*INDUCIBILITY OF TRYPTOPHAN SYNTHETASE IN PSEUDOMONAS PUTIDA**

By I. P. Crawford and I. C. Gunsalus

DEPARTMENT OF MICROBIOLOGY, SCRIPPS CLINIC AND RESEARCH FOUNDATION, LA JOLLA, CALIFORNIA, AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ILLINOIS, URBANA

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The pathway of tryptophan synthesis in various microorganisms[1-4] is shown in Figure 1. Although this reaction sequence is invariant in the organisms studied so far, the enzymes for the reactions appear to associate in different combinations.[5] This pathway offers several advantages for a study of the regulation of anabolic enzymes, among them opportunity to introduce the intermediate compounds anthranilate and indole into cells, and ease of detecting as accumulation products anthranilate, 1-(o-carboxyphenylamino)-1-deoxyribulose (CDR), indoleglycerol (InG), and indole.

Bacteria of the *Pseudomonas* group were shown by den Dooren de Jong (thesis, Technische Hogeschool, Delft, The Netherlands, 1926) to use a wide variety of organic compounds as single sources of carbon and energy. In recent years, studies of the pathways of oxidation of numerous substrates and the regulation of relevant enzymes have appeared (e.g., refs. 6 and 7). In the main, the enzymes for catabolic substrates are under inductive control, frequently in sequential blocks induced coordinately.[8] The regulatory mechanisms in pseudomonads, however, have not been shown to be identical with those of the enteric bacteria.[9, 10]

Regulation of anabolic enzymes in the *Pseudomonas* group has been little studied. We have obtained a number of tryptophan auxotrophs in *Pseudomonas putida*, a typical oxidative fluorescent pseudomonad, and examined its tryptophan synthetic pathway. This paper presents the results of our initial observations using mutants grown under conditions of tryptophan excess or deprivation. The results clearly show that the last enzyme in the pathway, tryptophan synthetase (TS), is controlled through induction by its substrate, indoleglycerolphosphate (InGP). This finding is in sharp contrast with the control mechanism operating in *E. coli*, where all the enzymes of the pathway are coordinately controlled through repression by tryptophan.[1, 11, 12]

Materials and Methods.—Organisms: The parent strain used in this study, *P. putida* C1S, was derived from the terpene oxidizer described earlier,[13] from which it differs in clone type (translucent rather than opaque) and sensitivity to bacteriophage Pf.[14] Tryptophan auxotrophs were identified by replica plating following treatment with the mutagen N-methyl-N-nitrosoguanidine.[15] Strains were numbered after determination of their enzymatic defect. All mutants used in this study have reversion frequencies within the range expected for point mutants. The organisms were maintained by monthly transfer on slants of L-agar.[16]

Growth conditions: Inocula were prepared in tubes of L-broth shaken at 30°. Cells for extract preparation were grown in 1-liter vol of medium E[17] agitated at 30° in dimpled 2-liter Erlenmeyer flasks. Although the citrate (*ca.* 10 mM) present will serve as carbon source for *P. putida*, we consistently added 11 mM D-glucose. Growth when limited by carbon source reached a final density of 300–320 Klett units (Klett-Sumerson colorimeter, 66 filter). This corresponds to *ca.* 0.8 mg/ml dry weight of cells, or a molar growth yield on citrate = 62; glucose = 90.[19] The specific supplements used, L-tryptophan, indole, and anthranilate, will not support growth of *P. putida* strain S inoculated into minimal medium lacking glucose or citrate, nor is indole or anthra-

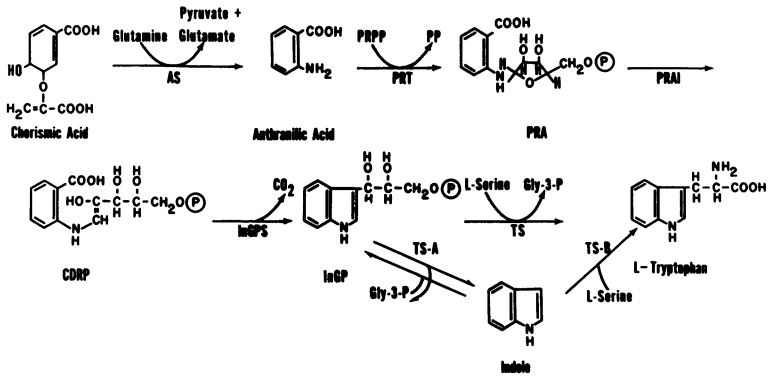

FIG. 1.—Schematic of tryptophan synthetic pathway as demonstrated in *Escherichia coli*[1, 18] and *Neurospora crassa*.[2] Chorismic acid is the last intermediate common to the synthesis of the aromatic amino acids. Abbreviations not in text: PRPP, phosphoribosyl-5-pyrophosphate; PP, inorganic pyrophosphate; Gly-3-P, glyceraldehyde-3-phosphate. Reaction TS-A is catalyzed by the A (or α) subunit of the *E. coli* enzyme, and TS-B by the B (or β_2) subunit. In all systems so far analyzed the three TS reactions are catalyzed by a complex protein constructed from different subunits.

nilate formed from L-trypsophan. We conclude that this strain possesses neither the pathway of oxidative degradation of tryptophan nor tryptophanase.

Extract preparations: Wild-type or mutant cells growing in excess tryptophan were harvested during exponential growth at a turbidity of 180–220 Klett units (66 filter), yielding about 4 gm wet weight of cells per liter. Mutant cultures grown on limiting supplement were supplied a level sufficient to allow about the same cell yield and incubated 1–3 hr after depletion of the supplement. Cells were sedimented by centrifugation at 4°, washed once with 0.1 *M* potassium phosphate, pH 7.0, and suspended in 3× their wet weight of this buffer. After disruption with a Branson sonic probe, cell debris was removed by centrifugation at 40,000 × *g* for 30 min at 4° and the supernatant solution was dialyzed 16–24 hr against 0.1 *M* potassium phosphate, pH 7.0, containing 1 mM β-mercaptoethanol. Dialyzed extracts were stored at 4°.

Enzyme assays and substrates: The anthranilate synthetase (AS), phosphoribosyl transferase (PRT), and indoleglycerolphosphate synthetase (InGPS) assays were those used in studies of *E. coli*.[12] The tryptophan synthetase reactions were assayed as described by Smith and Yanofsky,[18] reaction A (TS-A) with the addition to the reaction mixture of 1.2 *M* NH_2OH (freshly prepared from the hydrochloride by neutralization with NaOH), and reaction B (TS-B) substituting 0.1 *M* potassium phosphate, pH 7.8, for the NaCl-supplemented Tris buffer used for *E. coli*.

Anthranilate accumulation in cultures was quantitated by fluorescence after extraction from acidified supernatant fluids into ethyl acetate and return of the extracted anthranilate into aqueous 0.1 *M* Tris buffer, pH 7.8. Other accumulation tests were performed qualitatively.[18]

Chorismic acid was purified from the culture fluid of an *Aerobacter aerogenes* mutant and crystallized from an ether-petroleum ether mixture.[20] Crystals were dissolved in 0.2 *M* Tris, pH 7.5, containing 0.1 mM EDTA. The preparation of 1-(o-carboxyphenyl)-1-deoxyribulosephosphate (CDRP) and InGP has been described.[18] Protein was determined colorimetrically[21] with bovine serum albumin as standard.

Results.—Location of enzymatic defects: Eight tryptophan auxotrophs were examined for growth on minimal medium supplemented with anthranilate or indole and for accumulation of pathway-related substances (Table 1). Two strains, S21 and S62, appeared to be somewhat leaky as shown by slight growth in minimal medium. None of the auxotrophs isolated grew rapidly on indole, though the concentration used is not inhibitory to wild type or to auxotrophs growing on L-tryptophan. Only cultures depleted of tryptophan showed significant accumulation of precursor-related substances. All culture fluids exhibiting fluorescence were

TABLE 1

STRAINS OF *Pseudomonas putida* C1S* USED

Strain	Growth†			Accumulation‡		
	Anthranilate	Indole	Tryptophan	Anthranilate	Indoleglycerol	Indole
Tryptophan auxotrophs						
S1	++++	+	++++	0	0	0
S2	++++	+	++++	0	0	0
S11	0	+	++++	+	0	0
S21	Trace	+	++++	+	0	0
S31	0	+	++++	+	0	0
S51	0	0	++++	+	+	+
S61	0	0	++++	+	+	0
S62	Trace	Trace	++++	+	+	0
Double mutants						
S1-*i*1	0	++++	++++	0	0	0
S1-*i*4	++++	++++	++++	0	0	0
S11-*i*1	0	++++	++++	+	0	0
Prototrophs						
S (wild type)	++++	++++	++++	0	0	0
S1-*i*4R1	++++	++++	++++	0	0	0

* The wild-type, abbreviated S in this paper, was isolated from a(+)-camphor enrichment.
† Graded + to ++++ by colony size after 48 hr at 30° on minimal agar plates supplemented with 5 μg/ml anthranilate or indole or 10 μg/ml L-tryptophan. Similar growth response was observed in tubes of minimal medium. Strains S21 and S62 showed traces of growth on unsupplemented minimal medium.
‡ Determined qualitatively after 24 hr of growth in tubes of minimal medium plus 5 μg/ml L-tryptophan.

examined by paper chromatography[22] for the presence of CDR; this compound was not observed, though enzymatic analysis would predict its accumulation, especially by strain S31.

Table 2 displays the results of enzymatic analysis of dialyzed sonic extracts of seven of the strains shown in Table 1. For simplicity, the wild-strain S grown in excess L-tryptophan was used as standard; averaged specific activities from two such extracts are given in line one of Table 2. All other assays are expressed as the ratio of specific activity to that of the wild type. The mutants each lack one enzymatic activity, though strain S61 lacks both the A and B portions of TS. Measurements on S62 indicated its similarity to S61; therefore, it was not studied further. Since both the dissociating (*E. coli*) and nondissociating (*N. crassa*) types of TS have been shown to mutate in a single step to enzymes lacking TS-A, TS-B, or both, the enzymatic deficits in strains S51 and S61 are not surprising.

TABLE 2

ENZYMATIC SURVEY OF TRYPTOPHAN AUXOTROPHS, *Pseudomonas putida*

Strain S	Enzymatic Activity Relative to Wild Type Grown in Excess Tryptophan											
	AS 0.15		PRT 0.39		PRAI 2.1		InGPS 0.65		TS-A 0.017		TS-B 0.17	
(sp. act.)*	*xs*	*lim*	*xs*	*lim*	*xs*	*lim*	*xs*	*lim*	*xs*	*lim*	*xs*	*lim*
S	1†	1.5	1	1.1	1	1.1	1	1.2	1	7.1	1	6.7
S1	0	0	1.1	4.2	0.7	1.1	1.4	4.7	1.4	2.0	0.9	0.9
S2	0	0	0.5	2.6	0.6	0.9	0.7	3.4	0.7	1.1	0.9	0.8
S11	1.0	8.8	0	0	+	+	0.8	2.3	0.4	0.2	0.5	0.6
S21	0.7	15.7	0.9	5.5	0	0	1.0	3.4	0.7	0.8	0.5	1.1
S31	0.9	4.5	1.0	3.0	1.2	1.5	0	0	3.9	3.9	2.8	2.3
S51	1.5	4.0	1.0	2.3	+	+	1.5	3.8	3.5	19.8	0	0
S61	1.7	18.2	1.4	5.8	1.1	1.4	1.8	6.7	0	0	0	0

* Average from dialyzed extracts from two 14-hr cultures grown in minimal medium supplemented with 50 μg/ml L-tryptophan. Units = 0.1 μmole substrate used or product formed in 20 min at 37°; sp. act. = units/mg protein.
† Specific activities relative to wild type grown as described above. The column heading *xs* refers to growth in the presence of 50 μg L-tryptophan/ml; the column heading *lim* refers to growth in unsupplemented minimal medium for strain S, 5 μg L-tryptophan/ml for all other strains. The plus symbol indicates qualitative determination of the presence of isomerase (see text).

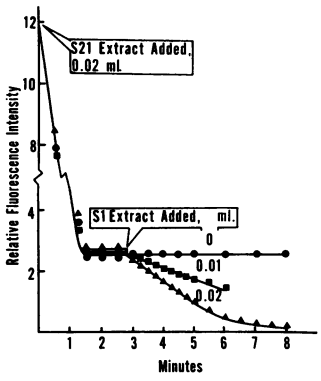

FIG. 2.—Fluorimetric assay for phosphoribosylanthranilate isomerase (PRAI). Reactants in μmoles/ml were: Tris, pH 7.5, 10; $MgSO_4$, 2; PRPP, 0.2; anthranilate, 0.015; and 0.02 ml of dialyzed extract of S21 (20.6 mg protein/ml). After 2.8 min at 37°, the indicated amount of S1 extract (17.0 mg protein/ml) was added. The relative fluorescence (excitation 315 mμ, emission 400 mμ) was measured in arbitrary units. For quantitation one assumes the anthranilate was all converted to PRA in 1.5 min. After adding PRAI-containing extract at 2.8 min, the disappearance of this "PRA" fluorescence during the first minute is used to calculate isomerase activity.

Mutant S21 was of especial interest for it lacked only phosphoribosylanthranilate isomerase (PRAI), permitting a rapid quantitative assay for this activity. When both PRT and PRAI are present in a PRT assay, the fluorescence of anthranilate disappears completely. With S21 extract the rate of fluoresence disappearance decreases after 50 per cent of the anthranilate is used and levels off with about 23 per cent of the fluorescence remaining, a result of the weaker fluorescence of phosphoribosylanthranilate (PRA) which accumulates in the absence of PRAI.[23] On addition of S11 extract or a purified *N. crassa* PRAI-InGPS preparation (kindly supplied by J. A. DeMoss), this residual fluorescence disappears at a linear rate.

A kinetic assay for PRAI based on its absence in S21 extract is shown in Figure 2. To check the expected stoichiometry the progress of the reaction measured by fluorescence was compared at various times to the amount of substrate consumed and InGP formed (Table 3). (InGPS is present in excess in the S21 extract. In the absence of L-serine, InGP accumulates quantitatively because it cannot be converted to L-tryptophan; the TS-A reaction rate is negligible under these conditions.)

The influence of tryptophan excess and deprivation on the enzymatic activities of both wild and mutant strains is also shown in Table 2. In the wild type, only TS-A and TS-B are increased upon omission of L-tryptophan from the medium. In the mutants, AS responds most to tryptophan limitation; PRT and InGPS appear to be affected in parallel and to a lesser extent, whereas PRAI does not vary much. It is perhaps significant that the PRAI level is 3× that of the next most active enzyme. Surprisingly, no increase over the basal level of TS-A and TS-B occurred when auxotrophs blocked in reactions before TS were harvested after tryptophan limitation. The TS mutant lacking reaction B activity (S51), however, did show elevated TS-A activity after tryptophan depletion.

Enzyme activity changes with time during tryptophan deprivation: To be certain that the activity changes recorded in Table 2 were meaningful, we followed the progress of enzyme formation in cells of strain S21 harvested near the end of growth on limiting tryptophan (Fig. 3). Control plating showed that none of the cultures had reverted. The contrast between the increment in TS and that of AS, PRT, and InGPS is striking, suggesting a strongly preferential synthesis of the latter three enzymes during times of tryptophan scarcity. Figure 3 also shows that anthranilate begins to accumulate only after tryptophan depletion at about 12 hr.

Assays of AS mutants grown on anthranilate: If the synthesis of more than basal levels of TS depends in some way on the presence of a pathway intermediate, the growth of strain S1 or S2 on anthranilate should cause elevation of both the TS-A

and B levels to those found when strain S is grown in minimal medium. The data in Table 4 show this to be the case, regardless of the amount of anthranilate used. An additional experiment was performed with S2 cells grown in the presence of both limiting tryptophan and excess indole. We reasoned that even though indole is poorly used for growth, it might have some effect on the regulation of TS. (The data of Table 2 implicate InGP or indole as the intermediate most likely to increase TS synthesis.) Indole gave an effect similar to anthranilate, though slightly less pronounced for TS-A activity.

TABLE 3

STOICHIOMETRY OF PHOSPHORIBOSYLANTHRANILATE ISOMERASE REACTION

Time (Min)	PRA Used*: By fluorescence decrease (μmoles)	PRA Used*: By degradation (μmoles)	InGP formed* as indolealdehyde after HIO_4 (μmoles)
2	0.014	0.012	0.012
4	0.022	0.022	0.020
6	0.031	0.030	0.026
8	0.039	0.036	0.034
10	0.046	0.045	0.043

* The reaction mixture contained in μmoles/7 ml: Tris, pH 7.8, 70; PRPP, 3.5; $MgSO_4$, 14; anthranilate, 0.7; and isomeraseless S21 extract (8.4 mg protein). After 5 min incubation at 37° to allow PRA formation, isomerase was added as S1 extract (1.48 mg protein). At 2-min intervals: (1) relative fluorescence was measured (excitation 315 mμ, emission 400 mμ) to estimate PRA disappearance; (2) samples were taken for residual PRA estimation by degradation to anthranilate (0.5 ml added to 0.1 ml 1 *M* acetic acid and incubated 15 min at 37°); anthranilate formed by PRA breakdown extracted into 5 ml ethyl acetate and quantitated by fluorescence; (3) samples were taken for InGP estimation (0.4 ml added to 0.2 ml 1 *M* sodium acetate buffer pH 4.8, treated with periodate, and determined as indolealdehyde as for the InGPS assay[16]).

Selection and characterization of indole-utilizing variants: The preceding experiment hints that indole fails to support rapid growth of auxotrophs blocked early in the pathway for a reason other than poor permeation. We therefore selected variants of strains S1 and S11 able to grow rapidly on indole by spreading about 10^8 cells on minimal agar plates containing 5 μg/ml indole. After 48 hr at 30°, 5–100 discrete colonies formed on the background film of growth. These were purified by several sequential single-colony isolations. After identifying those mutants which by growth and accumulation tests were revertants of the original mutation, each indole-utilizing variant was numbered -*i*1, -*i*2, etc. Table 5 shows an enzymatic analysis of three such variants grown under several conditions. Surprisingly, two of the variants, S11-*i*1 and S1-*i*1, had lost all TS-A activity. Concomitant with the gain in ability to grow on indole, strain S1-*i*1 lost the ability to grow on anthranilate (Table 1). The level of TS-B activity in both S1-*i*1 and S11-*i*1 was somewhat elevated, though in S1-*i*1 growth on indole was re-

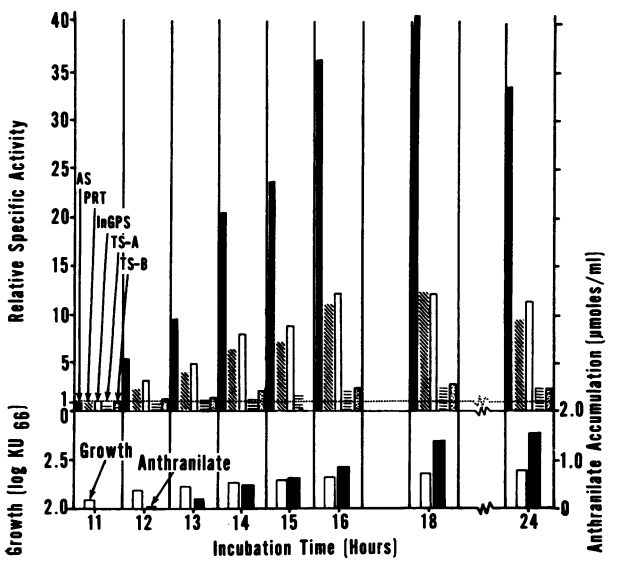

FIG. 3.—Strain S21, anthranilate accumulation and enzyme levels. Eight replicate 1-liter cultures were grown for various times at 30° in minimal medium containing 5 μg/ml L-tryptophan. Enzyme levels are specific activities relative to those of wild type grown with excess (50 μg/ml) L-tryptophan.

TABLE 4

ENZYME LEVELS OF ANTHRANILATE SYNTHETASELESS MUTANTS (S1 AND S2) GROWN IN PRESENCE OF ANTHRANILATE OR INDOLE

Strain and Growth Condition*		AS	PRT	PRAI	InGPS	TS-A	TS-B
S1	5T	0†	4.2	1.1	4.7	2.0	0.9
	30A	0	1.2	+	1.4	7.7	5.6
S2	5T	0	2.6	0.9	3.4	1.1	0.8
	3A	0	1.1	+	1.6	8.2	4.8
	30A	0	1.1	1.0	1.2	5.5	5.4
	5T, 30I	0	0.6	1.0	0.7	3.8	5.4

* A = Anthranilate; I = indole; T = L-tryptophan; numbers preceding indicate concentration in μg/ml of the substance added to minimal medium.
† Specific activity relative to strain S (wild type) grown in excess tryptophan (see footnote to Table 2). The plus symbol indicates qualitative determination of the presence of isomerase (see text).

TABLE 5

ENZYME LEVELS OF INDOLE UTILIZING MUTANTS OF STRAINS S1 AND S11 UNDER VARIOUS GROWTH CONDITIONS

Strain and Growth Condition*		AS	PRT	PRAI	InGPS	TS-A	TS-B
S11-*i*1	5T	3.7*	0	+	2.0	0	6.4
	50T	1.4	0	+	0.9	0	7.4
	3I	15.6	0	+	6.1	0	7.3
	30I	1.2	0	+	1.0	0	5.1
S1-*i*4	5T	0	3.4	1.3	4.4	118	55
	50T	0	0.8	0.7	1.0	84	50
	30I	0	0.7	+	0.9	63	47
	30A	0	1.1	+	1.3	89	51
S1-*i*4R1	0T	1.0	1.1	0.9	1.5	86	47
	50T	0.7	1.0	0.9	1.3	75	48
S1-*i*1	5T	0	3.7	1.1	3.3	0	1.3
	50T	0	0.8	0.6	0.8	0	0.9
	30I	0	0.9	1.3	0.9	0	4.2
	5T, 30A	0	5.5	1.5	9.3	0	207
	50T, 30A	0	1.0	1.0	1.4	0	176

* Symbols and entries as for Table 4.

quired for the increased activity. We tentatively concluded that the poor growth of strains S1 and S11 on indole rests on feeble TS-B activity within the cell. Possibly a mutation in the TS-A catalytic site improves efficiency in the remaining TS-B reaction.

A second type of mutation allowing rapid growth on indole is presented in Table 5; in strain S1-*i*4 both TS activities are increased 50- to 100-fold over the basal level. This level is independent of the nature and quantity of supplement (L-tryptophan, indole, or anthranilate), though the regulation of the early enzymes of the pathway remains normal. It is tempting to regard strain S1-*i*4 as a "constitutive overproducer" of TS, especially since its prototrophic revertant, S1-*i*4R1, retains high levels of the two TS activities after reversion of the AS defect.

Proof of the inducibility of TS: The finding of two independent and apparently total blocks in strain S1-*i*1 allowed a convincing test of the hypothesis that formation of increased levels of TS requires the presence of a pathway intermediate as inducer. As the data in Table 5 show, when this strain is grown on tryptophan, either limiting or in excess, the TS-B activity observed is not significantly greater than the basal level observed in strain S1 (Table 1). A medium with added anthranilate allows formation within the cell of PRA, CDRP, and InGP in turn. If the latter is an inducer, high levels of TS-B activity should appear. Conditions of

"gratuity" exist in this experiment, for it is immaterial to the growth of the cell whether anthranilate is present or TS-B activity elevated.

This experiment was run with 30 μg/ml anthranilate in the presence of both limiting and excess L-tryptophan (Table 5). The activities observed for TS-B were over 200× the basal level. One must attribute these extremely high levels of TS to the presence of intermediates formed from anthranilate. The anthranilate-grown cultures gave a strong qualitative test for InG in their culture fluids. When viewed beside the evidence in Table 2, this experiment implicates InGP as the primary inducer for TS. There appears to be little or no repressive effect of tryptophan on TS synthesis; the difference between 176 and 207× the basal activity may reflect the shorter growth time at harvest of the culture in excess tryptophan, slightly lower levels of InGP in the cells, or both.

Discussion.—The pathway of formation of tryptophan from chorismate appears to be the same in *P. putida* as in all other microbes examined. The enzymatic reactions shown in Figure 1 can be assayed in extracts of the pseudomonad under conditions similar to those used for *E. coli*. Mutation can bring about the elimination of any one of the enzymatic activities; in *P. putida* this may occur without affecting the other activities of the pathway. One of the next ventures in characterizing the pathway in *P. putida* should be to determine if all the enzymatic activities we have studied are catalyzed by independent protein molecules. Characterization of TS should be facilitated by mutants like S1-*i*1 and S1-*i*4 with high levels of TS activity.

At present we do not have a method of genetic recombination in *P. putida* with which to determine whether any of the genes controlling enzymes in this pathway are closely linked. For the related organism *P. aeruginosa*, Fargie and Holloway[24] report that three classes of tryptophan auxotrophs are unlinked in transduction tests. One class satisfies the criteria for mutants defective in TS. The other two classes are blocked between anthranilate and InGP; one is reported to lack PRT and the other PRAI. All tryptophan pathway mutants in *E. coli*,[1] *Salmonella typhimurium*,[25] and *B. subtilis*,[4] are closely linked. It would not be surprising if the regulatory difference observed between *P. putida* and the enteric bacteria correlates with a different chromosomal organization.

In *E. coli* K12, synthesis of anabolic enzymes is regulated by end-product repression. Even where noncoordinate regulation is found, as in the arginine pathway,[26, 27] excess product inhibits synthesis of all pathway enzymes. Although Gorini[27] reported preliminary experiments suggesting that glutamine may have an "inducing effect" on arginine synthetic enzymes, marked antagonism between inducer (glutamine) and repressor (arginine) was found.

In contrast, in *P. putida* the level of TS is controlled solely by induction, and its inducer appears not to affect the production of enzymes earlier in the pathway. A double mutant blocked in AS and TS-A permitted us to control the presence of intermediates between the blocks. Our data allowed us to conclude that InGP is the primary inducer for TS, although indole may also have a weak effect, and that tryptophan does not exert repression. Possibly regulation of the enzymes catalyzing the first four reactions of the pathway is established through repression by tryptophan. In one instance we observed a 40-fold increase in the basal level of AS when a slightly leaky mutant was maintained many hours under conditions of tryptophan deprivation. But lacking mutants blocked in the aromatic amino acid

pathway before chorismate and having no measure of the intracellular levels of compounds earlier in the pathway which could be inducers, we cannot rule out induction as the mechanism of control of these enzymes as well.

In the tryptophan anabolic pathway, as in the catabolic pathways studied,[7] blocks of enzymes within the pathway may respond to the presence of one or another small effector molecule. Superimposed upon this control of enzyme synthesis is feedback inhibition of enzyme activity. (D. Joseph in the laboratory of one of us has demonstrated that AS activity is very sensitive to inhibition by L-tryptophan.) Data available for at least one bacterium, *Chromobacterium violaceum*, suggest that a mechanism for regulation of the rate of enzyme synthesis in an anabolic pathway may not be a completely essential part of the genetic make-up of a cell, for the amount of tryptophan available to this organism does not seem to influence its enzyme levels.[28] It seems plausible to us that the method used by organisms to regulate enzyme synthesis may prove to be a useful parameter in the determination of relatedness among bacteria.

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