 168 and 23. Thus any mutant derivative of the original isolate 168 (10) is a member isolate of strain 168. Mutations located in *trp*R are presumed to specify the aporepressor entity (11, 12) in isolates which form enzymes of tryptophan synthesis constitutively. Genotypes are given according to the nomenclature of Demerec *et al.* (13). For convenience of notation the high level chorismate mutase phenotype of strain 23 or of hybrid NP 40 is designated chorismate mutase-H (the low level species being masked). The low level chorismate mutase phenotype of strain 168 is designated chorismate mutase-L. Strain 23 and the hybrid strain possess both chorismate mutase-H and chorismate mutase-L (6). The experimental distinction between chorismate mutase-H and chorismate mutase-L is based on the measurement of specific activity in crude extracts.

Nutritional Conditions—Unless otherwise indicated, growth experiments were done at a temperature of 37° with vigorous aeration by shaking in air or water. Minimal medium supplemented with 0.5% glucose and a trace element mixture has been described elsewhere (14). Amino acid supplements were present at an initial concentration of 50 μg per ml. Cultures for enzyme extracts were generally harvested between mid and late logarithmic phase of growth at turbidities ranging between optical density values of 0.500 and 1.000 at 600 nm.

Preparation of Cell-free Extracts—Cell pellets were resuspended in the appropriate buffer and incubated with 100 μg of lysozyme per ml at 37° until lysis was complete (usually 10 to 15 min). At this time, 20 μg of DNase per ml in 0.5% $MgSO_4$ were added and incubation was continued until there was no obvious visocity (usually 10 min). The resulting preparation was centrifuged at 8000 × *g* for 20 min at 4°. Small molecules were usually removed by gel filtration through columns of Sephadex G-25 previously equilibrated with buffer. Extracts used to assay the activity of DAHP synthase were dialyzed overnight against 1000 volumes of cold buffer (two equal volume changes). Protein concentrations were determined by the method of Lowry *et al.* (15) with bovine serum albumin as a standard protein.

Enzyme Assays—Assay methods for the activities of DAHP synthase (16), anthranilate synthase (4), prephenate dehydrogenase (17), and prephenate dehydratase (3) have been described. The buffer used in the assay of prephenate dehydrogenase was modified to include 30% glycerol, which helped considerably in maintaining the stability of the enzyme.

Chorismate mutase was assayed by either of two methods, the results of which were in good agreement. (*a*) The rate of chorismate disappearance at 37° was followed continuously with a Gilford recorder throughout an interval of 4 min at 274 nm, the absorbance maximum for chorismate. The reaction mixture consisted of 500 nmoles of chorismate, 100 μmoles of Tris-HCl buffer (pH 7.0), and 0.1 ml of enzyme in a final volume of 1.0 ml. (*b*) The reaction mixture containing 0.66 μmole of chorismate, 15 μmoles of Tris-maleate buffer (pH 6.4) or glycine-NaOH buffer (pH 8.9), together with 0.1 ml of enzyme was incubated

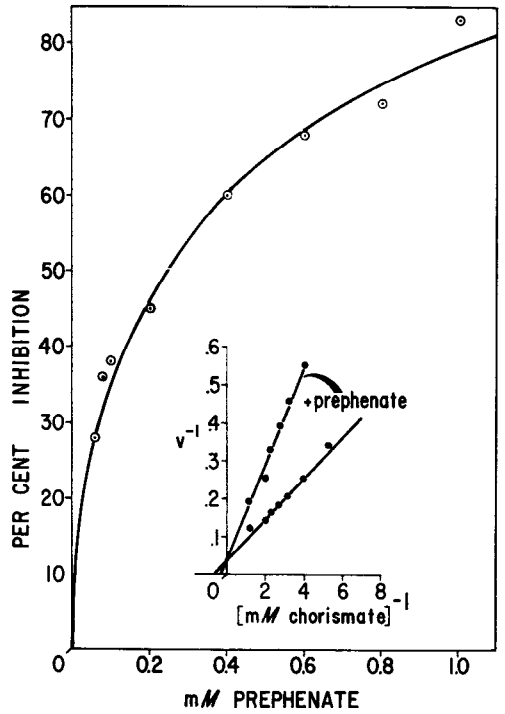

FIG. 2. Inhibition of the activity of chorismate mutase-H by prephenate in *Bacillus subtilis* NP 93. Per cent inhibition equals $(v_0 - v_i)/v_0$ where v_0 = velocity in the absence of prephenate and v_i = velocity in the presence of inhibitor. Chorismate was used at a final concentration of 0.4 mM. All reaction mixtures contained 490 μg of protein from a crude extract in a final volume of 1 ml. Initial reaction velocities were followed continuously as loss of chorismate at 274 nm as described under "Materials and Methods." The *inset* is a double reciprocal plot showing inhibition by prephenate to be competitive. Prephenate was used at a final concentration of 0.2 mM. Velocity is expressed as the reciprocal of the nanomoles of chorismate utilized per min in 1.0 ml of reaction mixture.

for 15 min at 37° in a final volume of 0.5 ml. The reaction was stopped with 0.15 ml of 20% trichloroacetic acid and incubated for an additional 10 min at 37°. The precipitated protein was spun off and the supernatnat was diluted $\frac{1}{4}$ in N NaOH and read at 320 nm, the absorbance maximum of phenylpyruvate. The sensitivity of the assay at 274 nm was only appropriate for use in the assay of chorismate mutase-H.

Results of gel chromatography and paper chromatography were in agreement with the findings of Lorence and Nester (6). Chorismate mutase-H exists in two molecular weight forms, one of which may be a dimer of the other. Both species of enzyme formed a product which chromatographed on paper (18) with authentic prephenate. When eluates of the high molecular weight species of enzyme were pooled and rechromatographed, dissociation occurred to yield the lower molecular weight species.[2] Both molecular weight forms had similar catalytic properties; both were product-inhibited by prephenate in crude extracts similar to the results shown in Fig. 2.

Biochemicals—Barium chorismate, ranging in purity from 80 to 90%, was prepared by the method of Gibson (19). Prior to use, it was converted to the potassium salt by adding twice the equivalent amount of K_2SO_4. Prephenate, likewise, was prepared by the method of Gibson with a purity of 91%. ^{14}C-Shikimate was obtained from New England Nuclear with a specific activity of 1.86 μCi per μmole. Erythrose-4-phosphate was purchased from Sigma as the sodium salt. Amino acids, acid-hydrolyzed casein (10% solution), PP-ribose-P, DL-5-methyltryptophan, anthranilate, and D-tyrosine were obtained from Calbiochem. All other biochemicals were purchased from Sigma.

Analogue Technique—The intracellular formation of tyrosine or tryptophan was estimated by relative sensitivity to growth inhibition by the antimetabolites, D-tyrosine or 5-methyltryptophan. D-Tyrosine is a false feedback inhibitor of prephenate dehydrogenase and probably acts as a false repressor, and is incorporated into protein (17, 20, 21). 5-Methyltryptophan acts as a false feedback inhibitor of anthranilate synthase in *B. subtilis* (4) but is a very poor repressor of enzyme synthesis (see Fig. 6). On agar plates isolate NP 42 is growth-inhibited (replica plating) by as little as 0.4 μg per ml of D-tyrosine, a concentration which does not inhibit the growth of isolate NP 40. In contrast, isolate NP 42 is fairly resistant to growth inhibition (replica plating) by 50 μg per ml of 5-methyltryptophan, a concentration which strongly inhibits the growth of isolate NP 40. Of course, even greater differences are observed if the mutants are compared by more sensitive techniques such as streaking or plating out individual colony formers.

Genetics—Genetic crosses were done by means of deoxyribonucleate transformation according to previously described methods (14). Whenever crosses involved the recovery of amino acid excretors with an appropriate analogue as the selective agent in the medium, it was necessary because of cross-feeding to purify recombinants by a rigorous technique. Cells of the recombinant colony were transferred to another plate of the selective medium with a sharp toothpick which was lightly stroked across the agar surface in such a fashion as to produce isolated colonies. Three subsequent purifications were done in the same manner, each time picking individual colonies which developed at the end of the streak.

Origin of Culture Isolates—Isolate NP 42 was obtained by deoxyribonucleate transformation with the use of limiting concentrations of deoxyribonucleate extracted from a multiple mutant derivative of strain 168 (our acquisition number, I-15), possessing only the chorismate mutase-L species of enzyme with recipient cells of E-35, a tyrosine auxotroph blocked in the gene for prephenate dehydrogenase (*tyr*A1) and selecting for tyrosine-independent recombinants. The presence of only chorismate mutase-L in this recombinant was indicated through the determination of its phenotype by replica plating to agar plates containing 5-methyltryptophan and D-tyrosine as discussed under "Materials and Methods." Further verification of the chorismate mutase-L phenotype was made by enzyme assay (*i.e.* low specific activity). Isolate NP 1 was selected as a spontaneous back mutation of mutant 168; its growth rate in minimal glucose medium is equal to that of isolates NP 40 and NP 42. Isolate NP 165 was obtained by adding limiting concentrations of deoxyribonucleate from NP 164 to competent recipient cells of tyrosine auxotroph E-35. Tyrosine-independent recombinants carrying only chorismate mutase-L were detected by their decreased resistance to D-tyrosine (See Fig. 10). Other stock histories are available in the references cited in Table IV.

Measurement of Tyrosine and Tryptophan Concentrations—Extracellular concentrations of tryptophan and tyrosine were measured by their fluorescence with an Aminco Bowman spectro-

[2] S. L. Stenmark and R. A. Jensen, unpublished data.

TABLE I

Strain comparisons of enzyme activity levels

Isolate[a]	Relative specific activities[b]				
	Chorismate mutase	DAHP synthase	Anthranilate synthase	Prephenate dehydrogenase	Prephenate dehydratase
NP 42	1.0	1.0	1.0	1.0	1.0
NP 40	12.5	0.5	1.8	0.9	1.8
NP 93	14.5	1.1	1.7	0.7	1.0
NP 46	9.3	0.7	2.3	0.5	0.8

[a] Strains are all described in Table IV except for isolate NP 46, a strain of *B. subtilis* synonymous with ATCC 6051.

[b] The various specific activities of enzymes in isolate NP 42 are arbitrarily assigned a value of 1.0. Actual specific activities in isolate NP 42, expressed as nanomoles per min per mg of crude extract protein, are 63.9, 17.3, 0.12, 16.2, and 0.20 from left to right above.

photofluorometer. Culture samples of 2 ml were removed during the course of growth and turbidity was measured at 600 nm. Cells were pelleted by centrifugation and discarded. Samples of the supernatant were examined for tryptophan and tyrosine concentrations. Tryptophan concentration was determined in 1 M glycine buffer, pH 11.0, at an excitation wave length of 282 nm and an emission wave length of 355 nm (both uncorrected). Under these conditions tyrosine does not fluoresce appreciably. Another portion of the same sample was examined in a 0.1 M Tris buffer, pH 7.5, at an excitation wave length of 275 nm and an emission wave length of 325 nm. The fluorescence under these conditions results from both tryptophan and tyrosine. Therefore, knowing the tryptophan concentration, the fluorescence was corrected to allow a calculation of tyrosine fluorescence.

RESULTS

The presence of phenylalanine and tyrosine during growth would be expected to cause the intracellular accumulation of prephenate (1). Prephenate, in turn, not only feedback inhibits the activity of DAHP synthase (1) but also inhibits the activity of chorismate mutase (Fig. 2). The *inset* of Fig. 2 shows that the inhibition of chorismate mutase-H by prephenate is competitive with respect to chorismate. Since prephenate is a simple rearrangement of the chorismate molecule, it seems likely that prephenate acts directly at the substrate binding site in the manner of a classical substrate analogue. The inhibition is effective, and the data of Fig. 2 illustrate that strong inhibition can be achieved even at saturating concentrations of substrate.

Prototrophic derivatives of three distinctly different strains of *B. subtilis* are listed in Table I. These are strain 168, strain 23, and ATCC 6051 represented by our collection numbers NP 42, NP 93, and NP 46, respectively. Isolate NP 40 is a hybrid strain the genome of which is mainly that of strain 168. It carries genes from strain 23 coding the following enzymes: chorismate mutase, indoleglycerol phosphate synthase, and the genes for most if not all of the remaining enzymes of tryptophan biosynthesis. Except for chorismate mutase, the specific activities for the key allosteric enzymes of aromatic synthesis shown in Table I are similar in the three strains. Strain 168 (NP 42) has a level of chorismate mutase activity which is about 10% of that assayed in the other strains. The genes for DAHP synthase and prephenate dehydratase are unlinked to the gene for indoleglycerol phosphate synthase, the selective marker in the cross used to construct hybrid NP 40. Therefore, the genes for DAHP synthase and prephenate dehydratase in the hybrid are undoubtedly those of the recipient strain 168. The lower level of DAHP synthase in hybrid NP 40 is probably due to repression exerted by the elevated amount of tyrosine synthesized by this strain (22). This is further supported by the finding[3] that both isolates NP 40 and NP 42 have the same specific activities (*i.e.* 19 to 21 nmoles per min per mg of crude extract protein) for DAHP synthase when grown in the presence of tyrosine. With the exception of anthranilate synthase, most of these enzymes do not show large differences in specific activity between the

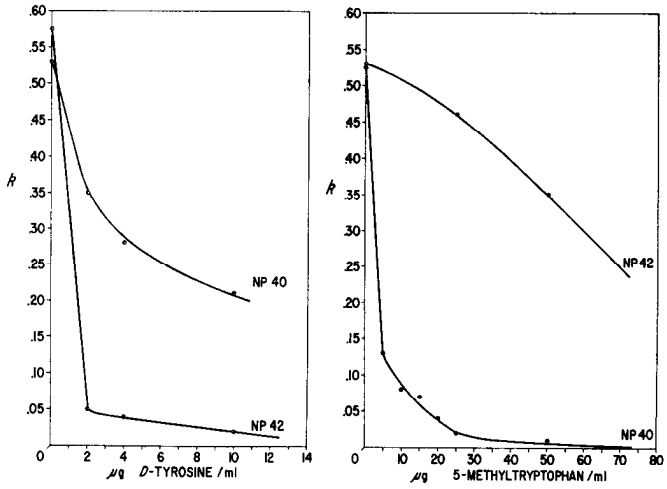

FIG. 3 (*left*). Growth inhibition by *D*-tyrosine in *Bacillus subtilis* isolates NP 40 and NP 42. The specific growth rate, k, expressed in reciprocal hours is plotted as a function of the concentration of D-tyrosine present in the growth medium. Cells were grown in 10 ml of minimal medium containing 0.5% glucose in 125 ml side arm flasks in a shaking water bath at 37°. Turbidity was measured with a Klett colorimeter (red filter). Each experimental point represents a value of k calculated from the slope of a growth curve, usually defined by turbidity measurements taken between 3 and 8 hours after inoculation. The inoculum was a culture in late logarithmic phase of growth in minimal medium at 37°.

FIG. 4 (*right*). Growth inhibition by 5-methyltryptophan in *Bacillus subtilis* isolates NP 40 and NP 42. The specific growth rate, k, expressed in reciprocal hours is plotted as a function of the concentration of 5-methyltryptophan. Other growth conditions are described in Fig. 3.

TABLE II

Effect of chorismate mutase-H on tyrosine and tryptophan production

Isolate	Specific excretion[a]	
	Tryptophan	Tyrosine
NP 42	2.1	0.9
NP 40	1.0	2.2

[a] Specific excretion is expressed as micrograms of tryptophan or tyrosine per mg of dry weight per hour. Concentrations were estimated by fluorescence measurements as described under "Materials and Methods."

[3] R. A. Jensen, unpublished data.

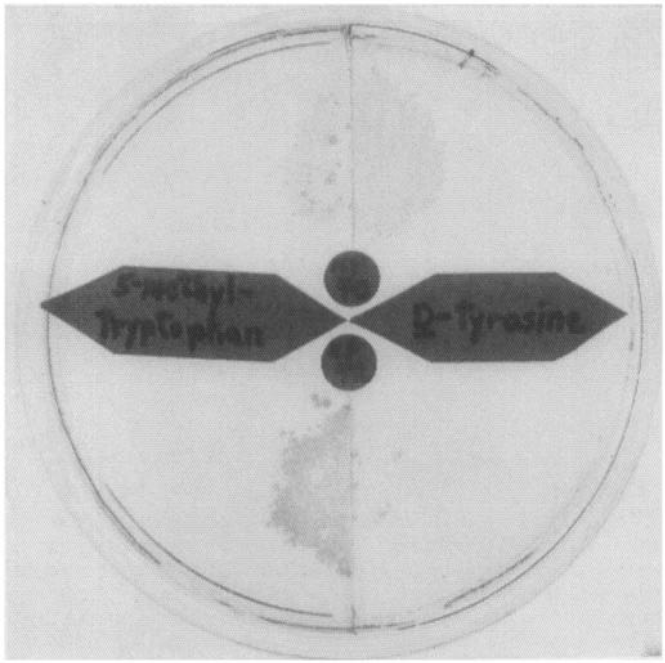

FIG. 5. Effect of chorismate mutase level on analogue resistance in isolates NP 40 and NP 42. Circular areas of a trypticase soy-yeast extract agar plate were inoculated with cells of NP 40 and NP 42. After 15 hours of incubation at 37° this master plate was replica plated to two plates, one containing 0.4 μg per ml of D-tyrosine and the other containing 50 μg per ml of 5-methyltryptophan. After 24 hours of incubation at 37° the agar was separated with a razor on a line bisecting the two areas of growth. The opposite halves of the two analogue-containing plates were fitted together to form the *mosaic* shown above in the photograph.

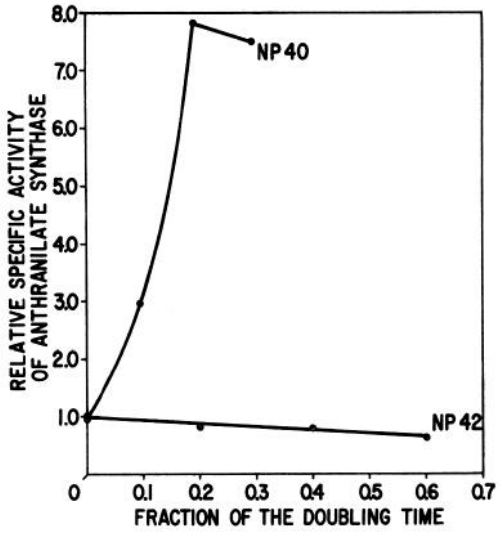

FIG. 6. The effect of 5-methyltryptophan on the synthesis of anthranilate synthase in isolates NP 40 and NP 42. Cultures were grown in 800 ml of minimal medium containing 0.5% glucose at 37°. At an A^{600} of 0.7, 50 μg of 5-methyltryptophan per ml were added to each culture. A 200-ml sample was taken from each culture and immediately centrifuged at 8,000 × *g* for 15 min in order to sediment the cells. Additional samples were taken every 20 min. Extracts were prepared from each sample and assayed for anthranilate synthase in the presence of glutamine and chorismate. The relative specific activity of anthranilate synthase is plotted against time expressed as fractional doubling time. Specific activity arbitrarily set at a value of 1 corresponds to 0.20 and 0.12 nmole of anthranilate produced per min per mg of crude extract in isolates NP 40 and NP 42, respectively. The doubling time in this experiment was 80 min.

limits of repression and derepression. Maximum factor differences for DAHP synthase, chorismate mutase, prephenate dehydrogenase, and prephenate dehydratase are 7, 2, 3, and 2 (7), respectively. Anthranilate synthase can vary in specific activity by a factor of at least 50.

Growth of these strains in minimal glucose medium resulted in specific activities for all of the enzymes listed in Table I which approach that of maximal repression. Accordingly, it can be concluded that under these conditions of nutrition none of the aromatic end products is limiting for protein synthesis. Furthermore, the presence of chorismate mutase-H in hybrid NP 40 does not perturb the routing of metabolites sufficiently to cause enzymes of tryptophan synthesis to derepress more than 2-fold. Hence, the increased tendency for shunting of chorismate toward tyrosine and phenylalanine must be offset in large part by allosteric control of prephenate dehydrogenase and prephenate dehydratase and by product inhibition of chorismate mutase-H. Nevertheless, the compensation is only partial, and the series of experiments described in Figs. 3 through 8 show that the quantitative distribution of common precursors into end products in isolates NP 42 and NP 40 is different. Isolate NP 42 is much more sensitive to growth inhibition by low concentrations of D-tyrosine (Fig. 3), but much more resistant to growth inhibition by 5-methyltryptophan (Fig. 4) than is isolate NP 40. Accordingly, one would anticipate an increased biosynthesis of tyrosine at the expense of a decreased endogenous formation of tryptophan in hybrid NP 40 possessing chorismate mutase-H. Table II contains data illustrating levels of tyrosine and tryptophan present in culture supernatants from the two isolates Exactly reciprocal results between isolates NP 40 and NP 42 with respect to relative concentrations of tyrosine and tryptophan were obtained. The ratio of tryptophan to tyrosine was 5-fold greater in isolate NP 42 than in NP 40. The ease with which the two chorismate mutase phenotypes can be distinguished on solid media containing analogues is shown by the replica plating results photographed in Fig. 5. This means that chorismate mutase-H can now be recognized phenotypically. Therefore, recombinant progeny in genetic crosses can be scored for genotype without the tedium of identification by enzyme assay (6).

The difference between isolates NP 40 and NP 42 with respect to endogenous levels of tryptophan is further illustrated by the results of the experiment shown in Fig. 6. When cells of the two strains growing in a minimal glucose medium were supplemented with 50 μg of 5-methyltryptophan per ml, derepression of anthranilate synthase occurred almost immediately in isolate NP 40. No derepression was seen in isolate NP 42 for the duration of the experiment. Eventually when the endogenous supply of tryptophan is exhausted, derepression of anthranilate synthase also occurs in isolate NP 42.[4] No significant inhibition of growth rate occurred over the time interval of the experiment shown in Fig. 6. (Characteristically, a time lag precedes the onset of inhibition of growth by the analogue; the diminished growth rate indicated in Fig. 4 at this analogue concentration of 50 μg per ml was obtained from the slope of the growth curve beginning with sampling times which were taken several hours after inoculation.)

[4] J. F. Kane, unpublished data.

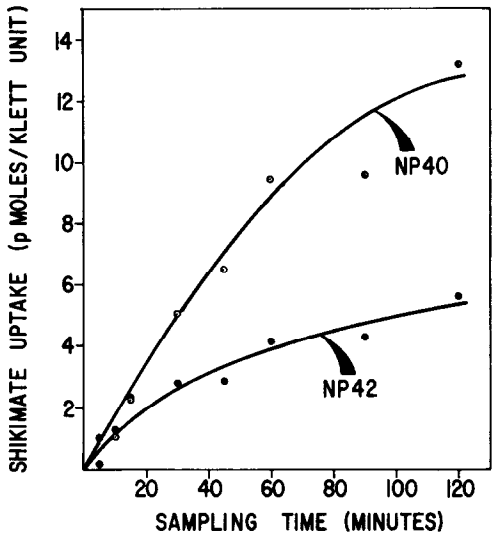

FIG. 7. Comparison of shikimate uptake in isolates NP 40 and NP 42. Side arm flasks of 125 ml volume containing 10 ml of minimal glucose medium were inoculated to an optical density of approximately 10 Klett units. Inocula were from overnight cultures grown at 37° with shaking in minimal glucose medium. The flasks were placed in a 37° shaking water bath and incubated until the turbidity was 45 to 50 Klett units. At this time 500 μg of unlabeled shikimate containing 0.2 μCi of ^{14}C-shikimate were added to each culture. Samples of 0.1 ml volume were withdrawn at the indicated times, filtered on a 0.45 μ membrane filter, and washed with 2.0 ml of minimal medium at 37°. The filters were air-dried and placed in vials. The radioactivity was measured with a Beckman CPM-100 liquid scintillation counter (efficiency, >95%).

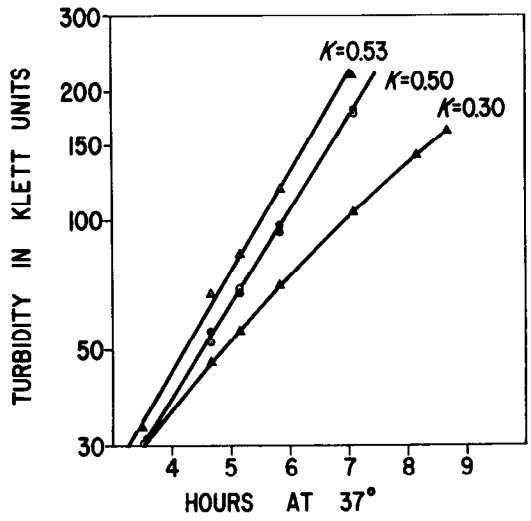

FIG. 8. Inhibition of the growth of isolate NP 42 by tryptophan and tyrosine. Cultures of NP 40 and NP 42 were inoculated from overnight minimal glucose grown cultures in the late logarithmic phase of growth into 125 ml side arm flasks containing 10 ml of minimal glucose medium at 37°. Turbidity was adjusted to 10 to 15 Klett units, and cultures were shaken in a New Brunswick psychrotherm at 37°. Sampling results read after 3½ hours of incubation are shown on the graph. Tryptophan and tyrosine, when present, were at concentrations of 50 μg per ml each. Symbols: isolate NP 40 in minimal glucose medium (⊙) or with tryptophan plus tyrosine (●), and isolate NP 42 in minimal glucose medium (△) or with tryptophan plus tyrosine (▲).

Still other differences correlate with the presence of high level chorismate mutase. Isolate NP 40 possesses a greater capacity for uptake of shikimate, a biosynthetic precursor of chorismate, than does isolate NP 42 (Fig. 7). It is possible that isolate NP 40 is also hybrid for some other gene involving the uptake of shikimate. Perhaps the greater activity of chorismate mutase acts to "pull" the preceding reactions including shikimate entry to completion. A second difference is that isolate NP 42 may exhibit growth inhibition by the amino acid combination of tryptophan and tyrosine (Fig. 8). There is some quantitative variation in this result from experiment to experiment. The history of the inoculum seems to be very important. As seen in Fig. 8, the inhibition does not take full effect until fairly late in the course of growth. This probably reflects some carry-over of phenylalanine in the inoculum and the time required for repression by tyrosine to deplete levels of phenylalanine precursors. Since the addition of phenylalanine overcomes the growth inhibition, it is assumed that inhibition results from a partial starvation for phenylalanine. In isolate NP 42 starvation for phenylalanine in the presence of both exogenous tyrosine and tryptophan may occur as the cumulative result of (*a*) the low level of chorismate mutase present to supply prephenate for phenylalanine synthesis, (*b*) the repressive effect of tyrosine on precursor formation (7), and (*c*) the inhibitory allosteric effect of tryptophan on prephenate dehydratase (2, 3, 5). None of the other possible combinations of aromatic amino acids or any single amino acid produced detectable inhibitory effects on growth rate in either isolate NP 40 or NP 42.

In contrast to the case of hybrid NP 40, the following series of experiments illustrate the unbalancing effect of chorismate mutase-H when it exists in combination with mutations conferring a loss of regulatory function in the specific pathway of either tyrosine or tryptophan synthesis. Isolates NP 100, F-2, and NP 101 in Table III are all *trp*R regulatory mutants (11, 12, 23, 33) in which all of the tryptophan biosynthetic enzymes are synthesized constitutively. A clear inverse correlation is observed between the level of chorismate mutase and the rate of tryptophan excretion. Since the *trp*R mutations in isolates NP 100 and F-2 were obtained independently, the strain differences

TABLE III

Effect of chorismate mutase-H on tryptophan production in trpR mutants

Isolate	*trp*R mutation site	Relative activity[a] of chorismate mutase	Specific tryptophan excretion[b]
NP 100	*trp*R2	1.0	40
F-2	*trp*R1	10	4.6
NP 101[c]	*trp*R1	1.0	50

[a] Chorismate mutase activity was determined at pH 8.9 as described in the text. The activity of isolate NP 100 was arbitrarily assigned a value of 1.0 and corresponds to a specific activity of 1.7 nmoles of prephenate per min per mg of extract protein.

[b] Specific tryptophan excretion is defined as the micrograms of tryptophan per mg of dry weight per hour.

[c] Isolate NP 101 was obtained by using a saturating concentration of donor deoxyribonucleate from isolate F-2 to tra sform recipient strain NP 35 (*tyr*A1) requiring tyrosine for growth. Recombinant NP 101 was selected as a tyrosine independent clone which also received the mutant *trp*R allele of the donor but not the gene specifying chorismate mutase-H.

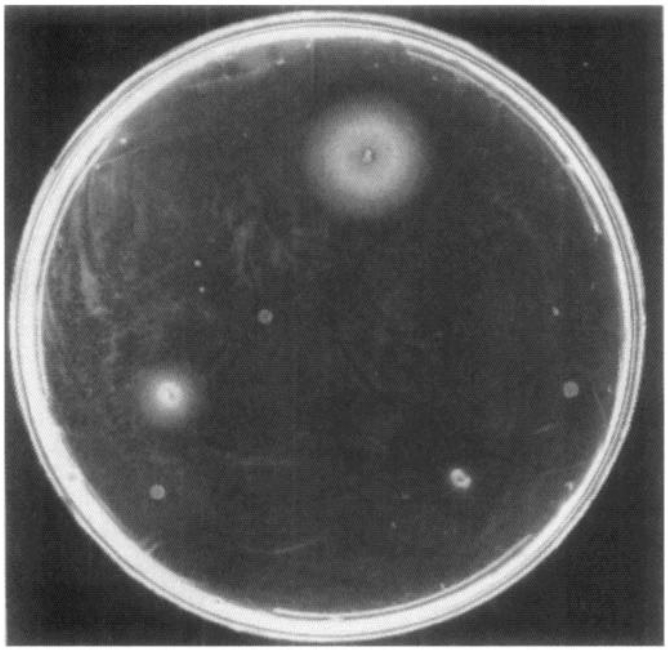

FIG. 9. Effect of the level of chorismate mutase upon tryptophan excretion in a regulatory mutant. A minimal salts agar plate containing 0.2% acid-hydrolyzed casein hydrolysate (Calbiochem) and 0.5% glucose was seeded with a lawn of a tryptophan auxotroph blocked in tryptophan synthase (our collection number, E-7). The surface of the agar was stabbed with toothpicks previously touched to cells of F-2 (*trp*R1, chorismate mutase-H) or NP 100 (*trp*R2, chorismate mutase-L). The plate was photographed after incubation for 20 hours at 37°. The *large halo* of background growth (*top center*) is supported by the tryptophan excreted by cells of isolate NP 100. The *smaller halo of growth* (*lower left*) is supported by the tryptophan excreted by cells of isolate F-2. Other colonies are revertants of the seed lawn population.

in tryptophan excretion could have been at least partly due to differences in the *trp*R mutations. Hence, the gene for chorismate mutase-H of isolate F-2 was removed by recombination to obtain isolate NP 101 in order to allow a valid comparison between isolates NP 101 and F-2. Isolate NP 101 was isogenic with isolate F-2 with respect to the *trp*R mutation (*i.e.* *trp*R1). Isolate NP 101 proved to be similar to isolate NP 100 in specific tryptophan excretion. Isolate NP 101 also exhibited the partial phenylalanine requirement characteristic of isolate NP 100. Thus, although isolates F-2 and NP 101 carry an identical *trp*R mutation, their abilities to overproduce tryptophan and their abilities to synthesize phenylalanine were markedly influenced by the level of the chorismate mutase present. This chorismate mutase-dependent difference in tryptophan excretion in *trp*R mutants is quite apparent on solid media. The photograph in Fig. 9 illustrates that isolate NP 100 is capable of supporting a much larger halo of background growth of a tryptophan auxotroph blocked in tryptophan synthase than is isolate F-2. Isolate NP 100 is such a powerful excretor that background feeding can be visibly discerned after about 5 hours of incubation at 37° compared to about 12 hours for isolate F-2.

Analogous results were obtained with strains having a mutant prephenate dehydrogenase that is resistant to feedback inhibition by tyrosine (Fig. 10). In this case the presence of chorismate mutase-H significantly increased the overproduction of tyrosine[4]

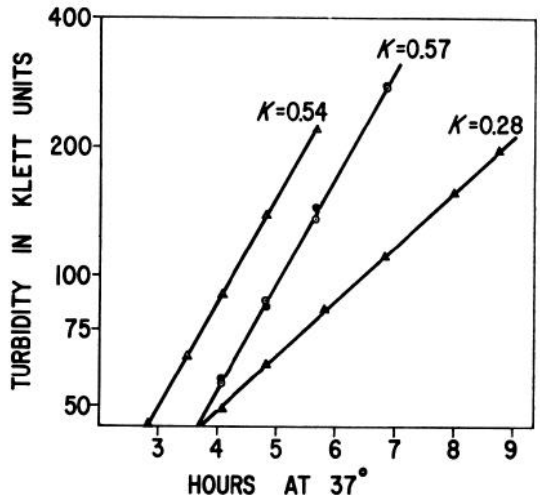

FIG. 10. Effect of the level of chorismate mutase upon the inhibition of growth by D-tyrosine. Isolates NP 164 and NP 165 both carry an identical mutation to feedback resistance of prephenate dehydrogenase by tyrosine (20). In addition NP 164 is a hybrid strain carrying the gene for chorismate mutase-H. The growth curves shown are plotted as turbidity as a function of time in hours at 37°. Other experimental procedures are described under Fig. 8. Symbols: growth of isolate NP 164 in minimal glucose medium (⊙) or minimal glucose plus 50 μg per ml of D-tyrosine (●), and growth of isolate NP 165 in minimal glucose medium (△) or in minimal glucose medium plus 50 μg per ml of D-tyrosine (▲).

in strains having a feedback-resistant prephenate dehydrogenase. Thus, NP 164 (chorismate mutase-H) was totally resistant to growth inhibition by D-tyrosine, even at high concentrations. On the other hand, when only chorismate mutase-L was present (in isolate NP 165), high concentrations of D-tyrosine still inhibited growth. Apparently, sufficient levels of prephenate could not be supplied to the mutant prephenate dehydrogenase by chorismate mutase-L to allow the required synthesis of L-tyrosine to confer total resistance to growth inhibition caused by the D isomer of tyrosine (21).

DISCUSSION

Genic Imbalance in Hybrid Strains—We infer that various strains of *B. subtilis* grown in minimal glucose medium synthesize aromatic amino acids in excess of their utilization for protein synthesis. Presumably the endogenous formation of aromatic amino acids and their subsequent entry into an internal pool is sufficient to maintain all of the pathway enzymes at near maximum levels of repression. Strain 168 of *B. subtilis* was metabolically unbalanced by transforming in a gene from strain 23 which specifies a chorismate mutase (chorismate mutase-H) having an activity which is 10-fold greater than is normal for strain 168 (chorismate mutase-L). Tryptophan and tyrosine are highly fluorescent compounds and these were assayed in culture supernatants as a measure of the altered metabolic routing of chorismate in hybrid NP 40. A 5-fold shift in the relative ratio of endogenous tyrosine to tryptophan was discerned, in comparison to the balanced strain 168. This difference in the endogenous levels of tryptophan and tyrosine was readily apparent by means of a sensitive technique involving relative resistances to growth inhibition by low concentrations of D-tyrosine or 5-methyltryptophan. The hybrid strain NP 40 carrying choris-

TABLE IV

Summary of some Bacillus subtilis isolates

Isolate designation	Literature citations	Chorismate mutase level	Strain[a] purity	Nutritional requirements	Resistance[b] characteristics
168	(10, 24)	Low	168	Tryptophan	
NP 93	(3, 17, 20, 24)	High	23	Prototroph	D-Tyrosine
SB 19	(7, 25, 26, 27)	High	Hybrid	Prototroph	Streptomycin
SB 491	(28, 29, 30, 31, 32)	High	Hybrid	Prototroph	Streptomycin
WB 746	(2, 6, 7)	Low	168	Prototroph	
WB 672	(2, 6)	High	23	Prototroph	D-Tyrosine
NP 40	(3, 4, 5, 14, 17, 20, 21, 22, 23, 33)	High	Hybrid	Prototroph	Streptomycin
NP 42		Low	(168)	Prototroph	
NP 1		Low	168	Prototroph	
SB 455 (F-2)	(4, 7, 31)	High	Hybrid	Prototroph	5-Methyltryptophan
NP 100	(4, 5, 23, 33)	Low	(168)	Phenylalanine[c]	5-Methyltryptophan
NP 101		High	Hybrid	Prototroph	5-Methyltryptophan
NP 164	(3, 5, 17, 20)	High	Hybrid	Phenylalanine[c]	D-Tyrosine
NP 165		Low	(168)	Prototroph	D-Tyrosine
SB 194	(31, 32, 34)	High	Hybrid	Tryptophan	

[a] Strains denoted (168) are pure with respect to the gene for chorismate mutase. Since their histories involve interstrain crosses, it is possible that they could be hybrid for other genes. It is certain that some of them carry the gene for indoleglycerol phosphate synthase from strain 23, for example.

[b] The "natural" resistance of strain 23 to D-tyrosine inhibition differs from the D-tyrosine resistance of isolates NP 164 and NP 165 which have feedback resistant prephenate dehydrogenases. Resistances to growth inhibition by streptomycin and 5-methyltryptophan are to concentrations of at least 1 mg per ml.

[c] The requirement for phenylalanine is partial (23).

mate mutase-H also maintains a significantly lower level of endogenous *p*-aminobenzoate than strains carrying only chorismate mutase-L.[5]

Nevertheless, the hybrid strain is by all ordinary standards a prototroph and displays no amino acid requirements to sustain normal growth rates. The relatively successful metabolic adjustment of the hybrid strain presumably is mediated by the allosteric control of prephenate dehydrogenase and prephenate dehydratase by tyrosine and phenylalanine, respectively. We suggest that the competitive inhibition of chorismate mutase-H by prephenate constitutes an important regulatory specificity in the over-all pattern of sequential feedback inhibition (2). In the unbalanced hybrid strain this control by prephenate would be especially important in preventing starvation for tryptophan due to the tendency toward decreased levels of chorismate and increased levels of prephenate. Since all three of the latter inhibitions are competitive with respect to substrate, the capabilities of the corresponding regulatory enzymes to adjust to the increased specific activity of chorismate mutase-H are necessarily incomplete.

The compensatory role of the normal allosteric and repressive control mechanisms are most apparent in various regulatory mutants possessing chorismate mutase-H. For example, when tryptophan enzymes were produced constitutively in a *trp*R mutant possessing chorismate mutase-L, tryptophan was excreted in a 13-fold greater amount than when the same *trp*R mutation was located in a hybrid strain possessing chorismate mutase-H (23). Similarly when the chorismate mutase-H locus was introduced into a mutant lacking feedback sensitivity of prephenate dehydrogenase for tyrosine, tyrosine production was increased very substantially. In both of these regulatory mutants the unbalancing potential of chorismate mutase-H is much more apparent than in hybrid isolate NP 40. Phenotypic correlates of the presence or absence of chorismate mutase-H are much more obvious in the background of certain regulatory mutants than in isolate NP 40. Thus the *trp*R1 mutant (NP 101) shows a partial requirement for phenylalanine in chorismate mutase-L backgrounds, but not in chorismate mutase-H (F-2) backgrounds. A regulatory mutant of prephenate dehydrogenase is fully resistant to growth inhibition by D-tyrosine in chorismate mutase-H backgrounds but is only partially resistant to inhibition by D-tyrosine in chorismate mutase-L backgrounds.

Phenylalanine Synthesis in Hybrid Strains—Phenylalanine is not measured as conveniently as are the fluorescent amino acids, tyrosine and tryptophan. However, β-2-thienylalanine is a potent antimetabolite of phenylalanine, and therefore the relative sensitivities of our strains to growth inhibition by this analogue were determined. One would expect that the results would parallel those obtained for tyrosine synthesis. Unexpectedly the presence or absence of chorismate mutase-H made very little difference with respect to the effect of β-2-thienylalanine on growth rate. If anything, the hybrid strain NP 40 was more sensitive to growth inhibition by this analgoue than isolate NP 42. Since both methionine and leucine influence β-2-thienylalanine sensitivity at analogue concentrations which are only partially inhibitory to growth (3, 5), a complicating consideration could be possible mutant differences in internal levels of methionine and leucine. However, a variety of unpublished results are consistent with an explanation which involves a preferential channeling of prephenate formed by chorismate mutase-H to prephenate dehydrogenase.

Ubiquitous Distribution of Hybrid Strains—In retrospect it is extremely unfortunate that the source of transforming deoxyribonucleate used in the early experiments of Spizizen was from strain 23, at that time thought to be a mutant variant of the same stock as strain 168 (10). As a consequence, hybrid strains were formed inadvertently and widely distributed, particularly SB 19 and SB 491 (Table IV) as well as numerous derivatives subse-

J. F. Kane and R. A. Jensen, unpublished data.

quently isolated from them in many laboratories. Many of these isolates together with appropriate literature citations are given in Table IV. Since the original mutant isolate of strain 168 is blocked in the gene for indoleglycerol phosphate synthase, it is especially likely that many strains are hybrid for genes mapping in the tryptophan gene cluster. An appreciation of the important implications of this situation may best be served by the following list of observations in the scientific literature which can now be explained as the result of the experimental use of hybrid strains.

1. The apparent pleiotrophic effect of a single mutation (*trp*R2) in isolate NP 100 whereby chorismate mutase activity was drastically diminished led us to suggest a role for chorismate as an inducer in the synthesis of the tryptophan enzymes (23). The apparent pleiotrophic effect on chorismate mutase was due to the presence of chorismate mutase-L in isolate NP 100 and to the presence of chorismate mutase-H in the reference prototroph (NP 40) used. Mapping procedures with donor genes of isolate NP 100 and various auxotrophic recipients possessing chorismate mutase-L gave the apparent result that the two characteristics could not be separated. When it became clear that isolates such as NP 42 or WB 746 rather than hybrid strains such as SB 19 or NP 40 were the appropriate reference prototrophs, a trivial explanation for the apparent pleiotrophic effect found in mutant NP 100 was provided. Accordingly, although the possibility remains that one or more pathway intermediates is involved in induction, the most compelling evidence in favor of the induction hypothesis was negated.

2. The wide range of specific activities for enzymes of tryptophan synthesis reported in the literature (12, 28, 30) may be taken as suggestive that these enzymes are not coordinately synthesized in spite of their operon-like genetic arrangement (35). The variable specific activities observed in different auxotrophs with the use of extracts prepared under the same nutritional conditions probably reflects the existence of hybrid mixtures of enzymes of tryptophan synthesis in many of these mutants. If the kinetics of derepression are followed in the same mutant strain, the enzymes of tryptophan synthesis are indeed synthesized coordinately (28).

3. The observation that a mutation causing histidine auxotrophy simultaneously led to an increased resistance to growth inhibition by 5-methyltryptophan (11) is now explained by the fact that the mutant (possessing only chorismate mutase-L) was compared with isolate SB 19. As shown in this paper a chorismate mutase-H hybrid such as SB 19 is more sensitive to 5-methyltryptophan due to the presence of chorismate mutase-H.

4. The variable map positions described for various *trp*R mutants (30) probably were due to the loss of *trp*R recombinants which did not receive the chorismate mutase-H gene from the donor. Such recombinants would produce small colonies due to the partial auxotrophy for phenylalanine and in some genetic crosses would undoubtedly have been partially or totally missed.

5. The surprisingly high levels of tyrosine production in isolate NP 40, presumed to be a pure strain (22), are now explainable by its identity as a hybrid strain possessing chorismate mutase-H.

Purity of Reference Prototrophs—Isolates WB 746 and NP 1 were selected as full revertants of isolate 168, and accordingly are pure strain representatives of strain 168. Although isolates SB 491 and NP 40 were also both isolated as back mutations of isolate 168, it is probable that both are in fact laboratory contaminants in synonymy with SB 19. In support of this is the observation that both isolates SB 491 and NP 40 are resistant to 1 mg per ml of streptomycin as is SB 19, even though selection for this mutation was never made in the isolations of SB 491 and NP 40.

Is Isolate 168 a Double Mutant?—The gene for chorismate mutase-H can be inserted into the genome of strain 168 by deoxyribonucleate-mediated transformation at normal efficiencies. Since this gene functions so well in strain 168, one can reasonably ask whether there is an allelic counterpart for the gene specifying chorismate mutase-H in strain 168. That is, could the original mutant 168 have been a double mutant, blocked in both indoleglycerol phosphate synthase and chorismate mutase-H? If this were true, an isolate such as NP 40 would resemble the ancestral parent strain. In this connection it is interesting that the chorismate mutase in a third strain of *B. subtilis* (NP 46, Table I) is present at the high specific activity levels of chorismate mutase-H. It is difficult to argue decisively for the physiological superiority of either isolate NP 40 or NP 42. Phenotypically, both are prototrophs displaying similar growth rates in minimal glucose medium. However, isolate NP 42 is susceptible to growth inhibition by the combination of tyrosine plus tryptophan under some conditions. Also the combination of chorismate mutase-L and the *trp*R mutation leads to a partial phenylalanine requirement, whereas the combination of chorismate mutase-H and *trp*R results in prototrophy. Isolate NP 42 is also incapable of achieving complete resistance to high concentrations of D-tyrosine unless the gene for chorismate mutase-H is introduced. Hence, hybrid NP 40 seems to be physiologically superior to isolate NP 42 in environments containing tryptophan plus tyrosine. Likewise, analogue-resistant derivatives of hybrid NP 40 seem to be physiologically superior in environments containing 5-methyltryptophan or D-tyrosine.

We examined eleven spontaneous revertants of isolate 168. With one exception all of the tryptophan-independent derivatives retained the low level of chorismate mutase. The exception proved to be resistant to 1 mg per ml of streptomycin and is assumed to be identical with isolate NP 40, arising as a laboratory contaminant. (It should be noted that the most troublesome and least obvious type of contamination is that by one's own laboratory strains.) Therefore, there is no reason to think that chorismate mutase-H is related to indoleglycerol phosphate synthase (the mutant gene in the original strain 168). We also tried without success to isolate chorismate mutase-H revertants from isolate NP 42 by selection on agar plates containing 0.4 μg per ml of D-tyrosine (see "Materials and Methods"). None of these mutants showed the phenotype expected of strains carrying chorismate mutase-H on 5-methyltryptophan media. All of the mutants recovered which were resistant to low concentrations of D-tyrosine retained the level of 5-methyltryptophan resistance that is characteristic of mutant NP 42.

It is possible, however, that in isolate 168 the gene for chorismate mutase-H was deleted, particularly since x-irradiation was used to mutagenize the parental cells (10). This would explain the inability to select chorismate mutase-H mutants from derivatives of strain 168 and the easy insertion of the gene by deoxyribonucleate-mediated transformation. Since, as far as we know, the original parent strain of isolate 168 is not available, th s question will probably never be resolved with finality. A discussion of the controversial origins of strains 168 and 23 is given in Reference 24.

Metabolic Interlock—For reasons of experimental practicality

the control of biochemical pathways is usually considered as if each pathway were a discrete entity, separate from other pathways. The implication is that the control of one enzymatic sequence (*e.g.* the pathway of tryptophan synthesis) is independent from metabolic events which may occur in other pathways. In point of fact, however, biochemical pathways are interrelated in a multitude of ways. It seems likely that a physiological or mutational change in one pathway might initiate perturbations of other pathways through an influence upon common metabolites such as initial substrates, end products, intermediates, cofactors, etc. In support of this point Zalkin (36) and Held and Smith (37) showed that alterations in the control of one terminal branch of the aromatic pathway strongly influenced the production of end products in the other branches, even though the specific control mechanisms in the latter branches were unchanged. Thus, even though the direct end product controls in a particular biochemical sequence of a mutant may remain intact, this is not necessarily sufficient to maintain the over-all level of balanced control found in the wild type organism. It seems likely that a widespread occurrence of interpathway relationships of regulation may characterize many biological systems, effecting a finely tuned biochemical integration.

Perhaps the simplest examples of metabolic interlock from the physiological point of view are the interactions between the separate branches of a multibranched biochemical pathway (5). Our previously described examples of metabolic interlock involved allosteric interactions (3–5). Other examples of interpathway regulatory interactions involve the control of the levels (*i.e.* the synthesis) of enzymes. For example, a regulatory element common to both the tyrosine and histidine pathways has been described in *B. subtilis* (38). Carsiotis *et al.* (39, 40) has also described a relationship between the histidine and tryptophan pathways in *N. crassa* which involves the regulation of enzymes for tryptophan synthesis. In this paper biochemical relationships exerted between the separate branches of the aromatic pathway were approached through the genetic alteration of the level of a key branch point enzyme, chorismate mutase. Our results with the hybrid strain of *B. subtilis* suggest that various allosteric specificities and ranges of enzyme levels may have a long history of selection for compatibility and integration with the over-all metabolic context of the cell. It will now be of interest to examine all of the various possible combinations of chorismate mutase-H and -L with other types of regulatory mutations which we have isolated. This ought to allow a more complete understanding of the control relationships exerted between the member branches of the aromatic pathway. Hopefully, the study of these strains will permit a more insightful evaluation of the regulatory relationships of aromatic amino acids with metabolites such as methionine, leucine, and histidine (3–5).

Chorismate Mutase, a Subordinate Enzyme—Chorismate mutase is located at a metabolic branch point, a widely acknowledged site of importance in biochemical control. Chorismate, the substrate for chorismate mutase, is also the last common precursor for the pathways of biosynthesis for tryptophan, folate, 2,3-dihydroxybenzoate, ubiquinones, and vitamin K. Accordingly chorismate mutase is in competition for chorismate with the initial enzymes of all of these pathways. Our results with the hybrid strain indicate that chorismate can be distributed appropriately into the multiple pathways competing for it without direct end product control of chorismate mutase. However, balanced competition of chorismate mutase with the other branch point enzymes is dependent upon the intact regulation of the regulatory enzymes in the terminal branches of the pathways. Thus, feedback inhibition of prephenate dehydrogenase and prephenate dehydratase would prevent the metabolism of prephenate by the tyrosine and phenylalanine pathways. Product inhibition of chorismate mutase by prephenate would tend to promote the intracellular accumulation of chorismate. Allosteric and repressive control of anthranilate synthase by tryptophan places substantial restraint upon the potential overcompetition for chorismate by anthranilate synthase. The exercise of these controls allows the wild type cell to compensate satisfactorily for experimental perturbation of chorismate mutase activity, *i.e.* introduction of chorismate mutase-H.

If repressive control of enzyme synthesis by tryptophan is removed by mutation, anthranilate synthase overcompetes for chorismate, resulting in a partial requirement for phenylalanine. The presence of chorismate mutase-H in the latter mutant actually promotes a balancing effect, abolishing the phenylalanine requirement and decreasing the tryptophan excretion. If feedback control of prephenate dehydrogenase by tyrosine is lost by mutation, a drain of prephenate in the direction of tyrosine is established. This secondarily diminishes product control of chorismate mutase by prephenate. It would appear that chorismate mutase-L is limiting in the latter mutant because tyrosine excretion was substantially increased by the introduction of chorismate mutase-H.

Hence, the exaggerated influence of chorismate mutase-H in strains carrying mutations in regulatory enzymes of the terminal branches of the aromatic pathway indicates the dependence of the *B. subtilis* cell upon the normal control of prephenate dehydrogenase, prephenate dehydratase, and anthranilate synthase in order to mediate the proper flow of chorismate to prephenate. The balanced control of chorismate metabolism depends upon (*a*) the restraint exercised upon the considerable catalytic potential of the tryptophan pathway through repressive and allosteric control by tryptophan and (*b*) by the product inhibition of chorismate mutase by prephenate (which would accumulate in the presence of phenylalanine and tyrosine). The kinetic parameters of chorismate mutase appear to be adjusted in the wild type cell to compete with the repressed level of anthranilate synthase observed under most growth conditions. The dependence of the regulatory properties of chorismate mutase upon the function of subsequent regulatory enzymes of the aromatic amino acid pathway exemplifies a highly integrative pathway-wide regulation. We refer to this type of enzyme as a subordinate enzyme.

Subordinate enzymes may represent a common device for achieving interlocking biochemical relationships of regulation. For example, the histidine pathway in *E. coli* connects with a salvage pathway (41) for an intermediate derived from the initial ATP substrate molecule, phosphoribosyl aminoimidazole carboxamide (PRAIC). A specific transformylase transfers a formyl group from N^{10}-formyltetrahydrofolate to form formyl-PRAIC. Formyl-PRAIC is converted to AMP and ultimately to ATP, completing the salvage pathway sequence. The transformylase is not regulated. This lack of regulation for the transformylase is entirely appropriate in wild type because a regulated rate of histidine synthesis will effect a stoichiometric relationship with the rate of transformylase function. This relationship is perturbed in certain auxotrophs which accumulate PRAIC behind an enzyme block. Accumulated PRAIC is wastefully

formylated in the absence of a regulatory control by histidine and the cell displays a drastic decrease in formylating capacity. A general detrimental effect upon protein synthesis ensues. Hence, the transformylase is a subordinate enzyme because its proper quantitative function as the initial enzyme of the salvage pathway is totally dependent upon the normal regulation of the histidine pathway. Disruption of the normal metabolite flow in the histidine pathway reveals a regulatory relationship which is not nearly so obvious in the wild type cell.

Ubiquity of Interpathway Regulation—Some of the most intriguing findings in the area of metabolic regulation involve interpathway control relationships. The coupling of RNA and protein synthesis in stringent strains of *E. coli* is an interpathway relationship of regulation. The energy charge effect on enzymes described by Atkinson (42) involves reinforcing, modifying control effects of energy metabolites upon enzymes. The effect of the formylating capacity of the cell upon the derepression kinetics of histidine enzymes in *E. coli* (41) and the role of adenosine 3′,5′-monophosphate in the phenomenon of catabolite repression (43) illustrate the modulation of specifically controlled pathways by more general cellular conditions. These effects all exemplify reinforcing, often subtle, influences which modify the specific control of a particular pathway in relationship to the general physiological state of the cell. We propose that the term metabolic interlock be used to describe regulatory relationships at the interpathway level. It seems likely that the emphasis in biochemistry will center more and more upon regulatory interactions between different biochemical pathways now that the regulation of specific pathways has been documented in such detail.

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