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Anthranilate Synthase/Anthranilate 5-Phosphoribosyl 1-Pyrophosphate Phosphoribosyltransferase from *Aerobacter aerogenes*

By A. F. EGAN* and F. GIBSON
Department of Biochemistry, John Curtin School of Medical Research, Institute of Advanced Studies, Australian National University, Canberra, A.C.T. 2601, Australia, and Microbiology School, University of Melbourne, Parkville, Vic. 3052, Australia

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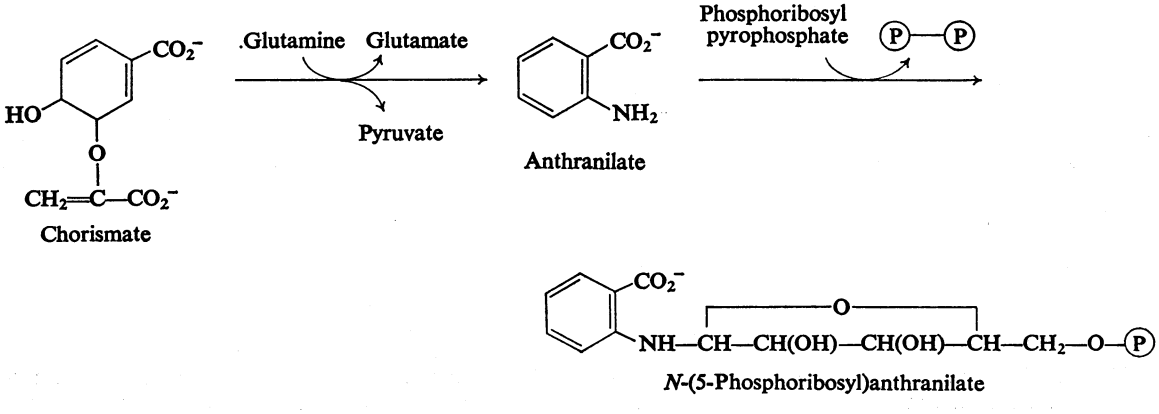
1. Anthranilate synthase and phosphoribosyltransferase from *Aerobacter aerogenes* purify simultaneously and sediment together on sucrose gradients, showing that they occur as an enzyme aggregate. Both activities of the intact aggregate are subject to inhibition by tryptophan. 2. By using appropriate auxotrophic mutants it was shown that an intact active enzyme aggregate is formed when the components come from separate mutant strains. An intact active aggregate can also be formed when one component is from *Escherichia coli* and the other from *A. aerogenes*. 3. Phosphoribosyltransferase of *A. aerogenes* is active when not in an aggregate with anthranilate synthase, but is not subject to tryptophan inhibition, indicating that the inhibitor site is on the anthranilate synthase component. 4. Anthranilate synthase can be active and sensitive to tryptophan inhibition when complexed with an inactive phosphoribosyltransferase. 5. Kinetic studies on the anthranilate synthase activity show that tryptophan is a competitive inhibitor with respect to chorismate and a non-competitive inhibitor with respect to either glutamine or NH_4^+ ions. This is consistent with a sequential mechanism of the ordered type in which chorismate is the first reactant.

The first two specific steps in the biosynthesis of tryptophan in *Escherichia coli* and *Salmonella typhimurium*, namely the conversion of chorismate into *N*-(5-phosphoribosyl)anthranilate (Scheme 1), are carried out by an enzyme aggregate consisting of two components (Ito & Yanofsky, 1966, 1969; Ito *et al.*, 1969; Bauerle & Margolin, 1966). Component 1 (anthranilate synthase) converts chorismate into anthranilate with NH_4^+ ions as nitrogen source. Component 2 has anthranilate 5-phosphoribosyl 1-pyrophosphate phosphoribosyltransferase activity. The aggregate of components 1 and 2 carries out the two reactions with glutamine as nitrogen source.

The present paper describes the partial purification of the anthranilate synthase/phosphoribosyltransferase complex from *Aerobacter aerogenes*, some kinetic studies on the anthranilate synthase activity

* Present address: Commonwealth Scientific and Industrial Research Organization, Division of Food Research, Cannon Hill, Queensland 4170, Australia.



Scheme 1. *Reactions in the conversion of chorismate into N-(5-phosphoribosyl)anthranilate*

and its feedback inhibition by tryptophan, and on the interactions between components 1 and 2 from tryptophan auxotrophs of *A. aerogenes* and *E. coli*. A preliminary report of part of this work has been published (Egan & Gibson, 1966).

Experimental

Chemicals

Reagent-grade 'Selectacel' DEAE-cellulose, obtained from Brown Co., Berlin, N.H., U.S.A., was prepared and regenerated by the procedures outlined by Peterson & Sober (1962). Sephadex G-200 was obtained from Pharmacia, Uppsala, Sweden. Acrylamide and *NN'*-methylenebisacrylamide were kindly provided by American Cyanamid Company, Melbourne, Vic., Australia, and were not further purified. Chorismic acid was prepared as described previously (Gibson, 1968) and was 94–98% pure. We are grateful to Dr. J. M. Edwards of the School of Chemistry, University of Melbourne, Melbourne, Vic., Australia, for the provision of some of the chorismic acid used in this work. Yeast alcohol dehydrogenase and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Inorganic chemicals were analytical-reagent grade and were not further purified. Amino acids were obtained commercially and not further purified.

Organisms

All auxotrophs of *A. aerogenes* were derived from strain NCW, a non-capsulated wild-type strain. Strains NC3, T21 and N5-36 are tryptophan auxotrophs of *A. aerogenes*. The first two are unable to convert chorismate into anthranilate and strain N5-36 is unable to convert anthranilate into *N*-(5-phosphoribosyl)anthranilate. Strain 62-1 is a multiply blocked aromatic auxotroph of *A. aerogenes* which has been previously described (Gibson & Gibson, 1964).

Strain C-47 is a tryptophan auxotroph of *E. coli* K-12 unable to convert indole glycerol phosphate into tryptophan. Strains E5972 and D9778 are tryptophan auxotrophs of *E. coli* K-12 with nonsense mutations in anthranilate synthase and the phosphoribosyltransferase, respectively (Ito & Yanofsky, 1966). We are grateful to Dr. C. Yanofsky, Dept. of Biological Sciences, Stanford University, Stanford, Calif., U.S.A., for provision of these strains.

Organisms were maintained by serial subculture at monthly intervals and kept on nutrient agar slopes.

Growth of cells and preparation of cell-free extracts

The minimal medium used was the glucose–citrate–mineral salts mixture described by Vogel & Bonner (1956) as medium E, supplemented with tyrosine and phenylalanine (each 0.2mM) and indole (2μg/ml). The concentration of indole used limited growth and allowed derepression of the enzymes of tryptophan biosynthesis. Glucose was autoclaved separately and added to the medium to give a final concentration of 0.5%.

The inoculum was about 10^9 cells from a 6h-shaken nutrient-broth culture per litre of medium. Cells were grown at 37°C as 1-litre batch cultures in 2-litre flasks and shaken on a New Brunswick Gyrotary shaker until 2–3h after growth had stopped. The cultures were centrifuged at 10000*g* for 15min, the cells resuspended and washed once with 0.9% NaCl solution and then suspended in potassium phosphate buffer, pH7.1 (0.1M), containing EDTA (0.5mM) and 2-mercaptoethanol (0.1mM) at 1g wet wt./4ml of buffer and broken in an Aminco French pressure cell at 1378×10^5 N/m^2 (20000lbf/in^2). Cell debris was removed by centrifugation at 30000*g* for 20min. The above operations were carried out at 4°C and the cell-free extract was stored at −15°C.

Enzyme assays

(*a*) *Anthranilate synthase.* Anthranilate formation was measured spectrofluorimetrically by two methods:

(i) Ethyl acetate extraction method. Chorismate (0.5μmol), $MgCl_2$ (10μmol), glutamine (5μmol), tris–HCl buffer, pH7.6 (20μmol), and enzyme in a final volume of 1ml were incubated for 20min at 37°C, acidified with HCl (100μmol) and extracted with 4ml of ethyl acetate. The ethyl acetate was decanted, and dried by the addition of about 0.1g of anhydrous Na_2SO_4. The fluorescence due to anthranilic acid in solution in the ethyl acetate was measured (excitation, 338nm; emission, 400nm, uncorrected). The concentration of anthranilic acid was determined from a standard curve, which was checked regularly.

This assay has the advantage that, after acidification, the samples may be kept and extracted later. It was used throughout the work except during the kinetic studies. The substrates are present at saturating concentrations to give near-$V_{max.}$ conditions. Specific activity is defined as units/mg of protein and 1 unit of activity is the production of 0.1μmol of product/20min at 37°C.

(ii) Initial-rate method (for kinetic studies). The substrates were present at the saturating concentrations shown below except for the variable substrate. Initial kinetic studies showed that it was possible to decrease the chorismate concentration from 0.5mM (as used in the extraction assay) to 0.2mM and still maintain saturation. The initial rate of appearance of anthranilate was followed fluorimetrically in the following reaction mixture, which

was prepared in a spectrofluorimeter cell kept at 30°C: chorismate (0.3 μmol), glutamine (7.5 μmol) or NH_4Cl (150 μmol), $MgCl_2$ (15 μmol), EDTA (0.15 μmol), tris–HCl buffer, pH 8.2 (30 μmol). Enzyme solution (2–5 μl) was added (final volume, 1.5 ml) and the rate of appearance of the fluorescence of anthranilate (excitation, 318 nm; emission, 400 nm, uncorrected) was recorded. The assay was always linear over the time of observation (up to 2 min).

(*b*) *Anthranilate phosphoribosyltransferase.* Assays for the transferase activity are discussed by Creighton & Yanofsky (1970). The most convenient and sensitive method of assay is to follow the initial rate of disappearance of anthranilate fluorimetrically. Two problems are encountered in using this method, however. First, phosphoribosylanthranilate fluoresces at the wavelengths used for anthranilate determinations (with about one-fifth of its intensity) and secondly, a concentration of anthranilate sufficient to saturate the enzyme cannot be used, since the fluorescence at this concentration is too high to be measured. Thus a suitable non-saturating concentration of anthranilate was chosen and the initial rate of decrease of fluorescence was followed. In each assay an enzyme dilution was chosen such that the rate of anthranilate disappearance was about the same in each experiment. Results are expressed on a relative basis, determined from the dilution used and the concentration of protein in the sample. The assay was performed as follows: anthranilate (0.01 μmol), $MgCl_2$ (15 μmol), tris–HCl buffer, pH 8.2 (30 μmol), and enzyme in a final volume of 1.4 ml were placed in the spectrofluorimeter and the fluorescence was measured. 5-Phosphoribosyl pyrophosphate (0.1 μmol) was added (final volume 1.5 ml) and the rate of removal of anthranilate was recorded. The assay was linear for up to 5 min with purified enzyme but only for 1 or 2 min with crude enzyme preparations.

Protein measurement

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin, dried before use, as standard.

Column fractionation procedures

The buffer used with all columns was potassium phosphate, pH 7.1 (0.02 M), containing EDTA (0.5 mM), 2-mercaptoethanol (0.1 mM) and NaCl (0.06 M).

Sephadex. A column (2.5 cm × 35 cm) was packed with Sephadex G-200 as recommended by the manufacturers, and the bed was stabilized by pumping buffer overnight at 9 ml/h. The sample was applied to the surface of the gel and 3 ml fractions collected.

DEAE-cellulose. The sample was applied directly to the surface of a column (2 cm × 20 cm) of DEAE-cellulose and allowed to sink in. A head of 10–15 ml of buffer was layered on the column, a flow rate of 50 ml/h was used and 10 ml fractions were collected. A linear gradient was generated with 500 ml of buffer in the mixing flask and 500 ml of buffer made 0.36 M with respect to NaCl in the inlet flask.

Polyacrylamide-gel electrophoresis

The gels were prepared and stained for protein as described by Davis (1964) and Ornstein (1964). To detect anthranilate synthase activity, the sample (50 μg of protein) was applied to the gel column in 25% (w/v) sucrose solution, instead of in the gel. Electrophoresis was carried out at 4°C, the gel removed and rinsed with water and then incubated in the reaction mixture used for the anthranilate synthase assay for 15 min at 37°C. The gel was again rinsed with water and examined under u.v. light.

Sucrose-density-gradient centrifugation

The method used was that of Martin & Ames (1961). All experiments were performed with a Spinco model L ultracentrifuge at 4°C in a SW. 39 rotor which was spun at 39000 rev./min for 12–14 h. A uniform gradient of sucrose varying from 5% (w/v) at the meniscus to 20% at the bottom of the tube was used. The sucrose was in solution in potassium phosphate buffer, pH 7.0 (10 mM), containing EDTA (0.1 mM) and 2-mercaptoethanol (1 mM). The samples were layered on to the sucrose gradient in a volume of 0.10 ml of the buffer used to prepare the sucrose solutions. The contents of each tube (4.55 ml) were collected, after centrifuging, in about 40 fractions by piercing the base of the tube and collecting six-drop fractions.

Results

Enzyme activity in extracts from cells of A. aerogenes 62-1

Crude extracts prepared from cells of *A. aerogenes* 62-1, grown on a limiting concentration of tryptophan or indole, readily converted chorismate plus glutamine into anthranilate, and the reaction was stimulated by Mg^{2+}. Dialysis against potassium phosphate buffer, pH 7.1 (0.01 M), containing EDTA (0.5 mM) resulted in a large decrease in activity, which could be restored by the addition of Mg^{2+}. The activity was stable in crude cell-free extract for up to 2 years when stored at −15°C. The enzyme showed a broad

plateau of maximum activity over the pH range 7.0–8.4 in potassium phosphate and tris–HCl buffers. For routine assays by the ethyl acetate extraction method, the reaction mixture was buffered at pH7.6. Anthranilate synthase activity was inhibited by various bivalent metals. The metal to be tested (1mM) was incubated with 2μl of derepressed cell extract in 0.5ml of 40mM-tris–HCl buffer (pH7.6) for 10min at 37°C. The substrates for the anthranilate assay were then added and the enzymic activity was measured by the ethyl acetate extraction method. $HgSO_4$ gave 100% inhibition; $CuSO_4$, 98%; $FeSO_4$, 92%; $CaCl_2$, 40%; $MnCl_2$, 30%. Under the same conditions $BaCl_2$, K_2SO_4 or NaCl had no effect on anthranilate synthase activity. *p*-Chloromercuribenzoate (100μM), when tested in a similar experiment, completely inhibited anthranilate synthase activity.

Anthranilate synthase activity is subject to repression and to feedback inhibition by tryptophan (Gibson & Gibson, 1964). Growth of cells in medium containing excess of tryptophan caused repression of the enzyme to about one-sixth the activity present in wild-type exponential-phase cells (specific activity, 0.6). Growth in medium containing a limiting concentration of indole caused a derepression of the enzyme activity of 31–52-fold. The phosphoribosyltransferase activity in unpurified enzyme was not examined in detail, but preliminary experiments showed that the activity was subject to repression and derepression by the presence of tryptophan in culture media to about the same extent as the anthranilate synthase activity.

Enzyme purification

Unless otherwise stated, all procedures were performed at 4°C.

Step 1. *Removal of nucleic acid.* Protamine sulphate was used to remove nucleic acid from the crude extract of derepressed cells of strain 62-1 (about 70ml). A 2% (w/v) solution of protamine sulphate was slowly added to the cell extract with continuous stirring (1ml per 6ml of crude extract). After addition was complete, stirring was continued for a further 10min and the precipitate was removed by centrifugation at 30000*g* for 15min and discarded.

Step 2. *Fractionation by a combination of $(NH_4)_2SO_4$ precipitation and heating.* Saturated neutral $(NH_4)_2SO_4$ was slowly added to the protamine-treated extract, which was continuously stirred, until 29% saturation was reached. The extract was then heated in a water bath (60–65°C) with continual stirring until the temperature reached 42–44°C (3–4min when the volume being treated was about 80ml). When this temperature was reached, the preparation was rapidly cooled in an ice–salt bath, and the precipitate was removed by centrifugation and discarded. Saturated $(NH_4)_2SO_4$ solution was added to the supernatant to adjust it to 44% saturation and stirring was continued for a further 10min. The precipitate was removed by centrifugation and dissolved in potassium phosphate buffer, pH7.1 (0.1M), containing EDTA (0.5mM) and 2-mercaptoethanol (0.1mM). The volume at this stage was about 20ml.

Step 3. *Sephadex G-200 gel filtration.* The solution from step 2 was passed through a Sephadex G-200 column as described above, and fractions around the peak of anthranilate synthase activity were pooled (usually 10–12 fractions, 30–36ml).

Step 4. *Gradient elution from DEAE-cellulose.* The combined Sephadex fractions were applied to a column of DEAE-cellulose and eluted with a linear gradient of NaCl as described above. Fractions around the peak of activity were pooled (usually about 15 fractions, 150ml).

Step 5. *Concentration of dilute partially purified protein.* The combined fractions from the DEAE-cellulose column were diluted with an equal volume of cold water and run on to a short column (1.5cm × 10cm) of DEAE-cellulose. The enzyme was then eluted with buffer containing 0.36M-NaCl. Fractions (5ml) were collected and the activity was present in fractions 3 and 4.

The results obtained in a typical purification are shown in Table 1. The purification was followed by measuring anthranilate synthase activity. Measurement of the transferase activity at various steps showed that anthranilate synthase and transferase activities purified together. As a routine the enzyme was purified to an activity 30–37 times that found in crude extracts prepared from derepressed cells of *A. aerogenes* 62-1. This represents 900–1200 times the specific activity of the enzyme in extracts prepared from wild-type cells, and up to 1400 times was obtained in individual fractions.

No other enzymic activities metabolizing chorismate were detected in the purified enzyme preparation.

Polyacrylamide-gel electrophoresis of the purified enzyme

The partially purified protein showed a slow-running major band on polyacrylamide-gel electrophoresis, with up to five minor bands. After electrophoresis the gels of the purified protein were incubated in the reaction mixture for the assay of anthranilate synthase activity, and a single strong blue fluorescent band was observed under u.v. light. The position of this band was noted and the gels were stained for protein. The band of enzymic activity corresponded to the major protein component.

Table 1. *Partial purification of the anthranilate synthase/phosphoribosyltransferase complex*

The procedure was developed and purifications followed by assaying for anthranilate synthase activity by the ethyl acetate extraction method. The transferase activity was determined after each procedure.

Procedure	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein concn. (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification	Activity relative to that in extracts from wild-type strain: Anthranilate synthase	Activity relative to that in extracts from wild-type strain: Phosphoribosyltransferase
Crude supernatant	72	350	25200	16.6	21.1	(100)	(1)	35	35
Protamine sulphate supernatant	78	260	20280	12.2	21.3	80	1	35	35
Heat and $(NH_4)_2SO_4$	22	650	14300	7.2	90	57	4	140	130
Sephadex G-200	33	373	10660	0.97	333	42	16	560	490
DEAE-cellulose	150	54	8060	—	—	32	—		
Concentration of protein	10	470	4700	0.64	734	20	35	1200	1100

Properties of the partially purified protein

Mg^{2+} ions were necessary for both anthranilate synthase activity and phosphoribosyltransferase activity; a mixture of ribose 5-phosphate and ATP would replace phosphoribosyl pyrophosphate in the latter reaction with crude cell-free extracts but not with the purified preparation. The purified enzyme was stable at 4°C, losing about 10% of the initial activity in 2 months. Freezing and thawing or dilution of the enzyme increased the rate of inactivation.

Source of the nitrogen atom of anthranilate and the effect of pH on the enzyme activity

Either NH_4^+ ions or glutamine readily served as the source of the nitrogen atom of anthranilate. Seventeen other amino acids, including asparagine, were tested and were inactive at a concentration of the L-isomer of 10mM in each case. D-Glutamine was also inactive.

With L-glutamine as the source of the nitrogen atom, the enzyme was active over the pH range 7–9 and showed a broad peak of maximum activity in tris–HCl buffer between pH7.9 and 8.6. With NH_4^+ ions as the source of nitrogen, the enzyme showed little activity below pH7.4, but the activity increased rapidly above this pH to a sharp peak of maximum activity at pH8.9, and then declined sharply (Fig. 1).

Inhibition of synthase and transferase activities by tryptophan

Fig. 2 shows the percentage inhibition of both the phosphoribosyltransferase and the anthranilate synthase activities of the purified aggregate by various concentrations of L-tryptophan. The synthase activity was completely inhibited by 0.1mM-tryptophan, whereas the transferase activity could not be more than 60% inhibited at concentrations up to 1mM-tryptophan. D-Tryptophan (1mM) did not affect the reactions.

Sedimentation characteristics of synthase and transferase on linear sucrose gradients

The synthase and transferase activities in a crude cell extract sedimented together in sucrose gradients to give a symmetrical peak with an $s_{20,w}$ value of 8.1S (mol.wt. about 170000) (Fig. 3). A similar result was obtained with the most purified enzyme preparations. The sedimentation characteristics of the purified enzyme were not affected by the addition of chorismate (10μM), glutamine (5mM), $MgCl_2$ (10mM) or tryptophan (200μM) to the sucrose gradient.

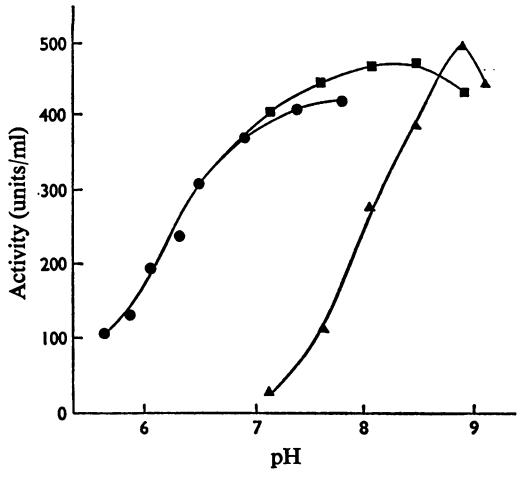


Fig. 1. *Effect of pH on the activity of purified anthranilate synthase with glutamine and NH_4Cl as nitrogen sources*

Activity was assayed by the initial-rate method as described in the Experimental section with all substrates present at saturating concentrations to give near-$V_{max.}$ conditions. ●, Potassium phosphate buffer, glutamine as substrate; ■, tris–HCl buffer, glutamine as substrate; ▲, tris–HCl buffer, NH_4Cl as substrate.

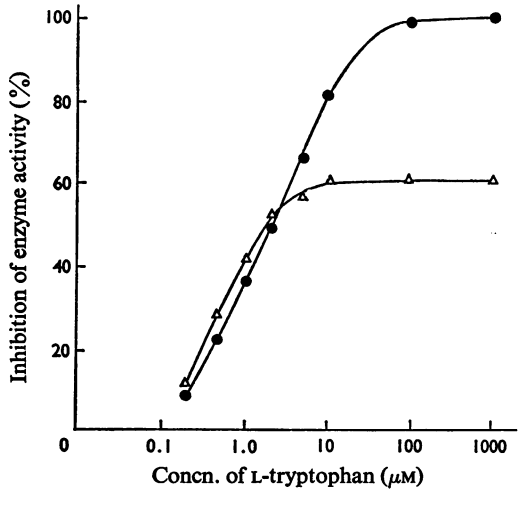


Fig. 2. *Inhibition of anthranilate synthase and phosphoribosyltransferase activities by* L-*tryptophan*

Anthranilate synthase activity (●) was measured by the initial-rate method but with a chorismate concentration of 5 μM. The transferase activity (△) was assayed with an anthranilate concentration of 5 μM. Purified enzyme (2 μg of protein) was added at zero time.

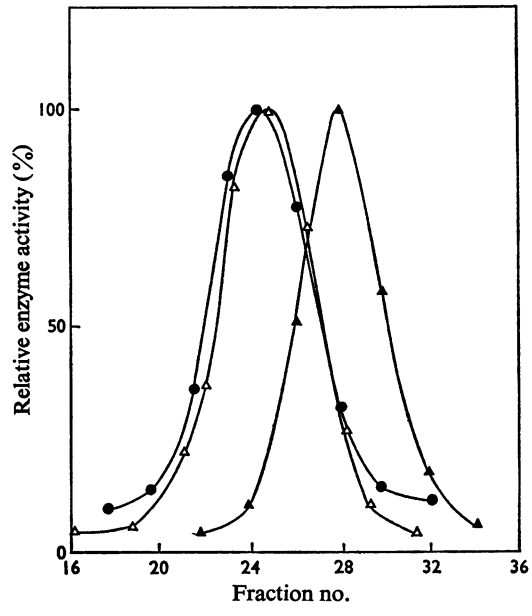


Fig. 3. *Sedimentation of synthase and transferase activities in crude extract*

Crude cell-free extract prepared from strain 62-1 (0.2 mg of protein) with added yeast alcohol dehydrogenase (0.1 mg) was centrifuged on a linear sucrose gradient as described in the text. The activity in any fraction is expressed as a percentage of the activity found in the most active fraction in each case. ●, Synthase activity; △, transferase activity; ▲, alcohol dehydrogenase activity.

Phosphoribosyltransferase and anthranilate synthase activities in tryptophan auxotrophs

A. aerogenes NC3 and *A. aerogenes* T21 are tryptophan auxotrophs which require either tryptophan, indole or anthranilate for growth. Crude extracts prepared after growth of these strains in minimal medium containing a limiting concentration of indole showed no anthranilate synthase activity, and the phosphoribosyltransferase activity was about one-tenth that found in *A. aerogenes* 62-1, i.e. derepression of only three- to five-fold over the activity observed in extracts prepared from wild-type cells harvested in the exponential phase of growth. When tryptophan (1 mM) was added, no inhibition of transferase activity was observed.

A. aerogenes N5-36 is a tryptophan auxotroph which requires either tryptophan or indole for growth. Crude extracts showed no transferase activity and about one-third of the derepressed synthase activity found in cells of strain 62-1. This activity from *A.*

Table 2. *Tryptophan inhibition of the transferase activity from tryptophan auxotrophs of A. aerogenes*

Crude extracts (16mg of protein/ml) prepared from cells of strains 62-1, N5-36, NC3 and T21 were incubated for 10min at 37°C. Mixtures containing equal volumes of extracts from strains NC3 and N5-36 and from strains T21 and N5-36 were also incubated. After incubation extracts were stored in ice, then diluted as required in 0.02M-tris–HCl buffer, pH8.2, and 0.3ml of each dilution was added to the reaction mixture and assayed for transferase activity as described in the Experimental section. The effect of 0.1mM-tryptophan on the activity was determined in each case. Strain 62-1 is included to show the inhibition by 0.1mM-tryptophan of the activity in a strain possessing an intact enzyme aggregate. Activities are expressed relative to that of strain 62-1 taken as 100 (— indicates no detectable activity).

	Transferase activity		
Strain	No tryptophan	+Tryptophan	Inhibition (%)
62-1	100.0	27.4	73
N5-36	—	—	
NC3	9.2	9.4	0
NC3+N5-36	9.7	4.8	50
T21	6.7	6.9	0
T21+N5-36	7.1	3.4	52

aerogenes N5-36 was sensitive to inhibition by tryptophan. Extracts from *A. aerogenes* NC3 and *A. aerogenes* N5-36 were mixed, the mixture was incubated at 37°C for 10min, and the effect of tryptophan (0.1mM) on transferase activity was determined (Table 2). The transferase activity in the mixture was sensitive to inhibition by tryptophan.

Sedimentation characteristics of the enzyme in tryptophan auxotrophs of A. aerogenes

The synthase of *A. aerogenes* N5-36, which lacks transferase activity, sedimented with an $s_{20,w}$ value of 7.2S. This corresponds to a molecular weight of about 150000, considerably smaller than the value for the intact enzyme aggregate. The transferases of *A. aerogenes* strains NC3 and T21, both of which lack synthase activity, sedimented with $s_{20,w}$ values of 4.1 and 4.3S, respectively. These values correspond to an approximate molecular weight of 90000, about one-half the value obtained for the aggregate.

When equal volumes of cell extracts from *A. aerogenes* strains NC3 and N5-36 were mixed and centrifuged, anthranilate synthase sedimented as a broad peak, its leading edge corresponding to an $s_{20,w}$ value of 8.2S and the trailing edge 7.2S. The phosphoribosyltransferase activity sedimented as two peaks of activity; the major peak sedimented with the anthranilate synthase and was sensitive to inhibition by tryptophan, and the minor peak, with an $s_{20,w}$ value of 4.1S, was not inhibited by tryptophan. Thus, although aggregation had occurred, it was not complete. The experiment was repeated by using 10vol. of strain-NC3 extract to 1vol. of strain-N5-36 extract. In this case, the transferase was present in large excess. The synthase sedimented as a single peak with an $s_{20,w}$ value of 7.6S, intermediate between the 7.2S value for strain N5-36 and the 8.1S for the intact aggregate (Fig. 4). Two peaks of transferase activity were again seen, a large peak ($s_{20,w}$ 4.1S; not inhibited by tryptophan) and a smaller shoulder, which had sedimented together with the synthase and was inhibited by tryptophan. Similar results were obtained when *A. aerogenes* T21 was substituted for *A. aerogenes* NC3.

Transferase and synthase activities in tryptophan auxotrophs of E. coli K-12

Strain C-47, a tryptophan auxotroph of *E. coli* unable to convert indole glycerol phosphate into tryptophan, was examined, and both transferase and synthase activities were sensitive to inhibition by tryptophan. Cell extracts were prepared from *E. coli* strains E5972 and D9778 grown under conditions in which tryptophan limited growth. The latter strains are both tryptophan auxotrophs with nonsense mutations in anthranilate synthase and phosphoribosyltransferase, respectively. The results of experiments in which extracts of these strains were mixed with extracts of the appropriate strains from *A. aerogenes* are shown in Table 3. Ito & Yanofsky (1966) had previously shown that the synthase is inactive in mutants lacking the transferase in *E. coli*. The present experiments show that the transferase from *A. aerogenes* NC3 activates the anthranilate synthase of *E. coli* D9778, and in doing so, itself becomes sensitive to inhibition by tryptophan. The reverse experiment was carried out, with the synthase from *A. aerogenes* N5-36 and the transferase from *E. coli* E5972. In this case, however, *A. aerogenes*

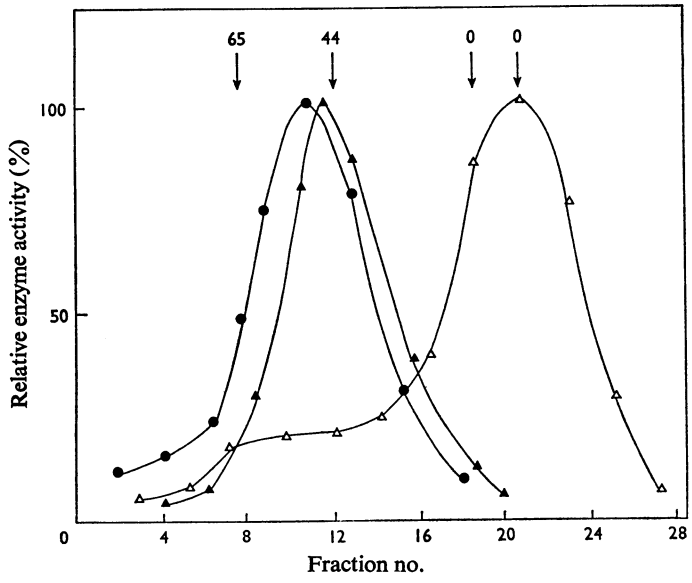


Fig. 4. *Sedimentation of synthase and transferase activities in a mixture of crude extracts from A. aerogenes strains NC3 and N5-36*

Crude cell-free extract prepared from strain NC3 (1.6mg of protein) was mixed with extract from strain N5-36 (0.76mg of protein) and 0.1mg of yeast alcohol dehydrogenase was added as a reference protein. The mixture was centrifuged on a linear sucrose gradient as described in the text. ●, Synthase activity; △, transferase activity; ▲, alcohol dehydrogenase activity. The activity in any fraction is expressed as a percentage of the activity found in the most active fraction in each case. The inhibition (%) by L-tryptophan (67μM) of the transferase activity of the fractions indicated are given.

Table 3. *Tryptophan inhibition of anthranilate synthase and phosphoribosyltransferase activities from tryptophan auxotrophs of E. coli and A. aerogenes*

Crude extracts (16mg of protein/ml) prepared from cells of strains C-47, NC3, D9778, N5-36 and E5972 were incubated at 37°C for 10min. Mixtures containing equal volumes of extracts of strains NC3 and D9778, and N5-36 and E5972, were also incubated. After incubation, extracts were stored in ice, diluted as required and assayed as described in Table 2. Strain C-47 is included to show the inhibition by 0.1mM-tryptophan of the activities in an *E. coli* strain possessing an intact enzyme aggregate. Activities are expressed relative to those of strain C-47 taken as 100 in each case (— indicates no detectable activity).

	Synthase activity			Transferase activity		
Strain	No tryptophan	+Tryptophan	Inhibition (%)	No tryptophan	+Tryptophan	Inhibition (%)
C47	100.0	9.2	91	100.0	51.9	48
NC3	—	—		8.5	8.7	0
D9778	—	—		—	—	
NC3+D9778	77.2	17.2	78	8.7	3.4	61
N5-36	29.6	3.7	88	—	—	
E5972	—	—		10.3	9.7	4
N5-36+E5972	28.7	3.0	90	11.8	9.4	21

N5-36 has an active synthase. Results from this mixing experiment were not as clear, the transferase in the mixture being only 21% inhibited by 0.1mM-tryptophan.

Sedimentation experiments similar to those described above for *A. aerogenes* were carried out. With *E. coli* C-47, the synthase and transferase activities sedimented with $s_{20,w}$ values of 7.9 and 8.2S, respectively. Thus, the transferase slightly preceded the anthranilate synthase, as was also found by Ito & Yanofsky (1966). $s_{20,w}$ values of 4.3S for the synthase of *E. coli* D9778 and 4.4S for the transferase of *E. coli* E5972 were reported by Ito & Yanofsky (1966). When a mixture of extracts from *A. aerogenes* NC3 (2vol.) and D9778 (1vol.) was centrifuged, aggregation occurred with the transferase sedimenting as a peak ($s_{20,w}$ 7.6S) with a trailing shoulder of lower molecular weight. Fractions in the main peak were sensitive to inhibition by tryptophan; those in the shoulder were not. The synthase was activated by the transferase and sedimented slightly ahead of it. In the reverse experiment, transferase from *E. coli* E5972, which was present in excess, aggregated with the synthase of *A. aerogenes* N5-36, and in doing so, became sensitive to inhibition by tryptophan.

Kinetic studies on the anthranilate synthase activity purified from A. aerogenes 62-1

Initial rates of reaction were measured as described in the Experimental section (assay ii). The substrate or cofactor whose concentration was not varied was kept at a saturating concentration. Kinetic studies were carried out at pH8.2, since the velocity of the reaction was slow with NH_4^+ ions as substrate at pH7.6.

The activity of the synthase was determined over a range of chorismate concentrations from 0.5μM to 0.1mM, with the concentration of glutamine constant at 5mM. Glutamine was then replaced by a saturating concentration of NH_4Cl (100mM). In each case the

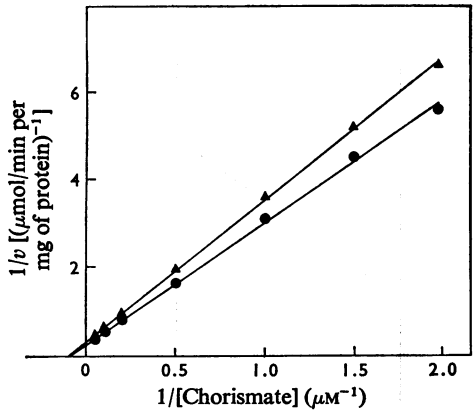


Fig. 5. *Double-reciprocal plots of initial velocity against chorismate concentration*

Anthranilate synthase activity was determined as described in the text, with both L-glutamine (●) and NH_4Cl (▲) as second substrates respectively. The apparent K_m value for chorismate is 10μM in each case.

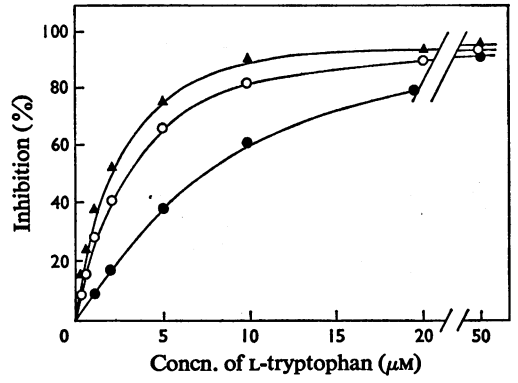


Fig. 6. *Inhibition of anthranilate synthase activity by L-tryptophan at three different chorismate concentrations*

Anthranilate synthase activity was determined as described in the text. L-Glutamine was the second substrate in each case. ▲, 1.5μM-Chorismate; ○, 5μM-chorismate; ●, 20μM-chorismate.

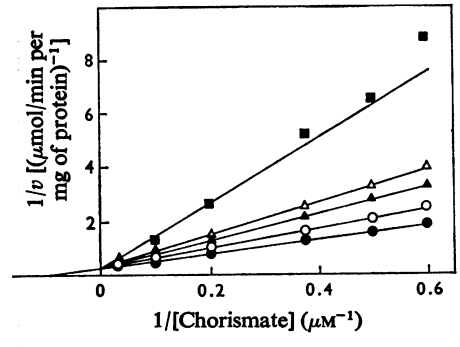


Fig. 7. *Effect of L-tryptophan on the double-reciprocal plots of initial velocity against chorismate concentration*

Anthranilate synthase activity was determined as described in the text. ●, No tryptophan; ○, 0.5μM-tryptophan; ▲, 1.5μM-tryptophan; △, 2μM-tryptophan; ■, 5μM-tryptophan.

reciprocal plot of velocity against chorismate concentration was linear (Fig. 5), indicating that Michaelis–Menten kinetics were obeyed. The apparent K_m value for chorismate was 0.01 mM in each case.

At a saturating concentration of chorismate (0.2 mM), the Lineweaver–Burk plot determined with glutamine as the variable substrate was linear and gave an apparent K_m value for glutamine of 0.51 mM. Kinetics were again linear with NH_4Cl as the variable substrate, and the apparent K_m value was 22 mM.

When the concentration of $MgCl_2$ was varied in the presence of saturating concentrations of chorismate and glutamine, the double-reciprocal plot appeared linear and the apparent K_m value was 5.3 μM.

Kinetics of feedback inhibition

The effect of tryptophan on the initial velocity of the reaction was determined. Fig. 6 shows the inhibition by tryptophan over a range of concentrations from 0.5 μM to 0.05 mM at each of three

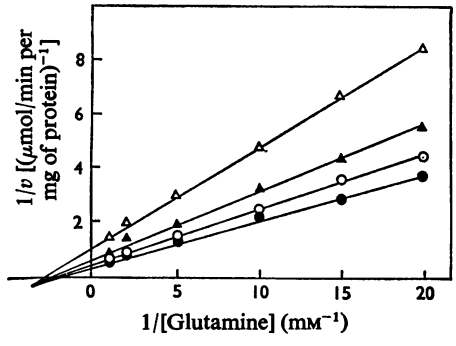


Fig. 8. *Effect of* L-*tryptophan on the double-reciprocal plots of initial velocity against* L-*glutamine concentration*

Anthranilate synthase activity was determined as described in the text. ●, No tryptophan; ○, 15 μM-tryptophan; ▲, 40 μM-tryptophan; △, 100 μM-tryptophan.

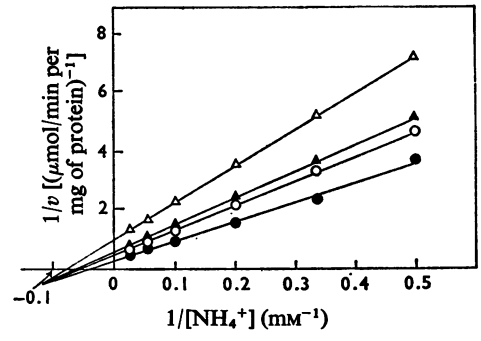


Fig. 9. *Effect of* L-*tryptophan on the double-reciprocal plots of initial velocity against* NH_4^+ *concentration*

Anthranilate synthase activity was determined as described in the text. ●, No tryptophan; ○, 15 μM-tryptophan; ▲, 40 μM-tryptophan; △, 100 μM-tryptophan.

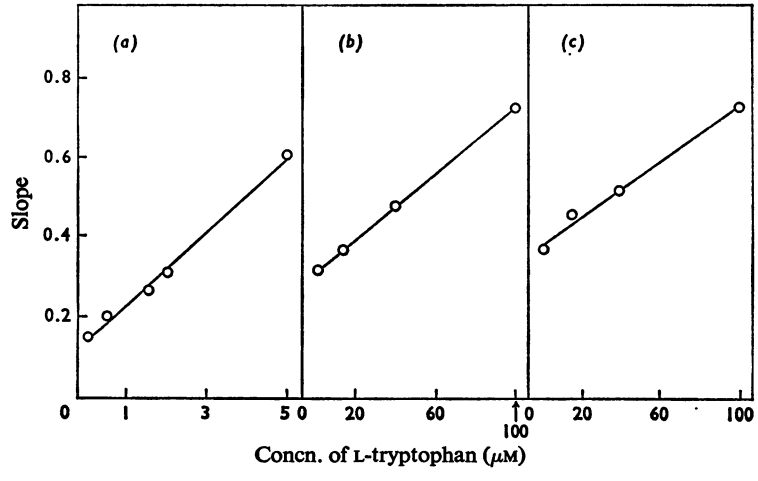


Fig. 10 (*a,b,c*). *Secondary kinetic plots of the results in Figs.* 7, 8 *and* 9 *respectively*

The slopes of the lines are plotted against inhibitor concentration in each case.

chorismate concentrations. Glutamine (5mM) was the second substrate. Even at the highest concentration of chorismate used (0.02mM, or twice the K_m value) almost complete inhibition of activity could be obtained. Four suitable tryptophan concentrations were chosen and the enzyme activity at each was determined at various non-saturating concentrations of chorismate. The double-reciprocal plots of this data were linear for all concentrations of tryptophan used and appeared to intersect on the ordinate (Fig. 7). The secondary plot, a plot of the slope of the reciprocal plots versus inhibitor concentration, also appeared linear (Fig. 10*a*). Similar results were obtained when NH_4Cl (100mM) replaced glutamine. In each case the results are consistent with linear competitive inhibition between chorismate and tryptophan.

With glutamine as the variable substrate and chorismate at a saturating concentration (0.2mM), the double-reciprocal plots were again linear and formed a family of straight lines which intersected at a point to the left of the ordinate and below the abscissa (Fig. 8). The secondary plot was also again linear (Fig. 10*b*). With NH_4Cl as the variable substrate instead of glutamine, similar results were obtained (Figs. 9, 10*c*). In each case the results are consistent with linear non-competitive inhibition between tryptophan and either glutamine or NH_4^+.

Discussion

The anthranilate synthase/phosphoribosyltransferase complex from cell extracts of *A. aerogenes* 62-1 has been purified up to 1400 times the activity present in cell extracts from wild-type *A. aerogenes*. It was shown previously that cell extracts from *A. aerogenes* convert chorismate into anthranilate with either glutamine or NH_4^+ ions as the source of nitrogen (Edwards *et al.*, 1964), and that NH_4^+ ions can serve as a nitrogen source *in vivo* without first passing through the amide nitrogen of glutamine (Gibson *et al.*, 1967). Present studies indicate that the purified enzyme aggregate can use either glutamine or NH_4^+ ions as the nitrogen source and that the maximum velocity of the reaction is similar for both substrates. The K_m value for NH_4^+ ions is some 40 times the K_m value for glutamine at pH8.2, but at this pH value only about 10% of the NH_4^+ ions are present as NH_3. The high pH optimum of the reaction with NH_4^+ ions suggests that NH_3 rather than NH_4^+ ions may be the active substrate.

The present kinetic studies on the anthranilate synthase activity show that tryptophan functions as a linear competitive inhibitor with respect to chorismate, indicating that substrate and inhibitor react with the same form of the enzyme and are mutually exclusive. This may mean that two compounds combine at the same or overlapping sites. These results differ from those of Baker & Crawford (1966) and Henderson *et al.* (1970) who, working with *E. coli* and *S. typhimurium* respectively, found that double-reciprocal plots of anthranilate synthase activity versus substrate concentration became non-linear when tryptophan was present. However, tryptophan inhibition has been found to be competitive with respect to chorismate for the un-aggregated purified component I of anthranilate synthase from *E. coli* (Ito *et al.*, 1969). Present studies indicate that tryptophan inhibition is non-competitive with respect to either glutamine or NH_4^+ ions, indicating that the mechanism of the reaction is possibly the same with either nitrogen donor as substrate. The inhibition pattern observed is consistent with a sequential mechanism of the ordered type in which chorismate is the first reactant, in accord with the type of mechanism postulated by Srinivasan (1965) and Nagano *et al.* (1970).

An early observation of the effect of tryptophan on the reactions of tryptophan biosynthesis was that the condensation of phosphoribosyl pyrophosphate and anthranilate was inhibited (Moyed & Friedman, 1959). It is therefore noteworthy that the phosphoribosyltransferase activity of the purified enzyme aggregate from *A. aerogenes* is sensitive to inhibition by tryptophan, but only with relatively high concentrations of tryptophan, and then only to a maximum of 60% inhibition.

The conclusion that the anthranilate synthase and the phosphoribosyltransferase occur as an enzyme aggregate in *A. areogenes* is supported by their simultaneous purification and experiments on sedimentation and tryptophan inhibition of the transferase activity. Thus both enzymic activities sedimented together in sucrose gradients and have $s_{20,w}$ values of 8.1–8.2S (corresponding to mol.wt. 170000). The transferases of two auxotrophs with no anthranilate synthase activity sedimented with $s_{20,w}$ values of 4.1–4.3S (mol.wt. 90000) and were not inhibited by tryptophan. Thus the transferase can be active when not associated with the synthase but is not then inhibited by tryptophan, indicating that the inhibition site is probably on the anthranilate synthase component. The latter result complements the results of Ito & Yanofsky (1966), who showed tryptophan inhibition of the synthase activity in cell extracts from mutants of *E. coli* lacking the transferase.

In the N5-36 strain of *A. aerogenes* lacking transferase activity the synthase activity sedimented with an $s_{20,w}$ value of about 7.2S (mol.wt. 150000), and thus is probably complexed with an inactive transferase. [The purified component I of anthranilate synthase from *E. coli* has an $s_{20,w}$ value of 4.4S (Ito & Yanofsky, 1966).] The normal transferase from *A. aerogenes* is apparently able to displace the inactive component from the aggregate from strain

N5-36 to form an active aggregate. Thus when extracts from strain NC3 or T21 were mixed with extracts from strain N5-36 and then centrifuged, the sedimentation velocities of both activities were increased. Further, if the transferase was present in excess two peaks of this activity were observed, the more rapidly sedimenting one being susceptible to inhibition by tryptophan. These experiments do not show whether the anthranilate synthase component is active in the absence of the transferase.

Ito & Yanofsky (1969) and Ito *et al.* (1969) studied the *E. coli* enzymes in detail. They purified component I (anthranilate synthase) and showed that it was active only with NH_4^+ ions as nitrogen donor but not with glutamine. The activity was inhibited by tryptophan. They also showed that component I is activated by component II (transferase) or by a presumed fragment of component II produced by a deletion mutant. The transferase was only inhibited by tryptophan in the intact complex.

The mixing of extracts of appropriate mutants of *E. coli* and *A. aerogenes* showed that an intact aggregate can be formed when one enzyme comes from one organism and one from the other (see also Ito, 1969). For example, the transferase from *A. aerogenes* (strain NC3) activated the synthase of *E. coli* (strain D778) and became sensitive to inhibition by tryptophan, showing that aggregation had occurred.

It is noteworthy that the initial stages in the three biosynthetic sequences from chorismate leading to the aromatic amino acids in *A. aerogenes* and *E. coli* are carried out by proteins or protein complexes which can be demonstrated to carry out two reactions in the sequence (Ito & Yanofsky, 1966; Cotton & Gibson, 1965; Pittard & Wallace, 1966). The presence of the first two activities of tryptophan biosynthesis in an enzyme aggregate suggested the possibility that anthranilate is not a true intermediate in the conversion of chorismate into *N*-(5-phosphoribosyl)-anthranilate, but rather that 5-phosphoribosylamine may react directly with chorismate. However, 5-phosphoribosylamine, synthesized from ribose 5-phosphate and ammonia as described by Goldthwait (1956), did not serve as a substrate when used to replace glutamine and phosphoribosyl pyrophosphate for the overall reaction (A. F. Egan, unpublished work).

The enzymes catalysing the early steps in tryptophan biosynthesis have now been examined in a number of bacteria. In *S. typhimurium*, the synthase and transferase occur as an aggregate similar to that in *E. coli* and *A. aerogenes* (Bauerle & Margolin, 1966; Henderson *et al.*, 1970). However, lack of aggregation has been demonstrated in *Chromobacterium violaceum* (Wegman & Crawford, 1968) in *Serratia marcesens* (Zalkin & Hwang, 1971) and in *Pseudomonas putida* (Enatsu & Crawford, 1968). In the latter organism Queener & Gunsalus (1970) were able to separate anthranilate synthase itself into two components. Whitt & Carlton (1968) found evidence for aggregation of the transferase with the subsequent two enzymes of tryptophan biosynthesis in *Bacillus subtilis*, but anthranilate synthase did not appear to be a component of this aggregate. Kane & Jensen (1970) have suggested that anthranilate synthase of *Bacillus subtilis* occurs in an aggregate, but that the transferase is not a component.

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