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The Regulation of Tryptophan Biosynthesis in *Pseudomonas aeruginosa*

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Summary. Eighteen auxotrophs of *Pseudomonas aeruginosa* requiring L-tryptophan for growth were isolated following nitrosoguanidine mutagenesis. Mutant blocks for each step of tryptophan biosynthesis were identified by enzymological assay. A regulatory mutant was characterized which was simultaneously constitutive for the gene products of *trpA*, *trpB* and *trpD*. Another class of regulatory mutant appears to synthesize tryptophan synthetase (i.e., *trpE* and *trpF* subunits) constitutively. The results implicate three control entities in the pathway of tryptophan biosynthesis: (i) The gene products of *trpA*, *trpB* and *trpD* are repressible by tryptophan, the range of enzyme specific activity varying at least fifty-fold. (ii) No regulation of the *trpC* gene product could be demonstrated, indicating that its synthesis is constitutive. (iii) The gene products of *trpE* and *trpF* are inducible by indoleglycerol 3-phosphate; the magnitude of induction can exceed 100-fold. These results together with some genetic data indicate a general similarity in gene-enzyme relationships between *P. aeruginosa* and *P. putida*. A number of specific differences that distinguish the two species are noted.

A mutant blocked in the common pathway of aromatic biosynthesis was used to prove that enzymes of tryptophan biosynthesis other than tryptophan synthetase are not inducible by precursors of the common pathway such as chorismate. It is concluded that the concentration of tryptophan that signals total repression of the gene products of *trpA*, *trpB* and *trpD* is lower than the concentrations necessary for maximal feedback inhibition of anthranilate synthetase and for abolition of the induction of tryptophan synthetase.

Introduction

The regulatory role of end product(s) in the repression of enzyme synthesis in biosynthetic pathways is now a familiar theme in microorganisms. Gross (1965) and Crawford and Gunsalus (1966) made the initial observations indicating that the mechanism governing enzyme synthesis may sometimes vary for enzymes in the same reaction sequence. Other examples of this pattern will undoubtedly accumulate in the literature (e.g., see Arfin *et al.*, 1969; Jones-Mortimer *et al.*, 1968; Lacroute, 1968). Hence, an end product-repressible enzyme may be followed by a substrate-inducible enzyme. Such regulatory schemes are perfectly consistent with our concepts of physiological economy, since end product control of the first enzyme step by repression and feedback inhibition will effectively decrease the subsequent formation of an inducer metabolite(s) in a biochemical sequence. Gross (1965) noted the advantage that such systems are responsive not merely to end product levels but also to the flux of carbon through the pathway and hence to variations in substrate levels. The demonstration of induction of tryptophan synthetase by indoleglycerol 3-phosphate (IGP) in *Pseudomonas putida* (Crawford and Gunsalus, 1966) and of the induction of the gene products of three

cistrons of leucine biosynthesis by α-isopropylmalate in *Neurospora crassa* (Gross, 1965) contributed substantially to the growing appreciation of the extraordinary diversity which characterizes microbial systems of regulation. This communication documents the regulation of the tryptophan biosynthetic pathway in strain 1 of *P. aeruginosa*, another genetic system suitable for defining gene-enzyme relationships.

Materials and Methods

Bacteria and Bacteriophage. *P. aeruginosa* strain 1 (Holloway, 1955) and bacteriophage F116 (Holloway *et al.*, 1960) were originally obtained from B.W. Holloway. Conditions for the growth of cultures and methods used for transduction and cross-feeding have been described previously (Calhoun and Feary, 1969; Calhoun and Jensen, 1970).

Analysis of Precursor Accumulation by Tryptophan Auxotrophs. Strains to be tested for accumulation of tryptophan precursors were grown overnight with shaking in 2 ml of minimal medium containing 100 μg of tryptophan per ml. A 0.5 ml portion of each overnight culture was inoculated into 15 ml of minimal salts-glucose medium (Jensen, 1968) containing 10 μg of tryptophan per ml. Cultures were incubated at 37° C on a gyrotary shaker (New Brunswick), and turbidity was followed using a Klett-Summerson colorimeter equipped with a green filter (#54). After 4–5 h of growth, turbidity increase ceased due to tryptophan starvation. After an additional four hours of incubation (derepression time), the culture was harvested by centrifugation. Indole was measured with *p*-dimethylaminobenzaldehyde according to Yanofsky (1956). IGP accumulation was detected with $FeCl_3$ reagent by the method of Yanofsky (1956). The concentration of anthranilate was quantitated by measuring absorbance in a Gilford spectrophotometer at 336 nm after extraction into ethyl acetate by the procedure of Smith and Yanofsky (1960). Qualitatively, the presence of anthranilate in culture fluids was readily recognized by its intense purple fluorescence under ultraviolet light.

Derepression Experiments. Levels of tryptophan biosynthetic enzymes in wild type and mutants were determined in extracts prepared from cultures grown in the presence or in the absence of tryptophan. Repression data were obtained from cultures grown at 37° C on a rotary shaker in minimal salts-glucose medium plus 100 μg of L-tryptophan per ml. Cultures were harvested in the late exponential phase of growth by centrifugation at 4° C, and the cells were washed once with cold medium. Derepression of enzyme synthesis in batch culture was accomplished by centrifuging cultures in the late exponential phase of growth and resuspending the cells in the original volume of fresh medium lacking L-tryptophan. The cultures were incubated at 37° C with vigorous shaking for 6 hours. The cell populations were harvested by centrifugation, washed with cold medium, and stored at —80° C until the time of extract preparation.

Preparation of Extracts. Crude extracts for enzymological assays were prepared from cells sedimented from 200 ml cultures. Cell pellets were suspended in 2–3 ml of 10 mM Tris· HCl (pH 7.8) containing 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol and were exposed to three 30-second bursts delivered from a sonic probe (Biosonik, Bronwill Instruments) at 4° C. Cell debris was removed by centrifugation at $40000 \times g$ for 15 minutes. Nucleic acids were removed by precipitation with freshly prepared protamine sulfate (0.2% final concentration). After 10 minutes at 4° C, the extracts were centrifuged at $40000 \times g$ for 25 minutes. The extract proteins were separated from small molecules by filtration through a small sephadex G-25 column equilibrated with the same buffer described above. All enzyme assays were performed on the same day of extract preparation. Protein concentrations were estimated by the method of Lowry *et al.* (1951).

Enzymological Assays. Anthranilate synthetase activity was assayed in the following manner. The 1.0 ml reaction mixture contained the following ingredients (μmoles): chorismic acid, 0.3; L-glutamine, 10; $MgCl_2$, 7; 2-mercaptoethanol, 7; Tris buffer (pH 7.8), 35; and a rate-limiting concentration of enzyme. The reaction was initiated by the addition ofchorismate to a reaction mixture previously incubated at 37° C for three minutes. The rate of anthranilate formation was followed continuously by measuring its fluorescence at an excitation wavelength of 313 nm and an emission wavelength of 393 nm using an Aminco-Bowman spectrophoto-

fluorometer. A standard curve was used relating known concentrations of authentic anthranilate to fluorescence.

Anthranilate-PRPP phosphoribosyl transferase (PR transferase) activity was measured as follows. The reaction mixture of 1.0 ml volume contained in μmoles: anthranilate 0.01; phosphoribosyl pyrophosphate, 10; $MgCl_2$, 7; 2-mercaptoethanol, 7; Tris buffer (pH 7.8), 35; and a rate-limiting concentration of enzyme. The reaction was initiated by the addition of enzyme preparation to the reaction mixture previously incubated at 37° C for three minutes. The rate of the reaction was followed continuously by observing the decrease in fluorescence of anthranilate using the procedure described for anthranilate synthetase.

The assay for N(5′-phosphoribosyl) anthranilate isomerase was identical to that used in *P. putida* (Crawford and Gunsalus, 1966) except that an extract of *P. aeruginosa* mutant P135 (Table 1) containing derepressed levels of the tryptophan enzymes (as in Table 3) was used to provide reaction conditions where isomerase activity is rate-limiting.

Indoleglycerol 3-phosphate synthetase was assayed by the method of Smith and Yanofsky (1962) except that the 0.5 ml reaction mixture contained in μmoles: 1-(*o*-carboxyphenylamino)-1-deoxy-ribulose-5-phosphate (CDRP), 1.2; Tris · HCl (pH 7.8), 20; $MgCl_2$, 4; 2-mercaptoethanol, 4; and a rate-limiting concentration of enzyme preparation. The substrate, CDRP, was synthesized chemically as described by Creighton and Yanofsky (1970).

Activity of the α-subunit of tryptophan synthetase was followed by measuring the appearance of indole. The reaction mixture of 1.0 ml volume contained 250–400 μg of crude extract protein from strain NG1 (source of excess β-subunit) plus the following in μmoles: pyridoxal-5′-phosphate, 0.2; L-serine, 20; hydroxylamine (pH 7.0), 1200; IGP, 0.3; Tris· HCl (pH 7.8), 20; $MgCl_2$, 4; 2-mercaptoethanol, 4; and a rete-limiting concentration of enzyme preparation. All reaction components were incubated for 5 minutes at 37° C except IGP and hydroxylamine. Then these components were added, and the reaction was allowed to proceed for 20 minutes. The indole formed was determined by the method of Meduski and Zamenhof (1968). The IGP substrate was enzymatically synthesized from CDRP using purified *Escherichia coli* IGP synthetase (27).

The 1.0 ml reaction mixture used to assay the activity of the β subunit of tryptophan synthetase contained in μmoles: indole, 0.15; L-serine, 80; Tris · HCl (pH 7.8), 40; $MgCl_2$, 8; 2-mercaptoethanol, 8; pyridoxal-5′-phosphate, 0.2; EDTA, 2; and a rate-limiting concentration of enzyme preparation. The reaction was initiated by the addition of enzyme to the remaining reaction mixture components preincubated for 10 minutes at 37° C. The reaction was terminated after 20 minutes and indole disappearance was determined by use of the assay described above for indole.

A unit of enzyme activity is defined as the production or utilization of one *n*mole of product or substrate per minute at 37° C. Specific activity is given as units per mg protein.

Isolation of Revertants of Strains trpE1 and trpE2. Mutant *trpE1* or *trpE2* (Table 1) was grown on a blood agar base medium at 37° C, and the cells captured in a small loop were spread confluently on the surface of an agar plate containing minimal salts-glucose medium. A 0.02 ml amount of sterile L-tryptophan (5 mg/ml) was spotted in the center of the plate. A revertant population typically develops around the periphery of the spot where growth is marginal. The density of the revertant population correlates with the decreasing concentration gradient of diffusing L-tryptophan. After 24–48 hours revertants were isolated by repeated streaking of single colony isolates on minimal salts-glucose agar. Such revertants were tested for cross-feeding of indole (a consequence of elevated levels of the α subunit of tryptophan synthetase) by picking to solid minimal salts-glucose medium seeded with a lawn of *trpF3* (lacking the α-subunit activity of tryptophan synthetase). Halos developed around picks in excellent correlation with enzymological determination of activity levels of the α protein of tryptophan synthetase.

Continuous Cultivation of Mutant trpA1 in the Chemostat. Mutant *trpA1* was grown to the late exponential phase of growth in 10 ml of minimal salts-glucose medium at 37° C on a rotary shaker. The cells were harvested, washed and resuspended in fresh medium containing 0.5 μg of L-tryptophan per ml. A sample of this suspension was used to inoculate the 350 ml working volume of a New Brunswick chemostat (Bioflow model C30) to an initial absorbance at 600 nm of 0.05. Continuous cultivation was carried out using the following settings: agitation, 600 rpm; air, 1.6; temperature, 37° C; and flow rate, 94 ml per hour. A steady state

was reached at an absorbance at 600 nm of 0.88 using a tryptophan concentration of 0.5 μg per ml in the medium reservoir.

Chemicals. The following chemicals were obtained from Calbiochem: anthranilic acid, indole, L-tryptophan, L-tyrosine and L-phenylalanine. DL-4-fluorotryptophan, DL-5-fluorotryptophan and all other biochemicals were obtained from Sigma Chemical Company. Chorismic acid (100% pure) was isolated from culture supernatants of strain 62-1 of *Aerobacter aerogenes* (Gibson, 1964) and prepared as the free acid (Edwards and Jackman, 1965).

Results

Characterization of Tryptophan Auxotrophs. The deficient enzyme activities of many of the 18 tryptophan auxotrophs listed in Table 1 were identified directly by enzyme assay. Growth responses of mutants (to indole, anthranilate or tryptophan), identification of accumulated precursors, and syntrophism between cross-feeding pairs of mutants were all wholly consistent with the location of blocks as established enzymologically. The mutants comprise six classes, each class lacking one of the enzymatic activities in the biochemical sequence between chorismate and L-tryptophan. The mutant genes are denoted *trpA* through *trpF*, the alphabetical order corresponding exactly to the reaction order in the biochemical sequence. *TrpD* mutants did not accumulate CDRP or its dephosphorylated derivative, CDR. This anomalous observation was also made in *P. putida* (Crawford and Gunsalus, 1966) although for reasons which are not clear, CDRP accumulation seems to be an exceedingly variable characteristic (Crawford, personal communication). The *trpF* mutants, which lack activity for the β-subunit of tryptophan synthetase, excrete indole and cross-feed each of the remaining five mutant classes listed in Table 1.

Tryptophan Degradation in Growing Cultures of P. aeruginosa? It was observed that mutant *trpA1*, which lacks anthranilate synthetase, requires less tryptophan for maximal growth than do mutants lacking other tryptophan genes. For example, at 10 μg tryptophan per ml, mutant *trpA1* grows from a small inoculum to an absorbance yield measured at 525 nm of about 1.8. In contrast, the other auxotrophs listed in Table 1 under similar conditions reach an absorbance yield of only 0.6 to 0.8, requiring higher concentrations of tryptophan to achieve the growth yield of mutant *trpA1*. It was further observed that when the wild type is grown in the presence of exogenous tryptophan, anthranilate accumulates in the culture medium. The presence of high levels of kynureninase activity in our strains (Pierson, unpublished results) suggests the presence of the pathway of degradation of tryptophan through kynurenine. However, the wild type cannot utilize tryptophan or anthranilate as a sole carbon and energy source, indicating that the oxidative pathway of tryptophan degradation known to be present in many pseudomonads is incomplete. Stanier and Hayaishi (1951) described wild type *Pseudomonas* isolates which can efficiently convert tryptophan to anthranilic acid, but which are severely restricted in the capacity to oxidize anthranilate. *P. aeruginosa* strain 1 is similar in this respect to the wild type isolates described by Stanier and Hayaishi (1951). Since *trpA* auxotrophs would be unique among tryptophan auxotrophs in ability to recycle anthranilate derived from degraded tryptophan for tryptophan biosynthesis, a reasonable interpretation is available to explain the lower growth requirement of *trpA* mutants for exogeneous tryptophan. This

Table 1. Tryptophan auxotrophs of *P. aeruginosa*

Mutant gene	Mutant designation	Growth requirements	Precursor accumulated	Enzyme defect
trpA	*trpA1*	anthranilate, indole, L-tryptophan		anthranilate synthetase[a]
trpB	*trpB1* *trpB2*	indole, L-tryptophan	anthranilate	5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase
trpC	*trpC1* *trpC2* *trpC3* *trpC4* *trpC5* *trpC6* *trpC7*	indole, L-tryptophan	anthranilate[b]	N(5′-phosphoribosyl) anthranilate isomerase
trpD	*trpD1* *trpD2*	indole, L-tryptophan	anthranilate	IGP synthetase
trpE	*trpE1* *trpE2*	indole, L-tryptophan	IGP	tryptophan synthetase (α-activity)
trpF	*trpF1* *trpF2* *trpF3* *trpF4*	L-tryptophan	IGP, indole	tryptophan synthetase (β activity)

Growth requirements and precursor accumulation were determined as described in the materials and methods. The enzyme defects inferred from the growth response and the precursor accumulated were confirmed by direct enzyme assays for at least one mutant representative of each class.

[a] Mutant *trpA1* is deficient in the large molecular weight component, and retains the small glutamine-binding subunit unchanged by mutation (J. Kane, unpublished observations).
[b] Phosphoribosyl anthranilate is very labile and accumulates almost entirely as anthranilate (Doy and Gibson, 1959).

relationship probably bears on the difficulty noted in causing derepression of [ABD] enzyme synthesis following tryptophan starvation of mutant *trpA1* in batch cultures.

Repression in Wild Type. The data in Table 2 show that the presence of exogenous L-tryptophan during growth in a minimals salts-glucose medium has no influence on the specific activities of enzymes [A] through [D]. Hence, enzymes [A] through [D] exists at maximally low levels during growth in minimal salts-glucose medium. In contrast the tryptophan synthetase activities (i.e., [E] and [F]) are reduced about ten-fold in the presence of exogeneous tryptophan.

Enzymological Profiles of Tryptophan Auxotrophs. Starvation of various tryptophan auxotrophs for L-tryptophan (Table 3) produces derepression for enzymes [A], [B] and [D], thereby demonstrating the repressibility of these enzymes.

Table 2. Repression of enzyme synthesis by endogenous tryptophan in wild type *P. aeruginosa*

Growth[a] condition	Specific activity[b]					
	[A]	[B]	[C]	[D]	[E]	[F]
−tryptophan	0.53	1.20	1.00	1.5	0.39	1.2
+tryptophan	0.49	1.25	1.04	1.6	0.05	0.10

[a] Growth was conducted at 37° C in 200 ml batch cultures, vigorously aerated and harvested in the late exponential phase of growth. The medium was a minimal salts-glucose preparation supplemented, where indicated, with L-tryptophan at a final concentration of 100 μg per ml. [b] Specific activities are expressed in units per mg of crude extract protein. Enzymes [A] through [F] are denoted to correspond to the gene designations and are in sequence: anthranilate synthetase, 5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase, N(5′-phosphoribosyl) anthranilate isomerase, IGP synthetase, and the α and β subunit activities of tryptophan synthetase, respectively. Repressed levels of enzyme activity are sufficiently low to approach the lower limits of assay sensitivity. Specific activities are given as averages from data obtained in two experiments.

Table 3. Enzymological analysis of tryptophan pathway enzymes in auxotrophic mutants

Mutant designation	Relative specific activity[a]											
	[A]		[B]		[C]		[D]		[E]		[F]	
	+	−	+	−	+	−	+	−	+	−	+	−
trpA1			1.0	2.4	0.6	0.6	1.0	1.7	N.T.	N.T.	3.5	2.4
trpB1	0.6	6.8			1.3	1.7	0.6	4.5	N.T.	N.T.	5.0	3.6
trpC4	0.4	35	0.7	15			1.5	7.5	N.T.	N.T.	4.4	6.0
trpD2	0.3	10	0.9	16	0.7	1.7			N.T.	N.T.	5.2	5.5
trpE1	0.5	8.5	0.9	8.6	1.2	1.3	1.0	10			590	759
trpF3	0.7	2.3	0.5	4.5	1.0	1.4	1.0	13	8.4	16		

[a] In the definition of relative specific activities the specific activities of extracts from wild type grown in the presence of excess L-tryptophan (as given in Table 2) are arbitrarily assigned a relative value of 1.0 for each of enzymes [A] through [F] (identified in Table 2). Shaded areas indicate the deficient enzyme activity of each mutant. N.T. denotes "not tested". Extracts were prepared from mutants grown in the presence of 100 μg per ml of L-tryptophan (columns denoted +) or from mutants starved for L-tryptophan (columns denoted −). Other details of experimental procedure are given under Methods.

Mutant *trpA1*, the only auxotroph blocked in anthranilate synthetase, could not be caused to undergo a convincing degree of derepression for enzymes [B] and [D] following starvation of batch cultures for tryptophan. This, however, was accomplished in a chemostat (see Methods) using continuous culture in the presence of a growth-limiting concentration of L-tryptophan. An extract prepared from a chemostat-grown culture possessed relative specific activities (defined as in Table 3) of 21, 0.9, 13, 1.0, and 0.7 for enzymes [B] through [F], respectively.

Enzyme [C] is not influenced appreciably by any nutritional manipulation or by mutation and therefore appears to be formed constitutively. A substantial elevation of enzyme activity [F] did not occur following tryptophan starvation in any of the mutants except *trpE1* and *trpE2*. A three to five-fold elevation in the basal level of [F] seems to characterize tryptophan auxotrophs for reasons that are not clear. The dramatic increase in activity of [F] is several hundred-fold in mutant *trpE1* (Table 3), and implicates the role of the accumulated precursor, IGP, as an inducer molecule. Similar results were obtained from mutant *trpE2*. The elevation of the [E] protein in mutant *trpF3* also is undoubtedly the result of induction by IGP.

Further Analysis of [F] Mutants. Curiously enough, [E]-deficient mutants *trpE1* and *trpE2* continue to express high induced levels of [F] in the presence of exogeneous tryptophan. This contrasts with [E]-deficient mutants of *P. putida* (Crawford and Gunsalus, 1966). The data given in Table 3 for [F]-deficient *trpF3* suggest a similar phenomenon for [E] since the level of [E] was elevated by a factor of 8.4 over that of wild type in the presence of excess L-tryptophan. Alternatively, degradation of tryptophan through anthranilate could lead to greater levels of IGP in the [F]-deficient mutant than in wild type. Revertants of *trpE1* and *trpE2* were purified on minimal salts-glucose medium. All revertants tested possessed elevated activity for [F] and levels of [E] equal to or lower than that of wild type. Activities of [E] and [F] were not influenced by the presence of exogeneous tryptophan during growth. A total of 20 *trpE1* revertants and 12 *trpE2* revertants were assayed for enzyme [F] activity, and all showed variable degrees of constitutivity. Complete enzymological profiles are shown for four representative revertants of *trpE1* in Table 4. It was found that the level of [F] in revertants of *trpE1* and *trpE2* correlated quite well with ability to cross-feed mutant *trpF3* on indole-supplemented minimal salts-glucose agar media. Subsequent testing by this methodology indicated that each of several hundred additional revertants tested possessed higher levels of [F] than wild type. In contrast to these data, results obtained with mutant *trpE1* shortly after its original isolation indicated that enzyme [F] initially displayed low uninduced levels following growth in the presence of tryptophan (Calhoun, unpublished observations). Hence, it appears

Table 4. Enzymological profile of some revertants of mutant *trpE1*

Mutant designation	Relative specific activity[a]					
	[A]	[B]	[C]	[D]	[E]	[F]
trpE1-b	0.8	1.3	1.4	2.4	2.4	62
trpE1-f	0.8	1.3	1.7	2.5	0.6	316
trpE1-j	0.9	1.2	0.9	0.9	0.1	17
trpE1-e	1.3	1.1			0.8	217

Extracts were prepared from cultures grown at 37°C in minimal salts-glucose medium supplemented with L-tryptophan at 100 μg per ml. The mutants are spontaneous revertants of *trpE1* (see Methods).

[a] The definition of relative specific activity is given under Table 3, and the enzymes [A] through [F] are identified under Table 2.

that mutants *trpE1* and *trpE2* acquired a second mutation causing constitutivity for [F] while carried in stock culture. A selective basis for this inadvertant selection may be increased leakiness of growth owing to elevated levels of [E], the deficient enzyme in this class of mutants (assuming that [E] and [F] are regulated coordinately).

A class of Constitutive Mutants which Excretes Tryptophan. Among 257 *P. aeruginosa* mutants isolated by selection for resistance to 10 μg of 4-fluorotryptophan per ml, 34 excrete tryptophan as judged by ability to feed the [F] class of tryptophan auxotrophs. Similarly, 6 of 40 mutants selected for resistance to 10 μg of 5-fluorotryptophan per ml excrete tryptophan. Thus, tryptophan excretors are a relatively common class of *P. aeruginosa* analogue-resistant derivatives. *P. aeruginosa* apparently differs from *P. putida* in this respect; among *P. putida* mutants selected for resistance to tryptophan and indole analogues, none excreted tryptophan (Calhoun and Jensen, 1972; Maurer and Crawford, 1971). Analogue resistant mutants of *P. putida* which excrete the tryptophan precursor, anthranilate, are of frequent occurrence. Many analogue resistant mutants in both species are constitutive for anthranilate synthetase, PR transferase, and IGP synthetase.

The analogue 4-fluorotryptophan was found previously to be a potent antimetabolite of tryptophan biosynthesis in *P. aeruginosa* by Calhoun and Jensen (1972). One of the best tryptophan excretors isolated by selection on analogue-containing medium was numbered 4FT1. The enzymological basis for tryptophan overproduction in mutant 4FT1 is the constitutive formation of the [ABD] enzymes (Table 5). The identity of tryptophan as an excreted metabolite by mutant 4FT1 is apparent from the ability to cross-feed mutant *trpF3*, an [F] mutant which cannot utilize anthranilate or indole to satisfy its growth requirement for tryptophan. A great deal of anthranilate is also produced by mutant 4FT1.

Table 5. Constitutivity of the ABD enzymes in regulatory mutant 4FT1

Growth Supplement	Relative specific activity[a]					
	[A]	[B]	[C]	[D]	[E]	[F]
None	97	61	1.2	33	4	
Phe plus tyr	136	70		17	5	7
Anthranilate	90	58		25	4	3

Extracts from 4FT1 were prepared from cultures grown in minimal salts-glucose medium at 37°C, and harvested in the late exponential phase of growth. Phenylalanine (Phe) and tyrosine (tyr), when present, were initially supplied at 100 μg per ml each. Anthranilate, when present, was supplied at a concentration of 100 μg per ml.

[a] The specific activities are relative values, compared to repressed wild type values, as described under Table 3.

The enzymological profile for mutant 4FT1 was not altered by any of various nutritional supplementations tested in the growth regimen (Table 5). For example, the presence of anthranilate (which bypasses the site of feedback inhibition) did not result in the induction of enzymes [E] and [F]. The presence of exogeneous

tyrosine and phenylalanine which would tend to shunt chorismate exclusively into the tryptophan pathway also failed to influence the levels of [E] and [F] in mutant 4FT1. Other experiments have shown that exogeneous tryptophan or the simultaneous presence of anthranilate, phenylalanine and tyrosine all fail to affect the levels of [E] and [F] in mutant 4FT1. The level of enzyme [C] was not influenced by the mutation in 4FT1.

Demonstration of Induction of [E] and [F]. Because *trpF* mutants possess constitutive levels of [E] (owing to a second mutation), they cannot be used to prove induction by accumulation of IGP. Regulation of tryptophan synthetase (i.e., [E] and [F]) by substrate induction seemed likely because of the existence of this pattern in the closely related *P. putida*. Experimental verification of induction under physiological conditions was accomplished by the use of mutant MC36, blocked in the common aromatic pathway at an enzyme reaction preceding shikimate (Calhoun *et al.*, 1972). Starvation for tryptophan in the presence of phenylalanine and tyrosine resulted in the derepression of [A], [B] and [D], but not of [E] and [F] (Table 6). The presence of anthranilate provides a supply of

Table 6. Physiological demonstration of an inducible tryptophan synthetase

Growth supplement[b]	Relative specific activity[a]					
	[A]	[B]	[C]	[D]	[E]	[F]
L-tryptophan	0.9	0.7	1.6	0.8	1.6	2.4
None[c]	61	28	1.9	2.7	4.2	5.5
Anthranilate	3.8	1.8	1.8	1.2	72	127

Mutant isolate MC36 (Calhoun *et al.*, 1972) requiring either shikimate or the full complement of aromatic amino acids for growth was grown at 37°C and harvested in late exponential phase for extract preparation.

[a] The definition of relative specific activity is given under Table 3, and the enzymes [A] through [F] are identified under Table 2.

[b] All cultures contained L-phenylalanine and L-tyrosine, each at a final initial concentration of 100 μg per ml.

[c] Starvation for L-tryptophan was carried out by resuspension of exponential phase cells in fresh medium lacking tryptophan but containing tyrosine and phenylalanine (see Methods).

precursor for IGP formation, and [E] and [F] activities are then dramatically elevated. Activities of [A], [B] and [D] in the latter experiment are near the repressed level, presumably owing to the endogenous formation of tryptophan from exogenous anthranilate. Starvation for phenylalanine and tyrosine in the presence of anthranilate (6 hours) prevented the expression of the induction phenomenon in mutant MC36. Possible explanations include the prevention of protein synthesis required for new enzyme synthesis and the tendency for chorismate to form prephenate in the absence of phenylalanine and tyrosine.

Linkage Arrangement of the Tryptophan Genes. Transduction mediated by bacteriophage F116 was used in genetic analyses. Previous studies indicate that a high degree of homology in chromosomal organization exists for structural genes of tryptophan synthesis between *P. aeruginosa* strain 2 and *P. putida*

(Chakrabarty and Gunsalus, 1970; Fargie and Holloway, 1965; Holloway, 1969; Holloway, 1971). In *P. putida* (Gunsalus *et al.*, 1968) and in *P. aeruginosa* strain 2 (Fargie and Holloway, 1965) the *trpC* locus has been reported to be linked to a gene of methionine biosynthesis. Accordingly, phage propagated on mutant *trpC2* was used to transduce a number of *P. aeruginosa* strain 1 methionine auxotrophs. Selection of recombinants was on minimal salts-glucose plates supplemented with tryptophan (100 μg per ml). Transductants were subsequently scored for the ability to grow on unsupplemented minimal plates to test for cotransfer of the unselected *trpC* marker. Since cysteine auxotrophs in *P. aeruginosa* can also utilize methionine as a growth factor (Calhoun and Feary, 1969), cysteine auxotrophs were included with the methionine auxotrophs in the testing for linkage to strain *trpC2*. Each of the recipient loci tested (expt. A, Table 7) is located in a different transduction group (Calhoun and Feary, 1969; Calhoun, unpublished observations). The results (expt. A, Table 7) indicate cotransfer of the *trpC* allele with the *cys 171* locus, but not with the other four methionine and cysteine loci tested. These results suggest that the marker recognized in *P. putida* and in *P. aeruginosa* strain 2 by a methionine requirement may, in fact, be a cysteine gene.

In experiment B of Table 7, phage propagated on representatives of each class of tryptophan auxotroph were used to test for linkage of the various tryptophan cistrons to mutant *cys 171*. The results indicate that although *trpC* mutants are closely linked to the *cys 171* locus, no linkage is apparent with the *trpA*, *trpB*, *trpE*, or *trpF* loci. Thus, the *trpC* locus in *P. aeruginosa* strain 1, as is the case in *P. aeruginosa* strain 2 and in *P. putida*, is apparently unlinked by transduction to the other genes of tryptophan biosynthesis.

Table 7. Transductional analysis of tryptophan auxotrophs of *P. aeruginosa*

	Phage donor	Bacterial recipient	Recombinants[a]		Cotransfer frequency
			Selected on tryptophan	Growth on minimal medium	
Expt. A	*trpC2*	met 117	18	18	0
	trpC2	met 16	60	60	0
	trpC2	met 239	28	28	0
	trpC2	cys 171	44	11	0.75
	trpC2	cys 410	52	52	0
Expt. B	*trpA1*	cys 171	49	49	0
	trpB1	cys 171	4	4	0
	trpC2	cys 171	46	21	0.54
	trpC3	cys 171	5	3	0.40
	trpC1	cys 171	19	4	0.79
	trpC4	cys 171	3	0	1.00
	trpE1	cys 171	52	52	0
	trpF3	cys 171	49	49	0

[a] Transducing phage F116 was used as described in Methods. Recombinants were selected for independence of the recipient strain requirement. The initial selection of recombinants was in the presence of tryptophan. Scoring for cotransfer of the unselected marker (i.e., tryptophan auxotrophy) was achieved by replica placing to minimal salts-glucose medium. The recipient strains were previously described (Calhoun and Feary, 1969).

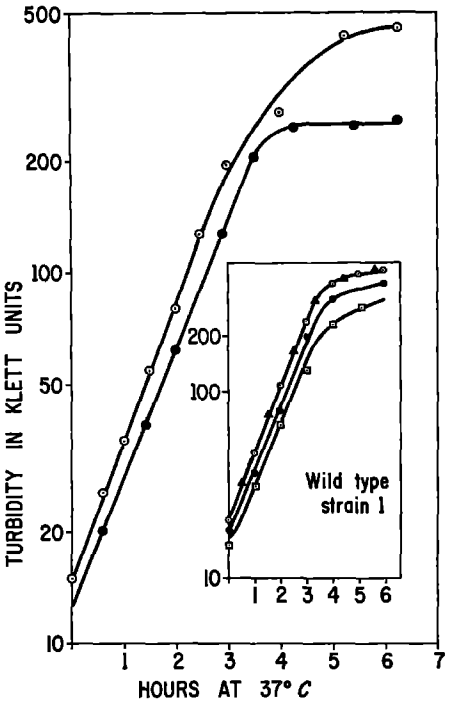

Fig. 1. Effect of indole on the growth rate of *P. aeruginosa*. A culture of late exponential phase cells of mutant *trpE1* or wild type (inset) in minimal salts-glucose medium was used to inoculate 10 ml cultures in 125 ml flasks equipped with sidearm Klett tubes. The separate flasks were inoculated in identical fashion to Klett values of 12. All cultures emerged from the lag phase of growth after one hour elapsed time (time zero on the abscissa scale). The cultures were shaken vigorously at 37° C. Symbols: ⊙ plus 100 μg L-tryptophan per ml; ● plus 50 μg indole per ml; ▲ unsupplemented minimal salts-glucose; and □ plus 100 μg indole per ml. The labels given for the ordinate and abscissa scales of the major figure also apply to those of the inset

Linkage of the *trpF* locus with a gene coding for an unidentified enzyme in the aromatic amino acid biosynthetic pathway has been reported in *P. putida* (Maurer and Crawford, 1971). Accordingly, phage propagated on mutant *trpF3* were used to transduce a number of aromatic amino acid auxotrophs of *P. aeruginosa* strain 1 isolated in this laboratory. Selection was on minimal salts-glucose medium supplemented with tryptophan, and transductants were subsequently tested for cotransfer of the *trpF* locus on unsupplemented minimal plates. Linkage of the *trpF* locus was indeed demonstrated with *aro* mutant BCM N5 (Calhoun *et al.*, 1972), but not with the other independently isolated aromatic amino acid auxotrophs.

Effect of Indole on the Growth of P. aeruginosa. Tryptophan auxotrophs of *P. putida* have been reported to lack ability to utilize indole from the growth medium (Crawford and Gunsalus, 1966). However, the concentration of indole tested was 5 μg per ml. Proctor and Crawford (personal communication) found that *P. putida* auxotrophs blocked early in the tryptophan pathway respond to concentrations of indole over 10 μg per ml. All of the appropriate classes of mu-

tants of *P. aeruginosa* (Table 1) display a growth response to indole (50 μg per ml) similar to such mutants of *E. coli* or of *Bacillus subtilis*. Fig. 1 illustrates the growth of wild type *P. aeruginosa* in the presence of indole as well as the growth response of one auxotrophic mutant. Indole may cause a slight degree of inhibition of growth. The effect is manifested as an increase in the duration of the lag phase of growth. The specific growth rate is not measurably altered in batch cultures. High concentrations of indole (100 μg per ml) may also depress the growth of *P. putida* (Proctor and Crawford, personal communication). The utilization of indole as a source of tryptophan appears to be quite adequate in *P. aeruginosa*. Numerous experiments consistently gave the results that the synthesis of enzymes [E] and [F] were unaffected by growth with exogeneous indole. Hence, IGP but not indole is the inducer molecule for enzymes [E] and [F].

Discussion

The General Pattern in Pseudomonads. The growth requirements, precursor accumulation, and enzymatic analysis of tryptophan auxotrophs indicate that the biochemical route of tryptophan synthesis in *P. aeruginosa* is similar to that described in other microorganisms, including *P. putida* (Calhoun and Jensen, 1972). No mutants were found to have the pleiotrophic effects that might implicate the existence of multi-functional protein complexes in *P. aeruginosa*.

Elevated synthesis of the tryptophan synthetase subunits cannot be achieved by starvation for tryptophan in mutants of *P. aeruginosa* lacking function in any of the first four cistrons of the tryptophan biosynthetic pathway. Dramatic elevations of both catalytic activities of tryptophan synthetase in a mutant blocked in the early common sequence of aromatic amino acid synthesis in the presence of exogeneous anthranilate provides evidence for a mechanism of induction by IGP. The negligible ability of indole, which is permeable to the cell membrane, to induce the synthesis of tryptophan synthetase under conditions in which anthranilate is effective undoubtedly indicates that IGP is the intracellular inducer molecule. The synthesis of enzyme [C] appears to be unregulated, while the synthesis of the [ABD] proteins is under repression control mediated by tryptophan. The occurrence of this unusual mixture of regulatory signals in both *P. putida* and *P. aeruginosa* provides further evidence for the validity of using regulatory patterns of some biochemical systems as criteria of evolutionary relationships (DeMoss, 1965; Jensen and Rebello, 1970). In this connection it is interesting that the genetic results confirm with strain 1 the conclusion made with strain 2 (Chakrabarty and Gunsalus, 1970; Holloway *et al.*, 1971) that the chromosomal arrangement of tryptophan-specific cistrons in *P. aeruginosa* resembles that of *P. putida*. Linkage of the *trpC* locus, but not of other tryptophan cistrons, to a cysteine (methionine) locus was found. The *trpF* locus is cotransducible with a gene involved with aromatic amino acid synthesis in both species. Further experiments will be required to determine the chromosomal positions of the other tryptophan genes in strain 1 of *P. aeruginosa*.

Relative Sensitivities of Repressible and Inducible Proteins to Regulatory Signals. A biochemical sequence in which an end product-repressible allosteric enzyme is followed by one or more substrate-induced enzymes provides some attractive

features. Such a control mechanism is responsive not only to end product levels, but also provides a modulating mechanism which depends on the availability of the initial and/or other substrates of the system. The direct repressive and allosteric control by tryptophan of early enzymes of the sequence should decrease the intracellular level of IGP, the inducer molecule for tryptophan synthetase. Since the synthesis of enzymes [A] through [D] are maximally repressed during growth in minimal salts-glucose medium, one can attribute the tryptophan-mediated decrease in specific activity of tryptophan synthetase solely to feedback inhibition exerted by tryptophan on the activity of anthranilate synthetase. Hence, during growth in minimal salts-glucose medium sufficient endogeneous tryptophan is formed to repress maximally the [ABD] enzymes and to partially feedback inhibit anthranilate synthetase. It follows that the degree of feedback inhibition which characterizes growth in minimal medium is sufficiently incomplete that enough IGP is formed to induce tryptophan synthetase ten-fold over the basal level.

Significance of High-Level Induction? It seems doubtful that the full potential of tryptophan synthetase to be induced by IGP is approached under most physiological conditions in wild type. The ten-fold differences seen in the data of Table 2 obviously represent a range of variation that is physiological. In both *P. aeruginosa* and *P. putida* highly elevated specific activities of tryptophan synthetase are only demonstrated under conditions that seemingly would not be frequent in nature: (i) in mutants lacking function for the α-subunit of tryptophan synthetase and accumulating IGP, (ii) in end product-starved mutants supplied with a precursor of IGP (e.g., anthranilate), and (iii) a class of regulatory mutants selected for ability to utilize indole (*P. putida*) or selected as *trpE* revertants (*P. aeruginosa*).

Another body of evidence casting doubt on the physiological importance of the ultra-induced levels of tryptophan synthetase that can be obtained is provided by characteristics of several classes of regulatory mutants. Mutant 4FT1 excretes tryptophan by virtue of a 15-fold elevation of the [ABD] enzymes. Tryptophan synthetase is not induced by endogeneous IGP in this constitutive mutant. Hence, elevation of the [ABD] enzymes is sufficient for tryptophan overproduction even though tryptophan synthetase is uninduced. On the other hand, constitutivity for tryptophan synthetase (as seen in revertants of *trpE1* and *trpE2*) is not sufficient to cause tryptophan excretion when the [ABD] enzymes are at repressed levels.

Significance of the Inducible Proteins. The following possibilities may warrant consideration. Perhaps the modern tryptophan synthetase of pseudomonads evolved from a catabolic protein(s). Inductive control may have been retained following certain modifications to integrate its function appropriately within the biosynthetic pathway. The great potential for inducibility may be an evolutionary remnant of the ancestral protein that is rarely expressed in the modern protein.

Perhaps the inducibility of tryptophan synthetase reflects a role in a detoxification mechanism. Phosphorylated compounds such as IGP are often toxic; the metabolism of IGP to tryptophan may have conferred selective advantage even in the presence of tryptophan. Such a mechanism could serve to prevent the potential toxicity of a compound like anthranilate which by-passes a site of allosteric regulation, being metabolized to phosphorylated intermediates such as phosphoribosylanthranilate, CDRP, and IGP.

The interesting possibility that repression control of the [ABD] enzyme group and induction control of the [EF] enzyme group are linked in mechanism cannot be excluded. It may be that the aporepressor molecule for the [ABD] group is a necessary entity in a positive control mechanism governing the synthesis of proteins [E] and [F]. Hence, alteration of repressor function in 4FT1 may pleiotrophically alter capability for induced enzyme synthesis. This would explain the following anomalous result. Mutant MC36 provided with phenylalanine, tyrosine and anthranilate displays induced levels of proteins [E] and [F]. Accordingly, one would expect mutant 4FT1 to show the same or higher levels of enzymes [E] and [F], perhaps even in the absence of exogenous anthranilate. Nevertheless, mutant 4FT1 grown in the presence of anthranilate, tyrosine and phenylalanine produces near-basal levels of the [E] and [F] proteins.

Constitutive Element of the Tryptophan System. A curious aspect of the control of tryptophan biosynthesis in pseudomonad species centers about the unregulated synthesis of N(5′-phosphoribosyl) anthranilate isomerase. The constitutive synthesis of this enzyme in the midst of contiguous enzymes which vary appreciably in specific activities is enigmatic. Although this question cannot be resolved here, one may consider the question of whether the enzyme level is "set" high or low with respect to the other enzymes of the pathway. The following observations seem to suggest that [C] is set low: (i) In minimal salts-glucose medium strain 1 excretes some anthranilate, but no detectable indole or tryptophan, (ii) CDRP (actually CDR) does not accumulate in *trpD* mutants, and (iii) mutant 4FT1 which synthesizes the [ABD] enzymes constitutively possesses near-basal levels of enzymes [E] and [F], a result consistent with the lack of enough IGP to induce tryptophan synthetase. The relative basal level of [C] may be even lower in *P. putida* since ABD constitutives comparable to 4FT1 excrete anthranilate but not tryptophan (Maurer and Crawford, 1971).

A priori one would expect anthranilate synthetase to be the limiting reaction of the tryptophan biosynthetic sequence under most conditions of growth. However, under conditions of starvation for tryptophan the isomerase activity may become the limiting reaction. This is consistent with the relatively high ratio of anthranilate to tryptophan overproduced by constitutive mutant 4FT1. The catalytic potential of [C] would tend to be maximized during tryptophan starvation. A derepression of enzyme [D] would tend by mass action to "pull" reaction [C] to completion while derepressed [A] and [B] proteins would tend to "push" it via an increased supply of subtrate.

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