JOURNAL OF BACTERIOLOGY, Oct. 1970, p. 254-263
Vol. 104, No. 1

Enzymes of the Tryptophan Pathway in *Acinetobacter calco-aceticus*

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Received for publication 27 March 1970

All enzymes of the tryptophan synthetic pathway were detectable in extracts from wild-type *Acinetobacter calco-aceticus*. The levels of these enzymes were determined in extracts from a number of auxotrophs grown under limiting tryptophan. In each case only anthranilate synthetase was found to be present in increased amounts, whereas the specific activities of the remaining enzymes remained unchanged and unaffected by the tryptophan concentration. Derepression of anthranilate synthetase was found to occur as the concentration of tryptophan became limiting. Anthranilate synthetase and phosphoribosyl transferase activities are both feedback-inhibited by tryptophan. Molecular weight determination carried out by gel filtration and zonal centrifugation in sucrose revealed that all the enzymes are less than 100,000, and no molecular aggregates of these enzymes were detected. The data indicate that tryptophan synthesis in *Acinetobacter* is regulated both by feedback inhibition of the first two enzymes of the pathway and by repression control of anthranilate synthetase.

An increasing number of reports have appeared in recent years concerning studies on the tryptophan biosynthetic enzymes from bacterial species outside the enteric group. In the latter, these enzymes are all derepressed under conditions of limiting tryptphan (3, 17, 19). However, the enzymes are not all synthesized coordinately (3). Variations in this pattern of regulation have been observed in *Chromobacter violaceum* (29) and *Pseudomonas putida* (6). The levels of the tryptophan enzymes in *C. violaceum* are unaffected by the tryptophan concentration (29). In *P. putida*, three enzymes, the first, second, and fourth, respond to changes in the concentration of tryptophan, whereas the level of the third enzyme remains unchanged and the last enzyme, tryptophan synthetase (EC 4.2.1.20), is induced by its substrate indoleglycerol phosphate (6). In all the cases, trptophan feedback inhibits the first enzyme of the pathway, anthranilate synthetase.

Analysis of extracts from these various organisms by zonal centrifugation in sucrose and gel filtration reveals the presence (or absence) of molecular aggregates of these enzymes which is characteristic for a group of bacteria. Hütter and DeMoss (18) demonstrated the usefulness of this procedure in studying phylogenetic relationships among the fungi. Whereas no such aggregates have been found in extracts prepared from *C. violaceum* (29) and *P. putida* (14), the same enzyme aggregates have been found in *Escherichia coli* (8, 9, 20) and *Salmonella typhimurium* (3). Hutchison and Belser (17) recently reported that the tryptophan enzymes from *Serratia marcescens* do not form aggregates which resemble those from *E. coli* and *S. typhimurium*. Since all three organisms are members of the enteric group, they suggested that there is considerable evolutionary divergence between other members of the enterics and *Serratia*.

This paper is concerned with a study of these enzymes in a gram-negative organism now termed *Acinetobacter* (4). This genus is composed of a collection of organisms having considerable biochemical diversity and which also have had a profusion of generic and specific names (4). We have selected as a representative strain one originally obtained from the American Type Culture Collection. One assumption was made: that tryptophan synthesis proceeds in this organism along the same pathway as that found in *E. coli* and *Neurospora crassa*. We will show that the concentration of tryptophan affects only the level of anthranilate synthetase and that the level of the other tryptophan synthetic enzymes remains unchanged. In addition, none of the tryptophan enzymes formed aggregates. This fact plus the unique pattern of repression of anthranilate synthetase found in this

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organism should prove useful in establishing phylogenetic relationships among other members of this diverse group of bacteria.

MATERIALS AND METHODS

Organism and cultural conditions. The organism used in this study was strain 15150 of the American Type Culture Collection. There it is listed as *Bacterium anitratum*; however, it has been designated as *Acinetobacter calco-aceticus* for these studies as suggested by the recent work of Bauman et al. (4).

Stock cultures of the parent and various mutants were maintained on Trypticase Soy Agar (BBL) slants at 4 C. They were routinely transferred at 6- to 8-week intervals.

Minimal medium was prepared by adding the following constituents to a final volume of 1 liter: sodium succinate, 5 g; sodium acetate, 1 g; $(NH_4)_2SO_4$, 1 g; L-glutamic acid, 1 g; 1 M Na_2HPO_4, 43 ml; Hutner's base (5), 20 ml. The *p*H of the medium was adjusted to 6.3 before autoclaving. Stock solutions of anthranilic acid, indole, and L-tryptophan were sterilized by filtration and added separately to sterile minimal medium for the growth of various mutants. Purified agar (Difco) was added at a concentration of 1.5% (w/v) to minimal and supplemented minimal medium for plates.

The tryptophan auxotrophs used in this study were obtained by nitrosoguanidine treatment of the wild-type strain, 15150.

Nitrosoguanidine mutagenesis. An overnight culture of cells grown in minimal medium supplemented with 0.05% acid-hydrolyzed casein was inoculated into the same medium and grown with shaking at 37 C to 2×10^8 to 4×10^8 cells per ml. A 5-ml sample was withdrawn and filtered through a sterile membrane filter (0.45 μm pore size) by use of a Sterifil apparatus (Millipore Corp.) Subsequent treatment and incubation of the cells was carried out by using this apparatus except where noted. The cells were washed either with 10 ml of TM buffer [0.05 M tris(hydroxymethyl)-aminomethane (Tris) + 0.05 M maleic acid, *p*H 6.0, + Hutner's base] or with 10 ml of 0.15 M sodium acetate, *p*H 5.0 (containing Hutner's base), both buffers at 37 C. The cells were resuspended in TM or acetate buffer and nitrosoguanidine (freshly prepared at 3 mg/ml) added at concentrations of 0.5 and 1.0 mg/ml, respectively. After a 30-min incubation period at 37 C without shaking, the cells were filtered and washed twice with 10-ml portions of the respective buffers. Five milliliters of minimal medium supplemented with 5 μg of tryptophan per ml was used to resuspend the cells, which were then transferred to 15 ml of the same medium in a flask. The cells were incubated for 18 to 22 hr, and a suitable dilution was plated onto agar plates of tryptophan-supplemented (5 μg/ml) minimal medium. In some cases, a penicillin selection step was included. Individual colonies were picked after 48 hr at 37 C to minimal agar and tryptophan-supplemented minimal agar. These were usually screened for a tryptophan requirement after 48 hr at 37 C.

Identification of intermediates. Mutant strains were grown for 22 to 24 hr at 37 C in minimal medium supplemented with 5 μg of L-tryptophan per ml. After centrifugation of the culture, 30 ml of the supernatant fluid was acidified with 0.60 ml of HCl and extracted twice with 10 ml of ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous Na_2SO_4, and concentrated to 2 ml with a gentle stream of air. Anthranilic acid was identified by its characteristic ultraviolet spectrum and by thin-layer chromatography on silica gel (Eastman Kodak No. 6061) with a solvent system (12) consisting of methanol-butanol-benzene-water (2:1:1:1). This same chromatographic system was also used to identify 1-(*O*-carboxyphenylamino)-1-deoxyribulose (CDR). The triphenyltetrazolium reagent (12) used to identify CDR on the chromatograms was also used to identify this compound qualitatively in the concentrated ethyl acetate extracts. Indole and indoleglycerol were determined by the procedures of Yanofsky (31, 32).

Cell-free enzyme extracts. Wild-type and mutant cells were grown in 250-ml volumes (per 1-liter flask) for 12 to 14 hr at 37 C in a gyratory water-bath shaker. The extracts used in the gel filtration and sucrose centrifugation experiments were prepared from mutants grown in minimal medium supplemented with 2.5 μg of L-tryptophan/ml.

Cells were harvested by centrifugation at 12,000 × *g*, washed once with 0.10 M Tris (*p*H 7.8), 0.01 M KCl, 0.001 M mercaptoethanol (TKM buffer), and resuspended in the same (usually 4 ml of buffer per 1 g wet weight of cells). This suspension was cooled in a stainless-steel or aluminum chamber held in an ice bath, and the cells were disrupted by ultrasonic vibration (Blackstone Ultrasonics, Inc.). This preparation was centrifuged for 45 min at 39,000 × *g* in a Sorvall RC2B centrifuge at 2 C. The clear supernatant fluid was the crude extract used in some of the experiments. A 30% solution of streptomycin sulfate was added dropwise (0.50 ml per 10 ml of crude extract) to the crude extract at 0 C. Fifteen minutes later, the extract was centrifuged for 20 min at 15,000 × *g*. The supernatant fluid was used for the zonal centrifugation experiments in sucrose and was fractionated with $(NH_4)_2SO_4$ for the gel filtration experiments.

The preliminary $(NH_4)_2SO_4$ fraction was made by the addition of 1.94 g of solid $(NH_4)_2SO_4$ per 10 ml of streptomycin-treated crude extract at 0 C. Fifteen minutes later, the preparation was centrifuged as above. The supernatant fluid was decanted, and to this was added 2.54 g of solid $(NH_4)_2SO_4$ per 10 ml. After centrifugation, the supernatant fluid was discarded, and the pellet was dissolved in TKM buffer. This fraction was used for gel filtration.

Enzyme assays. Unless stated otherwise, the activities of all enzymes were determined by incubating the reaction mixtures for 20 min at 37 C. One enzyme unit is defined as that amount of enzyme catalyzing the synthesis of 1 μmole of product or utilization of 1 μmole of substrate under the standard assay conditions stated. A Gilford recording spectrophotometer (model 2000) was used to determine absorbancy for all the assays except for indole determination in the tryptophan synthetase B assay which was done by

using a Klett-Summerson colorimeter. Specific activity is defined as enzyme units per milligram of protein. The procedure of Lowry et al. (23) was used to determine protein, with bovine serum albumin serving as a standard.

Anthranilate synthetase. The reaction mixture for anthranilate synthetase assay contained 100 μmoles of potassium phosphate (*p*H 7.3), 20 μmoles of L-glutamine, 10 μmoles of $MgCl_2$, 1 μmole of chorismate, and enzyme, and water to a final volume of 1.0 ml. After the 20-min incubation period, the reaction was terminated by the addition of 0.10 ml of 1 N HCl. The anthranilic acid synthesized was extracted by the addition of 1.5 ml of ethyl acetate. After thorough mixing and a brief centrifugation to separate the phases, the absorbancy of the anthranilic acid in the organic phase was measured at 336 nm, using a semi-micro absorption cell (10-mm path length). A molar extinction of 4,900 per cm (12) was used to calculate the amount of anthranilic acid formed in the reaction. Controls were included in every experiment and consisted of a complete reaction mixture minus chorismate. After the 20-min incubation period, chorismate was added, followed immediately by the HCl. Any absorbancy (336 nm) in the ethyl-acetate phase from the control tubes was subtracted from the complete reaction. Under these conditions this assay was linear with time for at least 30 min. This assay is not as sensitive as the fluorometric procedure of Ito and Crawford (19). Therefore, in most cases the least amount of anthranilate formed was 5 to 10 nmoles. It is difficult to determine accurately less than 2 nmoles of anthranilic acid formed by using this technique.

Phosphoribosyl transferase (PR-transferase). The volume of the reaction mixture for assay of PR-transferase was 1.0 ml and contained 0.2 μmole of anthranilate, 0.25 μmole of 5-phosphorylribose 1-pyrophosphate, 25 μmoles of Tris (*p*H 7.8), 2.0 μmoles of $MgSO_4$, and enzyme. The conditions of incubation, extraction of anthranilate remaining, and its subsequent measurement are the same as described for the assay of anthranilate synthetase. The amount of anthranilate remaining was subtracted from the control values to determine the amount utilized in the reaction. More than 8 nmoles of anthranilate was used in most cases.

Phosphoribosyl anthranilate isomerase (PRA-isomerase). The substrate *N*-(5′-phosphoribosyl)-anthranilic acid was prepared by the procedure of Creighton (7). The reaction mixture contained 70 μmoles of triethanolamine (*p*H 8.6), 0.53 μmole of PRA, water, and enzyme in a total volume of 1.5 ml. Incubation was carried out for 30 min at 37 C, and the amount of indole-glycerol phosphate (InGP) synthesized was determined on a 0.50-ml portion by the periodate method (27). This assay depends upon the conversion of the product of the isomerase catalyzed reaction, 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CDRP) to InGP by the indoleglycerol phosphate synthetase in the extract. A second assay was used to determine whether isomerase activity was present if no InGP was detected by the above assay. The assay used is a slight modification of that described by Creighton (7). The reaction contained 0.53 μmole of *N*-(5′-phosphoribosyl)-anthranilic acid, 300 μmoles of triethanolamine (*p*H 8.6), 300 μg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, 7.5 μg of phenazine methosulfate, 375 μg of gelatin, water, and enzyme to a total volume of 3.0 ml. Reduction of the tetrazolium dye was followed at 520 nm. Isomerase activity was indicated only as being present (+) or absent (−) when this assay was used.

Indoleglycerol phosphate synthetase (InGPS). The periodate method of Smith and Yanofsky (12) was used to determine the InGP synthesized. The reaction mixture contained 1 μmole of CDRP, 50 μmoles of Tris (*p*H 7.8), water, and enzyme to a final volume of 0.50 ml.

Tryptophan synthetase (EC 4.2.1.20). The tryptophan synthetase (β subunit) assay of Smith and Yanofsky (27) was used.

Alkaline phosphatase (EC 3.1.3.1). *E. coli* alkaline phosphatase was assayed by using the procedure of Garen and Levinthal (15). The rate of appearance of *p*-nitrophenol was determined at 410 nm. One enzyme unit catalyzed the formation of 1 μmole of *p*-nitrophenol per min. The enzyme stock used in the sucrose gradient experiments had a specific activity of 2.9.

Horseradish peroxidase. The assay used for horseradish peroxidase was devised by the personnel at Worthington Biochemical Corp., Freehold, N.J. The reaction contained 5.3 μmoles of H_2O_2, 0.50 mg of *O*-dianisidine, 30 μmoles of potassium phosphate (*p*H 6.0), water, and enzyme to a total volume of 3.0 ml. The rate of H_2O_2 decomposition was followed by the oxidation of *O*-dianisidine at 460 nm. One enzyme unit catalyzes the disappearance of 1 μmole of H_2O_2 per min.

Gel filtration. All experiments for gel filtration were carried out at 25 C in a Sephadex G-100 column (2.5 by 42 cm) equilibrated with TKM buffer. Elution was achieved in an ascending manner (TKM buffer) with flow rates (27 to 34 ml/hr) controlled by a peristaltic pump (Harvard Apparatus). Samples of the ammonium sulfate fractions varied from 2 to 4 ml and contained 20 to 108 mg of protein. Calibration of this column was carried out in six separate experiments by using a mixture of proteins of known molecular weight. Blue dextran 2000 was used to determine the void volume of the column. Elution of the blue dextran and marker proteins was monitored at 280 nm by use of an Isco (Instrumentation Specialties Co., Inc.) ultraviolet analyzer (model UA2). The procedure of Whitaker (30) and Andrews (1) was used in analysis by a semilog plot of molecular weight of the marker protein versus the ratio of the elution volume to the void volume.

Sucrose gradient centrifugation. Linear 5 to 20% sucrose (w/v) gradients were prepared in TKM buffer. Each gradient contained 4.5 ml of sucrose plus 0.50 ml of streptomycin sulfate-treated crude extract from either trp-3 or wild type (15150) cells. When peroxidase and alkaline phosphatase were included, 53 (50 μg) and 5.8 enzyme units (200 μg), respectively, were added to

the extracts to 0.50 ml. Centrifugation was carried out at 50,000 rev/min for 16 hr at 4 C with a Spinco L2-65B centrifuge (SW-65 rotor).

Fractionation of each gradient was achieved by infiltrating a 50% sucrose solution into the bottom of the tube with an Isco density gradient fractionator and collecting each fraction (seven drops) from the top.

Peroxidase and alkaline phosphatase activities were determined on a diluted (26×) sample of each fraction; otherwise the entire contents of alternate fractions were used to determine the activities of the enzymes from each gradient. Excess periodate (250 μmoles) was added for the oxidation of indoleglycerol phosphate (PRA isomerase and InGPS activities) to compensate for the sucrose present in the assays. The activities not determined in the gradients containing extracts prepared from 15150 and trp-3 cells were anthranilate synthetase and tryptophan synthetase (β subunit), respectively; other activities were determined in both.

Chemicals. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. Chorismic acid was isolated by the procedure of Gibson (16). The 5-phosphorylribose 1-pyrophosphate was obtained from P. L. Biochemicals, Inc., Milwaukee, Wis. Ribose-5-phosphate was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. We prepared CDRP chemically by using the procedure of Smith and Yanofsky (28). Horseradish peroxidase and alkaline phosphatase (*E. coli*) were purchased from Worthington Biochemical Corp., Freehold, N.J. Blue dextran 2000 and Sephadex G-100 were purchased from Pharmacia, Uppsala, Sweden. Bovine serum albumin, ovalbumin, chymotrypsinogen A, sperm whale myglobin, and horse heart cytochrome *c* were obtained from Mann Research Laboratories, Inc., New York, N.Y. Other chemicals used were of reagent grade.

RESULTS

Nature of the mutant classes. The properties of seven *Acinetobacter* tryptophan auxotrophs are summarized in Table 1. The most readily obtained class of mutants is that defective in the tryptophan synthetase reaction, and the most common defects in this class are those which have lost tryptophan synthetase A activity. The level of activity for the A reaction is low in wild-type cells and in all the tryptophan auxotrophs so far examined. Therefore, it was assumed that this was the site of the defect in those mutants accumulating CDR and indoleglycerol, if tryptophan synthetase B activity and the other enzymes of the tryptophan pathway were detected. This class of mutants resembles trp-3 with respect to the products accumulated and by their growth response to tryptophan. However, no tryptophan synthetase B activity could be detected in trp-3. Limiting concentrations of tryptophan in minimal medium affected only the total cell yield, and no effect on the growth rate could be seen relative to similarly grown wild-type or other mutant strains. This group of mutants differs in these respects from the tryptophanyl transfer ribonucleic acid synthetase mutants of *E. coli* recently described by Doolittle and Yanofsky (11).

TABLE 1. *Characteristics of Acinetobacter tryptophan auxotrophs*

Strain	Presumed defect[a]	Accumulation products[b]	Growth response[c]
26	AS	None	AA, Ind, Trp
27	InGPS	AA	Ind, Trp
3	TS-β	CDR, InG	Trp
10, 13, 15, 16	TS-α	CDR, InG	Trp

[a] Refer to Fig. 1 for enzyme notations. AS, Anthranilate synthetase; InGPS, indoleglycerol phosphate synthetase; TS-β, tryptophan synthetase (β subunit); TS-α, tryptophan synthetase (α subunit).

[b] AA, anthranilic acid; CDR, 1-(*O*-carboxyphenylamino)-1-deoxyribulose; InG, indoleglycerol.

[c] Determined on minimal agar containing 0.05% acid-hydrolyzed casein plus supplements: anthranilic acid (AA), 20 μg/ml; and tryptophan (Trp), 5 μg/ml.

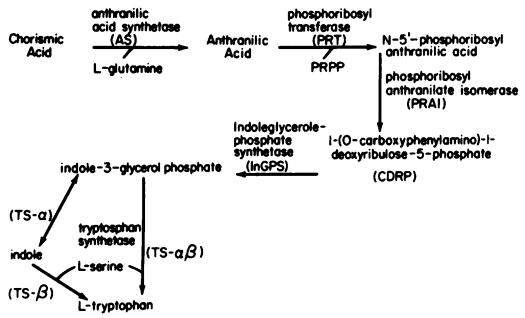

FIG. 1. *Tryptophan biosynthetic pathway found in the bacteria and fungi. TS-α and TS-β refer to the subunits of tryptophan synthetase and catalyze the A and B reactions, respectively.*

Extracts from trp-27 are inactive in the usual indoleglycerol-phosphate synthetase assay (27). However, they are capable of reducing a tetrazolium dye by using phosphoribosyl anthranilic acid as a substrate, thus suggesting PRA-isomerase activity (7). It is on the basis of these two assays that the block is presumed to be located at the synthesis of indoleglycerolphosphate from CDRP.

Strain 26 had no detectable anthranilate synthetase activity. The lack of any intermediates accumulating in the culture fluid and the growth response of this mutant to the various supplemented minimal media are also consistent with a lesion being located in the anthranilate synthetase gene.

None of the mutants isolated to date has been shown to accumulate indole in the culture fluids. In addition, the usual qualitative tests for the presence of CDR (12) and occasionally for indoleglycerol (32) in the culture fluids from many of these mutants have been weakly positive. Unequivocal, strong color reactions were seen when the tests were made on the ethyl acetate phase after extraction of the culture fluid.

Levels of enzyme activity in tryptophan auxotrophs. The effect on the enzyme levels in extracts from cells grown in the presence of low and high concentrations of tryptophan is summarized in Table 2. The specific activity of each enzyme from wild-type cells grown in the presence of 50 μg of tryptophan/ml was normalized to 1.0 for convenience. Anthranilate synthetase was the only enzyme having elevated levels of activity in extracts from mutant cells grown under derepressing conditions. The specific activity of most of the mutants tested, including some whose activities are not listed, had increased 3- to 10-fold. It was established by thin-layer chromatography and by the ultraviolet absorption spectrum that the ethyl acetate-extractable material from this enzymatic reaction was in fact anthranilic acid. The levels of the other enzymes of the pathway were not greatly affected by the tryptophan concentration. A very slight increase in the levels of PR-transferase, PRA-isomerase, and indoleglycerol-phosphate synthetase was seen in extracts from wild-type cells. Both indole-3-propionic acid and indole-3-acrylic acid were reported to cause derepression of the tryptophan operon in *E. coli* (2, 11). These compounds did not seem to derepress these enzymes in the *Acinetobacter*.

Derepression of anthranilate synthetase. Since anthranilate synthetase appeared to be the only enzyme repressed by tryptophan, experiments were carried out to illustrate its derepression and to establish whether the levels of the other enzymes remained constant with time. By using trp-3, the level of anthranilate synthetase began to increase as tryptophan became limiting (Fig. 2). It is clear also that the level of activities of the remaining enzymes in the pathway essentially remained unchanged and were not affected by the decreasing concentration of tryptophan during the course of the experiment. The activity of indole glycerolphosphate synthetase also remained constant but was not included in the figure.

Gel filtration and density gradient experiments. Extracts from mutant and wild-type cells were examined by gel filtration and centrifugation in sucrose to determine whether aggregation of the tryptophan enzymes occurs in these bacteria. A

TABLE 2. *Enzyme activities of Acinetobacter tryptophan auxotrophs relative to wild-type cell (15150)*

Strain	Tryptophan addition	Relative specific activities[a]				
		AS (0.0038)[b]	PRT (0.031)	PRAI (0.082)	InGPS (0.101)	TS-β (0.114)
15150	Excess limiting[c]	1.0	1.0	1.0	1.0	1.0
		2.24	1.25	1.54	1.4	0.80
26	Excess limiting	—[d]	0.78	1.34	1.12	0.82
		—	0.92	0.60	0.57	0.59
27	Excess limiting	—	1.58	+[e]	—	1.13
		10.3	1.98	+	—	1.15
3	Excess limiting	3.05	1.09	0.56	0.56	—
		22.90	1.10	0.92	0.92	—
10	Excess limiting	0.37	0.95	1.74	NT[f]	0.93
		2.96	1.10	1.67	NT	1.10
13	Excess limiting	0.92	0.91	0.44	0.77	0.75
		7.30	1.30	0.45	0.50	0.40
16	Excess limiting	3.16	1.09	0.61	0.60	1.27
		10.66	1.40	0.77	0.59	2.23

[a] Abbreviations: AS, anthranilate synthetase; PRT, phosphoribosyl transferase; PRAI, phosphoribosyl anthranilate isomerase; InGPS, indoleglycerol phosphate synthetase; TS-β, tryptophan synthetase (β subunit).

[b] Average specific activity (four experiments) of enzymes from strain 15150 grown for 20 hr at 37 C in minimum medium supplemented with 50 μg of L-tryptophan/ml.

[c] Minimal medium supplemented with 2.5 and 50 μg of tryptophan/ml for limiting and excess addition, respectively.

[d] Dash indicates no detectable activity.

[e] Qualitative determination of isomerase activity.

[f] Not tested.

typical gel filtration pattern is seen in Fig. 3. A similar elution profile was seen when we used an ammonium sulfate fraction made from trp-3 and wild-type cells. In the latter case, anthranilate synthetase was not assayed, and tryptophan synthetase activity was not determined in any of these experiments. The activities have not been resolved completely, however; little or no PR-transferase activity was seen in the region of maximal anthranilate synthetase activity. Although the PRA-isomerase and indoleglycerolphosphate synthetase activities nearly coincide, the evidence suggests that these two enzymes are not aggregated. Such an aggregate would elute prior to the PR-transferase enzyme, and no activity was seen in this region. No additional activity was recovered beyond fraction 65. Recently, we separated these two activities during the purification of indoleglycerolphosphate synthetase. This further suggests that the two activities are associated with

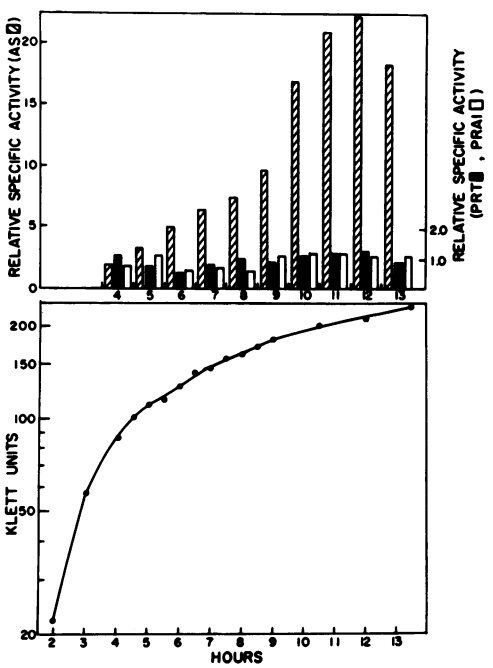

FIG. 2. *Derepression kinetics of anthranilic acid synthetase in strain trp-3 grown in minimal medium supplemented with 5 μg of L-tryptophan per ml. Cultures of 250 ml, each receiving a 5% (v/v) inoculum, were removed at the indicated time intervals and cooled quickly in ice. Extracts were prepared and assayed as previously described. Cell turbidity was determined by using a Klett-Summerson colorimeter (no. 54 filter). The specific activity of each enzyme is relative to wild-type cells grown in the presence of 50 μg L-tryptophan per ml.*

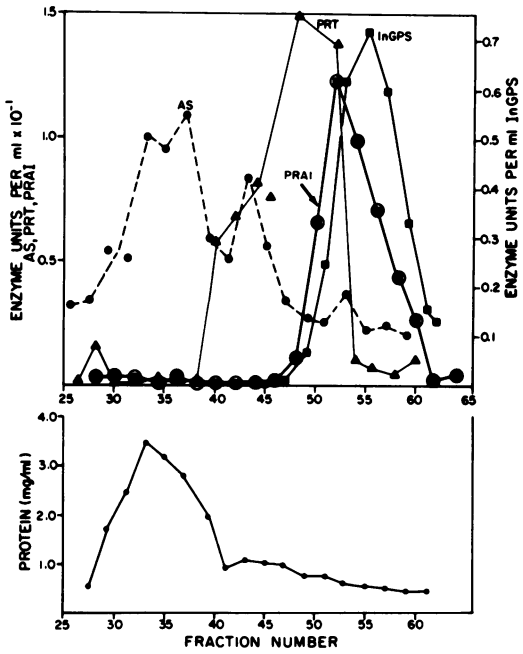

FIG. 3. *Gel filtration of an ammonium sulfate fraction from trp-16. The 4-ml sample introduced into the buffer stream contained 108 mg of protein. Elution was carried out in an ascending mode at a flow rate of 28 ml per hr. Each fraction contains 2.0 ml.*

different polypeptides, unlike the enzyme from *E. coli* (9).

A molecular weight range for the enzymes based upon three gel filtration experiments is seen in Fig. 4. The approximate molecular weights of the enzymes based on these experiments are: anthranilate synthetase, 80,000; phosphoribosyl transferase, 42,500; phosphoribosylanthranilate isomerase, 28,000; and indole glycerolphosphate synthetase, 23,000.

Zonal centrifugation in sucrose of streptomycin-treated crude extracts from mutant and wild-type cells gave essentially similar results (Fig. 5). PRA-isomerase and indoleglycerolphosphate synthetase activities were not resolved under these conditions. From these experiments their molecular weights were calculated (24) to be approximately 26,500, with *E. coli* alkaline phosphatase and horseradish peroxidase used as internal markers of known weight. PR-transferase and anthranilate synthetase activities are nearly completely resolved. By using this procedure, their molecular weights were calculated to be 51,000 and 93,000, respectively.

Anthranilate synthetase is the most labile of the enzymes, with recoveries from the G-100 column usually being less than 30%. Its molecular

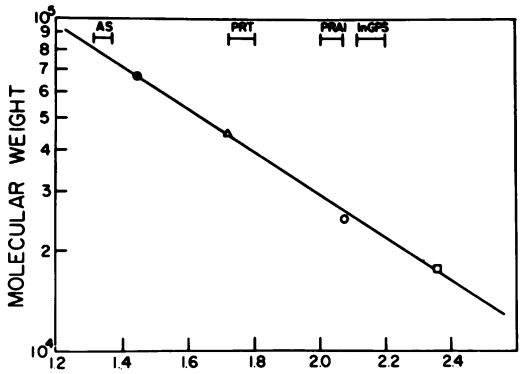

FIG. 4. *Elution of tryptophan enzymes from G-100 Sephadex relative to proteins of known molecular weight. The spread of the ratio of the elution volume (V_e) of the tryptophan enzymes to the void volume (V_0) was based upon three experiments. The data are plotted according to Whitaker (30) and Andrews (1). The marker proteins are bovine serum albumin (●), ovalbumin (△), chymotrypsinogen A (○), and myoglobin (□).*

weight is close to that of the corresponding enzymes from *C. violaceum* (29) and *P. putida* (14). This is considerably smaller than the component I-II complex of *E. coli* (21). Except for tryptophan synthetase (B reaction), the molecular weights of the other enzymes of the pathway are very close to those of the corresponding enzymes from *C. violaceum*.

The zonal centrifugation experiments indicate that the tryptophan synthetase activity (B reaction) was associated with a protein of molecular weight 58,000. This is considerably smaller than either the native tryptophan synthetase enzymes or the β subunits from *E. coli* (8), *C. violaceum* (29), or *P. putida* (14). Since the mutants presently available do not derepress (except for anthranilate synthetase), it has not been possible to attain sufficient activities of the A reaction for additional physical studies of this enzyme. We are presently attempting to isolate mutants that do produce greater quantities of tryptophan synthetase for such experiments.

Aromatic amino acid effects on anthranilate synthetase and phosphoribosyl transferase. Anthranilate synthetases from several organisms have been shown to be feedback inhibited in various degrees by tryptophan (5, 13, 21, 25, 29). Inhibition of PR-transferase activity by tryptophan has also been noted (3, 13, 21). Both of these reactions were examined by using crude extracts of trp-3 to determine whether the enzymes catalyzing these reactions in *Acinetobacter* behaved in a similar fashion. L-Tryptophan (0.2 mM) inhibits the anthranilate synthetase reaction approximately 70% (Table 3). This suggests that this enzyme is less sensitive to tryptophan inhibition than the anthranilate synthetase complex (Co I-Co II) from *E. coli* which can be inhibited 50% by 0.02 mM tryptophan when chorismate is 0.10 mM. However, recent experiments using a more sensitive assay indicate that 0.02 mM tryptophan will inhibit the reaction approximately 70% and increasing tryptophan concentration will give greater inhibition. Complete inhibition occurred at 0.5 mM tryptophan (*unpublished results*). Tyrosine and phenylalanine alone had either slightly stimulated trp-3 extracts or had little effect. In combination, however, they always stimulated this enzyme reaction. Maximal stimulation, up to 68%, was seen with a few extracts. Either of these

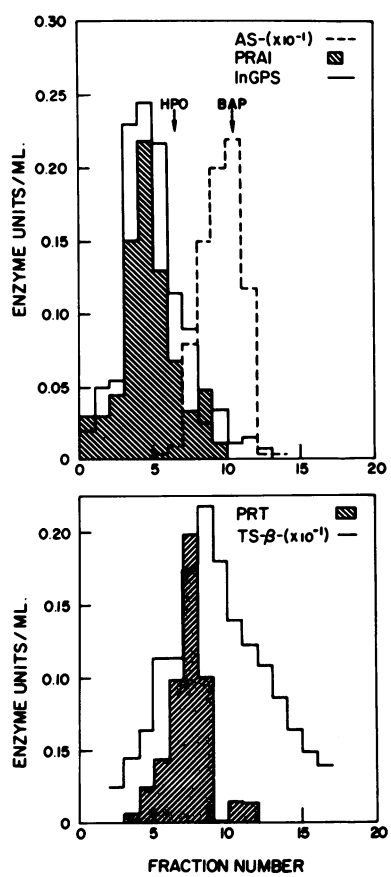

FIG. 5. *Zonal centrifugation of streptomycin-treated extracts from trp-3 and wild-type cells in sucrose (5 to 20%, w/v). Horseradish peroxidase (HPO) and bacterial alkaline phosphatase (BAP) activities were determined upon diluting 0.02 ml of each fraction with 0.50 ml of TKM buffer.*

two amino acids in combination with tryptophan had little effect on the activity of anthranilate synthetase. However, essentially complete inhibition of enzyme activity occurs when all three aromatic amino acids are present. It is possible that the apparent inhibition of anthranilic acid synthesis by tryptophan was an indirect effect caused by chorismate depletion as a result of chorismate mutase stimulation. However, under the conditions of the anthranilate synthetase assay, tryptophan had little effect on chorismate mutase. Tyrosine does slightly inhibit this enzyme under these same conditions. That the apparent stimulation of anthranilate synthetase by phenylalanine and tyrosine as measured by this assay was a result of anthranilic acid synthesis was confirmed by ethyl acetate extraction of the product at the end of the assay. The ultraviolet spectrum was characteristic of anthranilic acid. Similarly, no anthranilic acid could be extracted when inhibiting concentrations of tryptophan were used. These results do not rule out completely that at least partial inhibition of chorismate mutase by these amino acids may account for the apparent stimulation of anthranilate synthetase. Since crude extracts were used for these assays, it is possible that the degree of stimulation observed with these amino acids and the extent of tryptophan inhibition may differ markedly when compared with measurements made when using a purified enzyme. Purification of anthranilate synthetase is being undertaken for such studies.

The effect of the aromatic amino acids on phosphoribosyl transferase activity is seen in Table 4. Tryptophan, at a 0.5 mM, causes approximately 80% inhibition, and complete inhibition occurs at 2.0 mM tryptophan. The presence of either phenylalanine or tyrosine partially alleviates the inhibition by tryptophan. When all three amino acids are present, approximately 40% activity still remains. Tyrosine and phenylalanine alone or together do not affect this reaction. Whereas the anthranilate synthetase reaction in this organism may be less sensitive to tryptophan concentrations than the *E. coli* enzyme, the PR-transferase can be inhibited completely in the *Acinetobacter* by 2 mM tryptophan. PR-transferase activity (CO I-Co II complex) in *E. coli* and *S. typhimurium* is inhibited approximately 60% by 0.01 mM tryptophan, with little additional inhibition occurring even when the tryptophan concentration is raised to 1.0 mM (3, 21). In addition, in *E. coli* the CO-II protein alone will catalyze this reaction and is unaffected by tryptophan when not complexed with the CO-I protein (21).

TABLE 3. *Effects of aromatic amino acids on anthranilate synthetase activity*[a]

Additions	Enzyme units/ml	Activity remaining
		%
None	0.720	100
0.50 mM L-Phenylalanine	0.590	82
0.125 mM L-Tyrosine	0.567	79
0.20 mM L-Tryptophan	0.224	31
0.5 mM Phenylalanine + 0.125 mM tyrosine	0.768	107
0.2 mM Tryptophan + 0.125 mM tyrosine	0.172	24
0.2 mM Tryptophan + 0.5 mM phenylalanine	0.104	14
0.2 mM Tryptophan + 0.5 mM phenylalanine + 0.125 mM tyrosine	0.006	<1

[a] Each assay contained 0.5 mg of protein of a crude extract from trp-3 cells. The concentration of chorismate was 0.5 mM.

TABLE 4. *Effects of aromatic amino acids on phosphoribosyl transferase activity*[a]

Additions	Enzyme units/ml	Activity remaining
		%
None	0.353	100
0.50 mM Tryptophan	0.078	22
2.0 mM Tryptophan	<0.002	<1
1.0 mM Phenylalanine + 0.25 mM tyrosine	0.363	103
1.0 mM Phenylalanine + 1.0 mM tryptophan	0.178	51
0.25 mM Tyrosine + 1.0 mM tryptophan	0.100	28
0.25 mM Tyrosine + 1.0 mM tryptophan + 1.0 mM phenylalanine	0.140	40

[a] Each assay contained 0.95 mg of protein of a crude extract from trp-3 cells.

DISCUSSION

The unique feature of repression control of the tryptophan synthetic enzymes in *Acinetobacter* is that only the first enzyme of the pathway, anthranilate synthetase, is repressed by tryptophan. The specific activities of the remaining enzymes in the pathway remained essentially unchanged whether the cells were grown in limiting or excess tryptophan. This organism closely resembles *C. violaceum* in this respect, except that not even the level of anthranilate synthetase is affected by the concentration of tryptophan in the latter (29). Two other features in common are that the enzymes do not appear to form molecular aggregates and their molecular weights are nearly the same except for the tryptophan synthetase (β) protein. The fact that the remaining enzymes of the pathway are not derepressed when tryptophan becomes limiting might mean that sufficient quantities of these enzymes are normally synthesized to meet the needs of the cell. This resembles the situation found for regulatory mutants of *Bacillus subtilis* which are able to synthesize tryptophan,

phenylalanine, and tyrosine in excess without increasing the level of 3-deoxy-D-arabino-heptulosonic acid phosphate synthetase (26), the first common enzyme of the aromatic acid pathway Tryptophan feedback inhibits *Acinetobacter* anthranilate synthetase; however, the concentration of tryptophan needed to achieve significant inhibition is considerably greater than that reported for the same enzyme from other organisms (3, 19, 21, 29). The presence of all three aromatic amino acids resulted in essentially complete inhibition. There is no evidence that this mechanism of anthranilate synthetase inhibition plays any role in regulating tryptophan synthesis in vivo. It is difficult to see how such a mechanism would benefit the cell. The rate of tryptophan synthesis is also likely to be affected by the sensitivity of the PR-transferase reaction to tryptophan. The data presented showed that this enzyme reaction was more sensitive to inhibition by tryptophan than was the anthranilate synthetase. This might suggest that PR-transferase is the more important feedback-sensitive enzyme in regulating tryptophan synthesis; however, this situation may not prevail in vivo. The chorismate concentration in vivo is probably a great deal less than 0.5 mM, the concentration used in the assay. Since anthranilate synthetase has been shown to be competitively inhibited by tryptophan with respect to chorismate (21, 29), greater degrees of inhibition by lower tryptophan concentrations likely occur in vivo.

The tryptophan synthetase reaction has been shown to be catalyzed by an enzyme complex in several bacteria. In two cases the molecular weights of the B-proteins of the enzymes have been of the order of 80,000 to 90,000 daltons (8, 14). The molecular weight of the enzyme from *C. violaceum* is approximately 84,000, which suggests that the B-protein is much smaller than those from the *E. coli* and *P. putida* enzymes. If a similar complex exists for the *Acinetobacter* tryptophan synthetase, the relatively low molecular weight found (58,000) for the protein catalyzing the B reaction would represent the smallest B-protein so far reported. However, if this activity is due to a β-subunit, the B-protein (and β-subunits) would be larger than those of the *E. coli* tryptophan synthetase. The fact that this enzyme does not derepress has made it impossible reliably to detect α-subunit activity from gradients or columns. However, this enzyme warrants further investigation.

Studies on the tryptophan synthetic enzymes in fungi by Hütter and DeMoss (18) have shown that similar patterns of enzyme association reflect similar genetic background. These authors also suggest that organisms exhibiting the same pattern of organization of their tryptophan enzymes have evolved from one another. The latter observation is also supported by phylogenetic characteristics. This method of verifying phylogenetic relationships may prove to be a useful technique among the bacteria; it is consistent with the known close relationship of *E. coli* and *S. typhimurium*.

The nutritional studies recently conducted by Baumann et al. (4) on the oxidase-negative *Moraxella* group of bacteria has led to the formation of two taxonomic groups (A and B), each having three and four subgroups, respectively. The organism used in these experiments would represent subgroup A1 of Baumann et al. Similar studies with other strains in this and the other subgroups would further aid in establishing genetic relationships in this bacterial group.

There is as yet no genetic mapping data available in any of these organisms. However, recent reports of transformation by Juni and Janik (22) in these bacteria raise hopes that knowledge of the organization of the tryptophan structural genes in these bacteria will become available. We are presently attempting these experiments.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-07126 from the National Institute of Allergy and Infectious Diseases.

We thank R. H. Bauerle for *Aerobacter aerogenes* strain 62-1 used for chorismic acid production.

We also acknowledge the capable assistance of Faith M. Anderson in conducting many of the experiments.

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