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Comparative Regulation of Isoenzymic 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthetases in Microorganisms

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The independent control of regulatory isoenzymes by different metabolites constitutes one well-known pattern of control in branched metabolic pathways. This pattern was previously found to be widely distributed in the aromatic amino acid pathway of microorganisms in the case of the first enzyme of the sequence, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase. The comparative stability of the isoenzymes as well as the effect of aromatic amino acids in the growth medium upon the levels of the individual isoenzymes were shown for *Salmonella typhimurium*. Several lines of evidence are discussed to demonstrate the strong reliance of *Escherichia coli* upon the phenylalanine-sensitive isoenzyme for the ordinary biosynthetic needs of wild-type strains. The frequent occurrence of "dominant" isoenzyme species which resist repressive effects of the inhibitory end products was noted. The lack of an obligatory correlation of the level of an isoenzyme activity and the synthesis of the end product which specifically controls its activity is used to discount the possibility that each isoenzyme might feed a unique and separate metabolic pool of end-product precursor. An isoenzymic DAHP synthetase sensitive to feedback inhibition by low levels of tryptophan was fractionated from tyrosine- and phenylalanine-sensitive isoenzymes in cell-free extracts of *Neurospora crassa*.

The derivation of different end products from precursors that originate as the product of the activity of one or more shared enzymes has often been recognized as a dilemma with respect to the control of the common enzyme (13). One apparent resolution of this dilemma in microorganisms is the maintenance of differentially controlled isoenzymes. A previous comparison of numerous microorganisms revealed that such isoenzymic species of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase are of widespread occurrence (8). The regulation of the overall biosynthetic reaction is thought to be mediated through discrete enzyme molecules, each molecular form of the enzyme being subject to the controlling influence of its own metabolic signal. The product of the isoenzyme-catalyzed reaction, DAHP, could (i) enter a common metabolic pool or (ii) funnel into separate precursor pools for each of the end products. The results of Cotton and Gibson (4) with other branch-point enzymes of the aromatic pathway exemplify the latter case. Recent observations of multimolecular protein complexes (5) provide an attractive physical basis for such channeling effects. If, indeed, the fate of a DAHP molecule at the time of its formation is predetermined by the particular isoenzyme catalyzing its formation, then in a regulated system one would expect

$$\frac{\text{(isoenzyme-x activity)}}{\text{(total enzyme activity)}} = \frac{\text{(synthesis of end product-x)}}{\text{(synthesis of total end product)}}$$

This amounts to the exercise of independent control by each end product upon the activity of DAHP synthetase in proportion to the percentage of common precursors that are ultimately diverted to the synthesis of that specific end product. However, the latter simple relationship of isoenzyme level and the rate of synthesis of the controlling end product seems to occur rarely, if ever.

This paper details the characteristics of some microbial systems of enzymes that were assessed

by their physical fractionation from one another, by measuring quantitative effects of combinations of inhibitors, by the isolation of mutants which excrete aromatic amino acids, and by the use of nutritional procedures to manipulate the relative proportions of the isoenzyme proteins synthesized.

MATERIALS AND METHODS

Microorganisms. The sources of microorganisms and the nutritional conditions used in their culture were reported previously (8).

Extract preparation. The methods and experimental conditions attending cell disruption, dialysis, use of inhibitors, buffers, storage of extracts and substrates, and enzyme assay have been described (8). Extracts of *Neurospora crassa* were fractionated with $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$ slowly to the extract with gentle mixing at 4 C in the amount calculated to yield the percentage of saturation solution corresponding to the fraction of lowest salt concentration. After 30 min at 4 C, the precipitated protein was removed by centrifugation at 15,000 × *g* for 10 min. Subsequent fractions were salted out from the supernatant fluid in a similar way by the further addition of the calculated amounts of $(NH_4)_2SO_4$.

Sephadex G-100 fractionation. A gel column with dimensions of 2.5 × 60 cm was prepared according to the method of Andrews (1) and equilibrated with 0.04 M potassium phosphate buffer containing 0.1 M KCl, *p*H 6.8, at 4 C.

Isolation of regulatory mutants. Regulatory mutants were obtained by spontaneous mutation on plates of minimal medium supplemented with the indicated concentration of amino acid analogue, either β-2-thienylalanine (TA) or 5-methyltryptophan (5-mTrp). Cells in the logarithmic phase of growth in TSY broth (8) were washed in minimal salts and spread on the analogue-containing plates (about 10^8 cells per plate on TA; about 10^7 cells per plate on 5-mTrp). Tryptophan (Trp) and phenylalanine (Phe) excretors were readily detected as resistant clones surrounded by halos of background growth; nonexcretor clones displayed no halo effects. Excretors were purified on other analogue-containing plates by recloning about six times from isolated colonies. Especially rigorous purification was necessary because associated analogue-sensitive background cells tended to be quite resistant phenotypically. The latter resistance derived from the fact that Phe excreted by resistant cells strongly antagonized the inhibitory effects of the analogue, and from the tendency of excretors to grow more poorly than did closely associated sensitive cells. Excretor mutants also required more frequent transfer of stock cultures than did wild-type to prevent loss of viability.

RESULTS

Uniformity of inhibition data. The kinetic form of the inhibition curves obtained from extracts of *Escherichia coli* B and *Pseudomonas fluorescens* was typical of those systems where Phe or tyrosine (Tyr), or both, were identified as effectors regulating the activity of DAHP synthetase. The inhibition of initial reaction rate of DAHP synthetase in these bacterial strains is plotted as a function of inhibitor concentration in Fig. 1. The first-order kinetics of inhibition by Phe found with the enzyme of *E. coli* B can be observed in data obtained with the same strain by Previc and Binkley (11), and for the W and K-12 strains (9, 12; for simplicity, the minor inhibition exerted by Tyr was omitted from Fig. 1). *P. fluorescens* represents that class of microorganisms in which only one aromatic amino acid inhibits DAHP synthetase (8); Fig. 1 shows that Tyr inhibits enzyme activity almost totally.

Those bacterial enzyme systems in which Trp is the sole proven inhibitor make up a distinctive class (8). In representatives of this class, the affinity of DAHP synthetase for substrate was altered in the presence of Trp, a fact which was often qualitatively obvious because of the necessity to reduce substrate concentrations in order to demonstrate appreciable inhibition. Data obtained from *Streptomyces virginiae* (Fig. 2) were quite typical of this enzyme class. Such Trp-inhibited enzymes invariably displayed a non-Michaelian, sigmoid form of the substrate binding curve, which may implicate the occur-

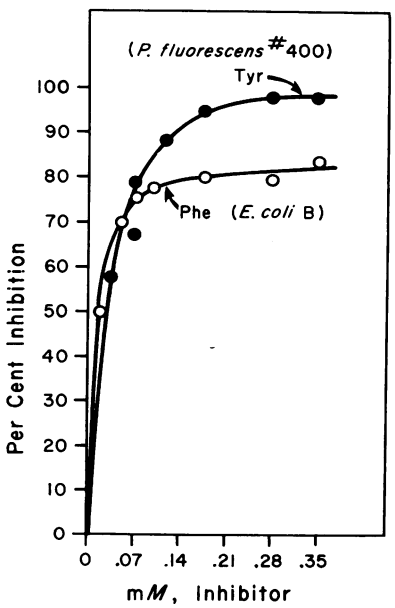


FIG. 1. *Inhibition curves for two major bacterial groups. Crude extracts prepared by sonic treatment and dialysis were used in the enzyme assay. The slight inhibition by tyrosine (Tyr) in Escherichia coli B is not shown, but was similar to published data (8).*

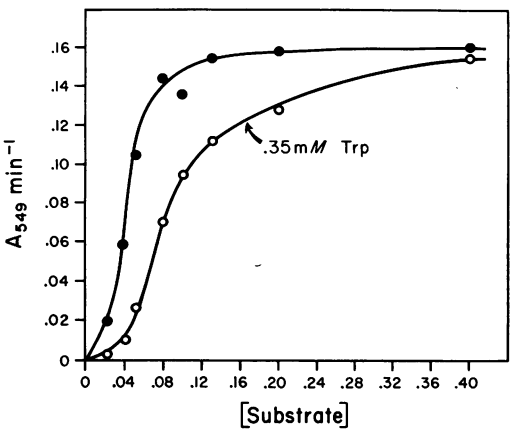


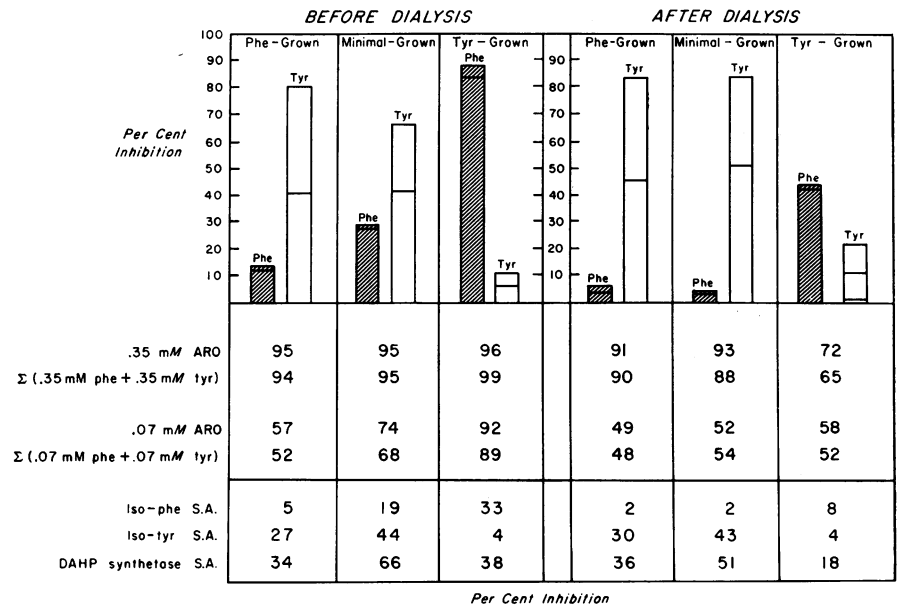
FIG. 2. *Sigmoid substrate saturation curve in Streptomyces virginiae. The reaction rate, A_{549} min^{-1}, is graphed as a function of substrate concentration. The mixture of substrates, erythrose-4-phosphate and phosphoenolpyruvate, was diluted to yield the concentrations denoted on the abscissa; the concentration unit is mM. The unlabeled curve is drawn through the experimental points obtained for the control reactions measured in the absence of tryptophan (Trp).*

rence of interactions between substrate binding sites (10). *Myxococcus xanthus* was the single exception found in a total of 16 species (representing seven genera) having the Trp-sensitive DAHP synthetase; its substrate saturation curve did conform to Michaelis-Menton kinetics.

Flux of isoenzyme levels in E. coli and Salmonella typhimurium. The isoenzyme DAHP synthetases of *E. coli* and *S. typhimurium* are similar in some of their properties. Both possess two easily detected isoenzymes: one specifically feedback-inhibited by Phe, the other by Tyr. In both microorganisms, inhibition by Trp was quantitatively minor or nonexistent. Also, in common, the Phe-inhibited isoenzyme (iso-Phe) was more sensitive in vitro to Phe than the Tyr-inhibited isoenzyme (iso-Tyr) was to Tyr, on a molar basis. The latter point is illustrated for *S. typhimurium* in Fig. 3, where a fivefold decrease of inhibitor concentration (from 0.35 mM to 0.07 mM) reduced the inhibition by Tyr about twofold but made very little difference in the inhibition produced by Phe. Iso-Phe was also the most labile enzyme in both microbes. They differed in that iso-Phe was strongly dominant in *E. coli*, whereas iso-Tyr tended to be dominant in *S. typhimurium*. [A dominant isoenzyme (8) is one which makes up the bulk of total enzyme activity under conditions of growth in minimal medium.] The isoenzymes of *S. typhimurium* showed particularly good inhibitor specificities. This was apparent from the good correspondence of the sum of individual Phe and Tyr inhibitions with the inhibition measured in the simultaneous presence of Phe and Tyr. ARP (mixture of L-Tyr, L-Phe, and L-Try, each at the indicated concentration plus the vitamins *p*-aminobenzoate and *p*-hydroxybenzoate at a 10-fold lower concentration) produced no greater inhibition than did a mixture of Phe and Tyr. The relative levels of these two isoenzymes could be readily altered nutritionally by supplying exogenous Phe or Tyr to the cultures from which the extracts were prepared. Iso-Tyr was repressed and iso-Phe was derepressed after growth in Tyr; analogously, iso-Phe was repressed and iso-Tyr was derepressed after growth in Phe.

Since 0.35 mM of either Phe or Tyr specifically inhibited most of the activity of the corresponding isoenzyme, the inhibition produced by one of the effectors was a satisfactory estimate of the fraction of total DAHP synthetase activity existing as the corresponding isoenzyme. Comparison of the product: (overall specific activity of DAHP synthetase) $\times$ (per cent inhibition in the presence of an inhibitor) of the crude, undialyzed extract with the dialyzed preparation, showed iso-Tyr to be stable to dialysis, whereas iso-Phe was quite labile. Since the loss in overall enzyme specific activity after dialysis approximated the observed loss in iso-Phe activity, interconversion of the isoenzymes under these conditions was unlikely. In Fig. 3, the partial inactivation of iso-Phe activity by dialysis accounts for the relatively greater inhibitory effect of Tyr after dialysis. The portion of iso-Phe activity that survived dialysis was partially desensitized, the latter accounting for the decreased inhibition exerted by ARO in the dialyzed preparation. These differences were most noticeable in extracts prepared from Tyr-grown cultures in which a large proportion of the original DAHP synthetase activity was unstable iso-Phe.

The greatest derepression of iso-Tyr synthesis was found in extracts of minimal-grown cultures (specific activity, SA = 44). On the other hand, iso-Phe synthesis was derepressed the most in extracts of Tyr-grown cultures (SA = 33). Comparison of levels of isoenzymes that characterized cultures grown in minimal medium, where Phe and Tyr are formed endogenously, with isoenzymic levels, when Phe or Tyr were supplied exogenously, gave the following results: (i) Tyr supplementation repressed iso-Tyr synthesis about 11-fold (40 units of specific activity) but derepressed iso-Phe about twofold (about 14 units of specific activity), and (ii) supplementation with Phe repressed iso-Phe and iso-



	Before Dialysis			After Dialysis		
	Phe-Grown	Minimal-Grown	Tyr-Grown	Phe-Grown	Minimal-Grown	Tyr-Grown
.35 mM ARO	95	95	96	91	93	72
Σ (.35 mM phe + .35 mM tyr)	94	95	99	90	88	65
.07 mM ARO	57	74	92	49	52	58
Σ (.07 mM phe + .07 mM tyr)	52	68	89	48	54	52
Iso-phe S.A.	5	19	33	2	2	8
Iso-tyr S.A.	27	44	4	30	43	4
DAHP synthetase S.A.	34	66	38	36	51	18

Per Cent Inhibition

FIG. 3. *Response of isoenzyme levels to exogenous supply of aromatic amino acids in Salmonella typhimurium. Inhibitions are represented in histogram form in which the upper bound of each bar designates results obtained by using an inhibitor concentration of 0.35 mM; the horizontal line within each bar denotes results obtained by using a lower inhibitor concentration of 0.07 mM. The data shown on the right side of the figure were obtained from the same three extracts used to secure the results shown on the left side. Below each set of histograms, in vertical order, is given: the per cent inhibition in the presence of 0.35 mM ARO (see footnote to Table 1 for definition), mixture of 0.35 mM phenylalanine (Phe) plus 0.35 mM tyrosine (Tyr), 0.07 mM ARO, mixture of 0.07 mM Phe plus 0.07 mM Tyr, specific activity of phenylalanine-inhibited isoenzyme (iso-Phe), specific activity of tyrosine-inhibited isoenzyme (iso-Tyr), and total specific activity of DAHP synthetase. The specific activities of iso-Phe and iso-Tyr were calculated from the inhibition data (at 0.35 mM). Since the total inhibition is less than 100%, the summed specific activities of iso-Phe and iso-Tyr are less than the total specific activity.*

Tyr about fourfold and 1.5-fold, respectively (a total of 31 units of specific activity). Therefore, iso-Tyr catalyzes the formation of precursors for some Phe synthesis in addition to precursors supporting Tyr synthesis. By comparing the level of iso-Phe, when Phe was supplied exogenously (SA, exogenous Phe), with that in minimal medium (SA, endogenous Phe), the factor was found to be about four (5/19). On the other hand, (SA, exogenous Tyr)/(SA, endogenous Tyr) was 11 (4/44). Exogenous Tyr spared the synthesis of total DAHP synthetase about 28 units of specific activity (66/38), and exogenous Phe had a sparing effect of about 32 units (66/34). Therefore, in spite of the surprising effects of exogenous Phe and Tyr upon the levels of the individual iso-Phe and iso-Tyr isoenzymes, the net effect of these amino acids upon total DAHP synthetase activity was an approximately equal ability of Tyr and Phe to repress overall enzyme synthesis 40 to 50%. Apparently, iso-Tyr represents more enzyme than required for the synthesis of Tyr, whereas iso-Phe represents less enzyme than required for Phe synthesis. These results need not imply that the repression of isoenzyme synthesis lacks specificity (see Discussion). The results demonstrate that the compositional distribution of the two isoenzyme DAHP synthetases in *S. typhimurium* can be readily altered in response to the exogenous levels of the controlling amino acids. In contrast, the proportion of isoenzymes in species of *Escherichia* were not varied so easily in batch cultures grown in the presence of exogenous Phe or Tyr. A previously reported derepression of iso-Tyr in *E. coli* was obtained by using limiting supplements of aromatic end products with auxotrophic mutants (2).

Another useful approach imposing a stress upon the biosynthetic output of the pathway involves the use of regulatory mutants. Mutants which overproduce one of the amino acid end products would utilize a greater supply of common intermediates, therefore requiring greater activity of the common enzymes, although not necessarily greater levels. By using lower con-

TABLE 1. *Derepression of tyrosine-inhibited isoenzyme in Escherichia coli strain B and various mutant derivatives*[a]

Designation	Phenotypic description	Growth regimen	Per cent inhibition					
			Concn 0.35 mM			Concn 0.07 mM		
			Phe	Tyr	ARO	Phe	Tyr	ARO
Ec-1.....	Wild-type	No additions	84	14	96	79	None	79
		+ TA	74	26	97	59	12	73
		+ TA + Tyr	91	None	95	78	None	78
Ec-2.....	Resistant: 100 μg/ml of TA Does not excrete Phe	No additions	84	13	96	76	3	81
Ec-3.....	Resistant: 100 μg/ml of TA Excretes Phe	No additions	36	60	96	35	33	70
		+ Phe	38	59	94	35	30	66
		+ TA	43	52	94	43	29	69
		+ TA + Tyr	92	7	94	80	1	79

[a] TA was used at a final concentration of 50 μg/ml. When TA was added to Ec-1, the growth rate was depressed about 90%. An extract was made from the latter culture after a 2-hr exposure to the analogue. TA and aromatic amino acids were present in cultures of Ec-3 at concentrations of 50 μg/ml. Cells were cultured in a minimal salts-glucose medium at 37 C with aeration. Abbreviations: TA = β-2-thienylalanine; Tyr = tyrosine; Phe = phenylalanine; ARO = mixture of L-Tyr, L-Phe, and L-tryptophan, each at the indicated concentration plus the vitamins *p*-aminobenzoate and *p*-hydroxybenzoate at a 10-fold lower concentration.

centrations of TA, Ezekiel (7) obtained a resistant mutant in which iso-Phe was insensitive to inhibition by Phe. A class of TA-resistant mutants which excretes Phe (Ec-3, Table 1) was derepressed for iso-Tyr. Neither the synthesis of iso-Phe nor of iso-Tyr (Ec-3, Table 1) was influenced by the addition of Phe or TA to the growth medium. The mutation in Ec-3 was not the formation of a constitutive iso-Tyr DAHP synthetase, since exogenous Tyr in the medium repressed the synthesis of iso-Tyr. The elevated level of iso-Tyr in this mutant, when grown in minimal medium, was undoubtedly the physiological consequence of the regulatory mutation in the Phe branch of the pathway which would result in the shunting common intermediates to the synthesis of Phe. This would tend to make the synthesis of Tyr limiting to growth and to result in the observed derepression of iso-Tyr. In contrast, Ec-2 (Table 1), a mutant that was also resistant to TA but did not overproduce Phe (perhaps a permeability mutant), was identical to the sensitive parent type in the activity and distribution of isoenzymes. The exposure of wild-type cells to TA (Ec-1, Table 1) led to a slight derepression of iso-Tyr, an effect which was reversed with the simultaneous addition of Tyr.

A 5-mTrp-resistant mutant of *E. anindolica* which excreted Trp (Ea-3) was also derepressed for iso-Tyr (Table 2). Again, a nonexcreting variant that was resistant to 5-mTrp resembled wild-type. Although total DAHP synthetase was elevated fivefold in the Ec-3 mutant of Table 1, total DAHP synthetase in Ea-3 approximately equaled that of the parent wild-type. Apparently the derepression of iso-Tyr was offset by a concomitant increased repression of iso-Phe. The addition of TA to Ea-3 repressed the synthesis of iso-Tyr nearly to the wild-type level; perhaps the regulatory mimicry of TA in the specific Phe embranchment diverted sufficient amounts of common precursors to Tyr to result in the observed repression of iso-Tyr. Since the output of the pathway was increased in Ea-3 due to the overproduction of Trp, while the total level of DAHP synthetase measured in vitro remained comparable to that of wild-type, it seems likely that the increased enzyme activity in vivo derived from a release of intracellular control by feedback inhibition. The conclusion that the full catalytic potential of DAHP synthetase is not normally expressed in vivo has been made elsewhere (3).

Three isoenzymes in N. crassa. Perhaps the most convincing documentation of an isoenzyme system is the physical fractionation of discrete isoenzyme activities along with the demonstration of separate inhibitor specificities. Some of the characteristics and kinetic properties of iso-Phe and iso-Tyr DAHP synthetases of *E. coli* were originally studied in this way (12). We found the *N. crassa* system to be of particular interest for isoenzyme studies because preliminary work with crude extracts showed that a third isoenzyme, which is Trp-sensitive

TABLE 2. *Enzyme levels of tyrosine-inhibited isoenzyme in Escherichia anindolica ATCC 6879 and various mutant derivatives*[a]

Designation	Phenotypic description	Growth regimen	Per cent inhibition							
			Concn 0.35 mM				Concn 0.07 mM			
			Phe	Tyr	Trp	ARO	Phe	Tyr	Trp	ARO
Ea-1.....	Wild-type	No additions	76	12	3	95	68	8	3	80
Ea-2.....	Resistant: 100 μg/ml of 5-mTrp Does not excrete Trp	No additions	85	16	None	95	70	10	None	77
Ea-3.....	Resistant: 100 μg/ml of 5-mTrp Excretes Trp	No additions + TA	48	58	None	97	44	42	9	88
			81	19	None	94	73	1	None	78

[a] TA was added to Ea-3 at a final concentration of 50 μg/ml. The growth rate was depressed about 80%. An extract was prepared from the latter culture after a 2-hr exposure to the analogue. Cells were cultured in a minimal salts-glucose medium at 37 C with aeration. Abbreviations: Trp = tryptophan; 5-mTrp = 5-methyltryptophan; for other abbreviations, see footnote to Table 1.

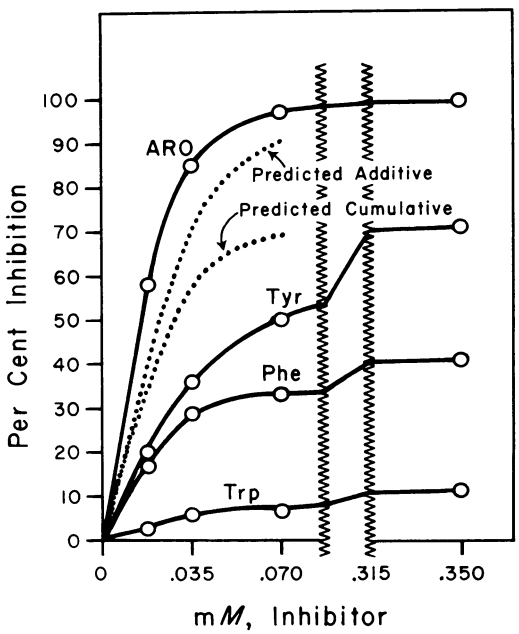


FIG. 4. *Inhibition of DAHP synthetase in Neurospora crassa. The inhibition observed in the presence of ARO is compared with the theoretical expectations for additive and for cumulative effects of inhibitor combinations as calculated from the individual inhibitions found in the presence of tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp).*

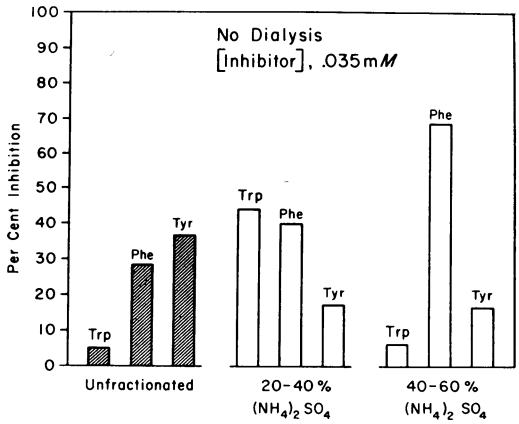


FIG. 5. *Levels of isoenzymes in $(NH_4)_2SO_4$ fractions of Neurospora crassa compared to those in an unfractionated extract. DAHP (3-deoxy-D-arabino-heptulosonate 7-phosphate) synthetase activity in the 20 to 40% $(NH_4)_2SO_4$ fraction was 11% of the total activity remaining after fractionation; the 40 to 60% $(NH_4)_2SO_4$ fraction made up 83% of the total activity.*

(iso-Trp), existed, in addition to the iso-Phe and iso-Tyr fractions.

The relative contribution of the three isoenzymes to total DAHP synthetase activity present in crude undialyzed extracts prepared from cultures grown in minimal medium can be assessed from the inhibition curves of Fig. 4. These isoenzymes are quite sensitive to the inhibitors, becoming saturated at concentrations exceeding 10^{-4} M. Inhibitions plotted as a function of ARO concentration fit the form of the curve constructed from the simple summation of the three individual amino acid inhibitions. The combination of inhibitors (ARO) tended to exert a quantitatively small but consistent synergism throughout the entire range of inhibitor concentrations. The histograms in Fig. 5 show that fractionation of a crude extract by $(NH_4)_2SO_4$ led to an enrichment for iso-Trp (20 to 40% of saturation) and for iso-Phe (40 to 60% of saturation). The 20 to 40% salt fraction contained 90% of the original iso-Trp

activity and made up about 11% of the original total activity. The enrichment of iso-Phe in the 40 to 60% fraction reflects primarily the lability of iso-Tyr to the fractionation regimen. Although iso-Trp was a quantitatively minor portion of the original activity present in crude, undialyzed extracts, it was also the most stable isoenzyme. Purification procedures produced preparations in which most or all of the remaining DAHP synthetase activity was sensitive to inhibition by Trp. Comparison of (A), Fig. 6, with the 20 to 40% $(NH_4)_2SO_4$ fraction of Fig. 5 shows that dialysis abolished all of the iso-Tyr activity which survived the $(NH_4)_2SO_4$ treatment. Even iso-Phe was partially labile to this treatment, and subsequent overnight storage of the dialyzed, 0 to 45% $(NH_4)_2SO_4$ fraction at −20 C led to a nearly complete loss of the remaining iso-Phe activity. A similar differential stability of the DAHP synthetase isoenzymes in *N. crassa* was found by Sephadex G-100 gel filtration (Fig. 7). The activity in the eluates corresponding to the high molecular weight peak of enzyme activity (about tube 35) proved to be 100% inhibited by L-Trp. The activities of the latter two peaks continued to decay rapidly after the initial enzyme assays of the eluates, and thus prevented the rigorous identification of the corresponding inhibitor specificities. However, it seems certain that the remaining two small peaks of activity corresponded to iso-Phe and iso-Tyr.

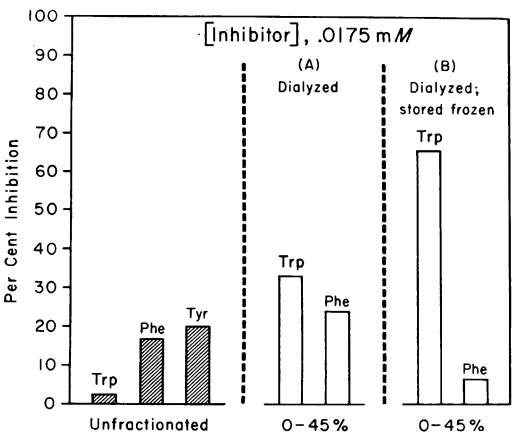


FIG. 6. *Stability of Neurospora crassa tryptophan-inhibited isoenzyme (iso-Trp) after freezing of 0 to 45% $(NH_4)_2SO_4$ fraction. The activity of 0 to 45% fraction after freezing (24 hr) was 52% of the total activity before freezing. Increase in relative inhibition by tryptophan (Trp) is approximately equal to the loss of total activity after freezing. The Trp-sensitive isoenzyme activity was stable to subsequent freeze-thaw treatments.*

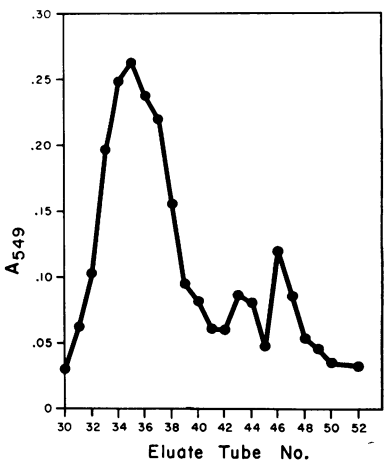


FIG. 7. *Fractionation of DAHP synthetase isoenzymes of Neurospora crassa on Sephadex G-100. A 4-ml amount of crude extract was applied to the top of the column. Bovine serum albumin was added to the eluates to facilitate the precipitation of the enzyme with $(NH_4)_2SO_4$. The protein was resuspended in 0.04 M phosphate buffer, pH 6.7, and the concentrated enzyme preparation was assayed for activity and inhibitor specificity.*

DISCUSSION

Isoenzyme systems of microbial DAHP synthetases so far studied can be assorted among a number of distinct subcategories (8): (i) those in which significant amounts of two or more isoenzymes are normally present in media unsupplemented with aromatic acids, (ii) those for which the latter statement potentially applies, but where one isoenzyme tends to predominate ("dominant" isoenzyme), and (iii) those for which only one enzyme species is detected. The latter could be an extreme expression of the dominant isoenzyme type, or it may indeed be a single enzyme and therefore qualitatively different from the foregoing isoenzyme systems.

Of those systems where fluctuation of isoenzyme levels was accomplished nutritionally, *S. typhimurium* was particularly responsive to such techniques. Relative proportions of iso-Phe or iso-Tyr could be adjusted almost at will by amino acid supplementation. It was concluded that iso-Tyr forms a portion of the precursors used in the ultimate synthesis of Phe in addition to those required for Tyr biosynthesis. This quantitative discrepancy between the level of an isoenzyme and its contribution to precursors for the cognate amino acid was much more exaggerated for species with strongly dominant isoenzymes. Of the latter, a collection of *E. coli* strains was studied in some detail.

The following facts are presented in support of the thesis that the iso-Phe of *E. coli* normally supplies most of the precursors for aromatic end products. (i) When cells are grown in minimal medium, iso-Phe is strongly dominant, yet exogenous Phe does not inhibit growth. (ii) Exogenous Phe does not derepress the synthesis of iso-Tyr or any other latent isoenzyme or isoenzymes (3, 11). (iii) It can be assumed that the cells are permeable to exogenous Phe, since it does partially repress the synthesis of iso-Phe. Since iso-Phe is the only significant source of precursors for aromatic end products under conditions of growth which require their endogenous synthesis, it must be partially active in vivo, even in the presence of exogenous Phe.

The above conclusion is also consistent with the following items. (i) The normal intracellular concentration of Phe (about 2 mM) was reported to be 3 to 4 times as great as that of Tyr in *E. coli* (5), e.g., the larger pool size may reflect a relatively less-sensitive regulation of Phe synthesis. (ii) Measurements of accumulated aromatic metabolites in whole cells of *E. coli* W demonstrated that Phe was much less effective than Tyr in inhibiting these accumulations in vivo; neither Tyr nor Phe was as effective in vivo as was the demonstrated potential for inhibition in vitro (2). (iii) Regulatory mutant strain Ec-3 (Table 1) in the presence of Tyr (which represses the synthesis of iso-Tyr) is still resistant to TA and continues to overproduce Phe even though the bulk of DAHP synthetase existed as iso-Phe under these conditions. Although iso-Phe of Ec-3 was as sensitive as the isoenzyme of the wild-type strain to feedback inhibition by Phe in vitro, its activity in vivo was nevertheless quite adequate to produce precursors for Trp and for the excess synthesis of Phe.

Physiologically, iso-Tyr appears to be derepressed only under conditions which place unusual synthetic demands upon the output of the pathway. For example, the increased quantitative output of the pathway due to the overproduction of Phe or Trp in various regulatory mutants of *E. coli* was correlated with the derepression of iso-Tyr. Therefore, iso-Phe seems to serve the ordinary biosynthetic needs of the cell with iso-Tyr potentially capable of providing additional activity whenever metabolic utilization of DAHP extends beyond the enzymic capacity of iso-Phe. Inspection of many bacterial systems of isoenzymic DAHP synthetases revealed that *E. coli* was by no means unique in this respect. Commonly, under conditions where all amino acids were formed endogenously (i.e., in minimal medium), one isoenzyme was dominant (8), and addition to the growth medium of the regulatory effector for the dominant isoenzyme did not necessarily derepress other isoenzyme activities. This deviation from a correlation between level of isoenzyme and its quantitative contribution to the eventual synthesis of its specific end-product effector varied from relatively little (*S. typhimurium*) to substantial (*E. coli*). The observed lack of any such obligatory proportionality indicates that the product molecules of the isoenzyme activities enter a common pool of end-product precursors.

Undoubtedly, the complete understanding of the control of DAHP synthetase must be made in the context of regulatory interactions involving other control foci in the pathway. For example, consider the branch point in the aromatic pathway where prephenate serves as the last common precursor for the synthesis of Tyr and Phe. If prephenate dehydratase had greater affinity for prephenate than did prephenate dehydrogenase, then, under conditions where the concentration of prephenate is not saturating, Phe would be made preferentially and the synthesis of Tyr would tend to become growth-limiting. Then, the data given in Fig. 3 would be consistent with the following set of physiological interactions. Addition of Tyr results in a greater content of Tyr in the metabolic pool than is normally maintained by endogenous synthesis, and iso-Tyr is largely repressed. However, since iso-Tyr also makes precursors that are used for a portion of Phe synthesis, the tendency is for Phe synthesis to become sufficiently limiting to account for the observed derepression of iso-Phe. On the other hand, exogenous Phe represses the synthesis of iso-Phe. Sparing of the Phe requirement and the resulting slowed activity along this preferential route would tend to shunt prephenate to Tyr, and would cause the additional repression of iso-Tyr. In addition to metabolic preference for one terminal route over another (e.g., by virtue of different K_m's of competing regulatory enzymes), the existence of strongly dominant isoenzymes can be related to factors such as: (i) differences in the size or spatial localization of intracellular pools of effector molecules, or both, (ii) differences in the threshold concentration of effector molecules required to exert regulatory effects, or (iii) combinations thereof. In short, other regulatory influences in the pathway could direct a variety of complex and indirect interactions which might ultimately influence the regulation of DAHP synthetase.

These studies document the first in vitro identification of a Trp-sensitive DAHP synthetase in a

proven isoenzyme system, that of *N. crassa*, which additionally possesses Tyr- and Phe-sensitive isoenzymes. By the use of crude extracts, Doy (6) recently concluded that *N. crassa* possesses a Trp-sensitive DAHP synthetase in which the inhibition depends upon the presence of Phe and Tyr (5). In our hands, iso-Trp activity was too low in crude extracts for quantitative work. Our partially purified preparations of iso-Trp were very sensitive to Trp alone. We found that crude extracts of a number of other microorganisms also display small but significant sensitivities to inhibition by Trp (8). Because of the latter findings, it seems possible that the "non-inhibitable" isoenzyme of *E. coli* which is repressible by Trp (2) might simply be an easily desensitized enzyme. As in other cases where additive inhibitory effects of aromatic amino acids occur, iso-Phe and iso-Tyr comprise the major part of the total DAHP synthetase activity.

The differential labilities of iso-Phe, iso-Trp, and iso-Tyr demonstrated in *N. crassa* as well as in the isoenzymes of *S. typhimurium* lend emphasis to the possibility that similar but more exaggerated labilities could explain results obtained with some or all of the microorganisms which possess that class of DAHP synthetase which is inhibited by a single amino acid effector. In addition to the different stabilities of isoenzymic proteins, they are inevitably distinguished by other differences, such as affinity constants for substrates and inhibitors, heat stability, *p*H and ionic strength optima, molecular weight, and so forth. Since differences of inhibitor specificities are only one of many such differences, it is possible that regulatory isoenzymes may have evolved primarily as a consequence of the selective value of multiple enzymic forms, whereby each molecular species was uniquely suited to different environmental conditions. The fact that the isoenzymes are differentially controlled by small molecules may conceivably have been an entirely secondary development in the course of evolution.

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