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Tryptophan Biosynthetic Pathway in the *Enterobacteriaceae*: Some Physical Properties of the Enzymes

MICHAEL LARGEN[1]* AND W. L. BELSER

Department of Biology, University of California, Riverside, California 92502

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Several physical properties of the first four enzymatic activities of the tryptophan pathway were examined using gel filtration and ion exchange chromatography. Five different patterns were noted. Differences in the anthranilate synthetase (AS) and phosphoribosylanthranilate transferase (PRT) defined these patterns. In all the organisms studied phosphoribosylanthranilate isomerase and indoleglycerol phosphate synthetase co-eluted from both diethylaminoethyl-cellulose and G-200 and thus probably are contained in a single polypeptide of 50,000 daltons. An AS-PRT complex was found in *Citrobacter* species, *Enterobacter cloacae*, and *Erwinia dissolvens*. In all the other bacteria examined AS and PRT were separate molecules. In *Serratia marcescens*, *S. marinorubra*, and *Enterobacter liquefaciens*, AS was 140,000 daltons and PRT was 45,000 daltons. In *Erwinia carotavora* and *Enterobacter hafniae* the AS was the same size as the *Serratia* species but the PRT was larger at 67,000 daltons. Two *Proteus* species had an AS and PRT of the same size as *E. carotavora* and *E. hafniae* but the *Proteus* AS was different in that it partially dissociated upon gel filtration. *Aeromonas formicans* was unique in its possession of an AS with a molecular weight of 220,000. The PRT of *A. formicans* was found to elute at 67,000 daltons. Possible paths of evolution of the tryptophan enzymes are discussed in terms of the reults of this study. The results presented here are also considered with respect to existing taxonomic schemes of the enteric bacteria.

In recent years molecular biological techniques have been used to elucidate phylogenetic relationships among various microorganisms. A variety of techniques have been used and have included the following: (i) nucleic acid studies, both the determination of the base composition of an organism's deoxyribonucleic acid (DNA) (41) and DNA-DNA and DNA-ribonucleic acid (RNA) hybridization (6, 7); (ii) peptide mapping and amino acid sequence determinations of homologous proteins in different species (5, 32); (iii) comparative examinations of the modes of regulation of various biochemical pathways (9, 25); and (iv) studies of genetic exchange among closely related organisms (26).

The pathway for the biosynthesis of the amino acid L-tryptophan has been a focal point for molecular biological studies of evolutionary relationships among microorganisms. These studies have examined various stages in the production of enzyme activity from the genetic material of the cell. DNA specific for the tryptophan genes has been used to estimate nucleotide sequence divergence of messenger RNA for the tryptophan enzymes in several of the enteric bacteria. Amino acid sequences have been determined for the α proteins of tryptophan synthetase from several enterobacterial sources (30), and from *Pseudomonas putida* (11). The general surface structure of the α and β_2 subunits of tryptophan synthetase of several members of the *Enterobacteriaceae* have been examined using immunochemical techniques (33, 39). Protein-protein interactions have been measured by enzyme complementation studies. In these studies tryptophan synthetase α and β_2 subunits from enteric bacteria have been used (33, 39) as well as the subunits of anthranilate synthetase from the enteric bacteria (23, 38) and from various pseudomonad bacteria (36). Protein-protein interactions were also examined in a study of the molecular distribution of the tryptophan enzymes in the fungi (20). Taking into account the total number of genes responsible for the coding of the tryptophan enzymes and the aggregations of the polypeptide products of these genes, five different distribution patterns were found. These results were considered with respect to existing phylogenetic schemes and were used to clarify some of the natural relationships among the fungi.

[1] Present address: Dept. of Biology, UCSD, La Jolla, Calif. 92037.

This report describes some physical properties of the tryptophan enzymes in a selected group of enteric bacteria as determined by G-200 Sephadex and diethylaminoethyl (DEAE)-cellulose chromatographic techniques. (This work was submitted in partial fulfillment of the requirements for the Ph.D. degree in biology by M.L.)

MATERIALS AND METHODS

Bacterial strains. The strains used in this study, together with their sources, are listed in Table 1.

Growth media. The minimal medium used was EMA medium; each liter contained Vogel-Bonner salts (42) and acid-hydrolyzed casein (1% wt/vol). After autoclaving, pantothenic acid (10 mg) and nicotinic acid (10 mg) which had been sterilized separately by filtration were added to each liter. D-Glucose at 0.5% (wt/vol), autoclaved separately, was added as a carbon source.

Growth conditions. Liquid cultures of all organisms except *Enterobacter hafniae* and *Erwinia carotavora* were grown at 37 C in a New Brunswick rotary shaker with a shaker setting of approximately 200 rpm. *E. hafniae* and *E. carotavora* were grown at 30 C. Growth was monitored turbidimetrically using a Klett-Summerson colorimeter with a filter no. 66 (660 nm).

Enzyme preparations for chromatography were prepared from 1-liter cultures of wild-type strains grown in EMA medium to approximately 200 Klett units, measured as described above.

Preparation of cell-free extracts. The buffer used for extract preparation and chromatography was 0.05 M potassium phosphate buffer (pH 7.6), supplemented with 10^{-4} M dithiothreitol (reduced form) and 10^{-4} M ethylenediaminetetraacetate (hereafter referred to as PED buffer). The procedures employed were described previously (19).

TABLE 1. *Strains used*

Organism	Strain no.	Source[a]
Enterobacter cloacae	NCTC 9394	NCTC
Enterobacter hafniae[b]	NCTC 9540	NCTC
Enterobacter liquifaciens	ATCC 14460	ATCC
Citrobacter freundii	NCTC 9750	NCTC
Citrobacter ballerupensis	NCTC 6021	NCTC
Proteus vulgaris		UCR
Proteus morganii	NCTC 235	NCTC
Serratia marcescens	ATCC 8195	UCR
Serratia marinorubra		UCR
Aeromonas formicans	ATCC 13137	I. P. Crawford
Erwinia carotovora	ICPB EC153	M. P. Starr
Erwinia dissolvens	ICPB ED106	M. P. Starr

[a] All cultures were assumed to be correctly labeled when received. Abbreviations for sources are: NCTC, National Collection of Type Cultures (England); ATCC, American Type Culture Collection; UCR, University of California Riverside Biology Dept. Collection; ICPB, International Collection of Phytopathogenic Bacteria.

[b] This culture was received as *Hafnia alvei*. The nomenclature adopted here is that currently accepted (M. A. Fife, W. H. Ewing, and B R. Davis, The biochemical reactions of the tribe *Klebsiellae*. 1965. U. S. Dept. of Health, Education, and Welfare.)

Enzyme assays. All assays were performed as described previously (19, 37). Unless otherwise indicated in the text, a unit of activity is defined as 1 nmol of substrate disappearing or 1 nmol of product appearing per min (m.i.e.u.).

Gel filtration chromatography. G-200 Sephadex columns were prepared by the manufacturer's specifications. The chromatography was performed in a column (1.5 by 65 cm) eluted with 0.05 M PED buffer in the upward mode. Flow rate was 5 to 6 ml/h. Fraction size was 2.0 ml. The column was calibrated with several proteins of known molecular weight: cytochrome *c*, chymotrypsin, bovine serum albumin, yeast alcohol dhydrogenase, and catalase (43).

DEAE-cellulose chromatography. DEAE-cellulose (BioRad Cellex D) was prepared by the method of Peterson and Sober (34). The column (1 by 30 cm) was equilibrated with 0.02 M PED buffer. The sample was applied in the same buffer. Elution was with a 400-ml, 0 to 0.5 M KCl linear gradient. The fraction size was 5.0 ml.

Chemicals. Chorismic acid was isolated from the culture filtrates of *Enterobacter aerogenes* 62-1 following the method of Gibson (15). It was isolated as the free acid, and assayed by its enzymatic conversion to anthranilate. *O*-carboxyphenyl-amino-deoxyribulose-5-phosphate was synthesized by previously described methods (40). Reduced dithiothreitol, bovine serum albumin, nicotinic acid, and pantothenic acid were from Calbiochem. Phosphoribosylpyrophosphate (PRPP) was from P-L Biochemicals or Sigma Chemical Co. Catalase, yeast alcohol dehydrogenase, alpha chymotrypsin, and cytochrome *c* were obtained from Worthington Biochemicals. Acid-hydrolyzed casein was from Nutritional Biochemicals Co.

RESULTS

The pathway for tryptophan biosynthesis in all microorganisms thus far studied is presented in Fig. 1. The appropriate enzymes are indicated below the arrows for each of the reactions.

Cell-free extracts prepared from wild-type organisms were subjected to G-200 Sephadex chromatography as described in Materials and Methods. There were five distinct patterns found upon elution of the column (Fig. 2); in addition to the two patterns described previously (19, 20) for the enteric bacteria, three new patterns were found.

The type 1 organization, characterized by a complex of the first two enzymes of the pathway anthranilate synthetase (AS) and phosphoribosylanthranilate transferase (PRT), has been shown previously for *Escherichia coli* (22, 24), *Salmonella typhimurium* (3), and *E. aerogenes* (13). It was demonstrated here for two *Citrobacter* species, *freundii* and *ballerupensis*, *Enterobacter cloacae*, and *Erwinia dissolvens*. It is evident from Fig. 2 that both AS and PRT

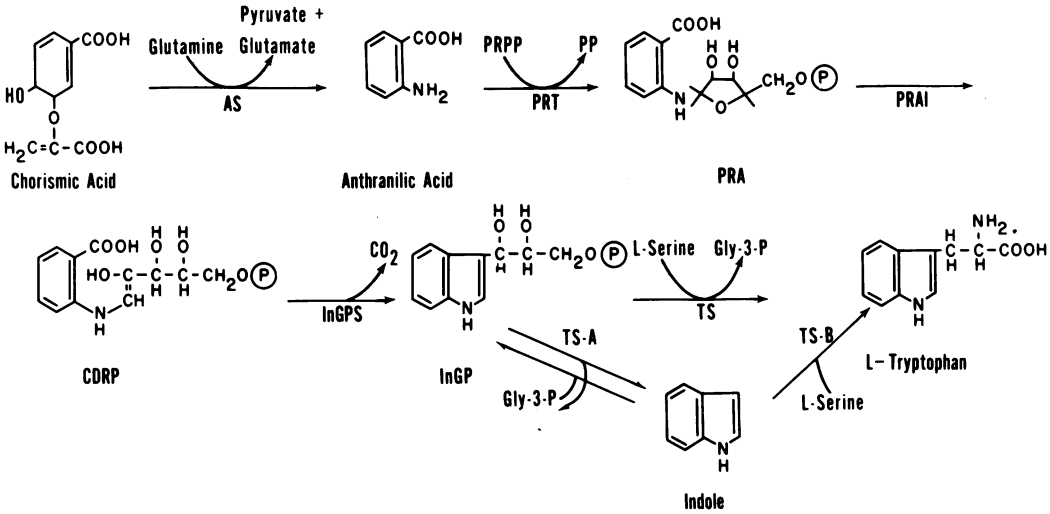

FIG. 1. *Pathway for L-tryptophan biosynthesis. Abbreviations for the enzymes are: AS, anthranilate synthetase; PRT, phosphoribosylanthranilate transferase; PRAI, phosphoribosylanthranilate isomerase; InGPs, indoleglycerol phosphate synthetase; TS, tryptophan synthetase; TS-A, tryptophan synthetase α protein; TS-B, tryptophan synthetase β_2 protein.*

are found in the excluded volume upon elution from G-200. According to the manufacturer's specifications, a spherical globular protein of molecular weight 400,000 or greater will be excluded from G-200. In Fig. 2 there is a small peak of AS at the trailing edge of the PRT peak. According to the calibration, this elution volume is characteristic of a protein of molecular weight 250,000. There is no distinct peak of PRT in this position, but it should be noted that the sensitivity of the PRT assay is much lower than that of the AS when monitored continuously. The fraction of the total recovered AS activity in this auxiliary peak is small for *C. freundii* and *E. dissolvens* but is much larger in *C. ballerupensis* and *E. cloacae*, amounting to 30 to 50% of the total AS activity. A typical experiment with *E. cloacae* is presented in Fig. 3. It can be seen that PRT is associated with the AS in both peaks. In at least three experiments for each of the type 1 organisms, the fraction of the total AS in the 250,000 molecular weight fractions was relatively constant (± 15%) and this auxiliary peak was always present. It is likely that this smaller species is homologous to the 260,000- to 290,000-dalton AS-PRT complex purified from *E. coli* (24) and *S. typhimurium* (21). It is possible that the molecular species in the excluded volume represents a multimer of the 250,000-dalton complex or is associated with some cellular component. Two species of AS-PRT complex have also been reported for *S. typhimurium* and *E. coli* (21). As is the case here, the larger species was found in the excluded volume of Sephadex G-200. It was proposed that this larger species was associated with the cell envelope.

To confirm the results of the gel filtration experiments, crude extracts of the type 1 organisms were applied to DEAE-cellulose columns, eluted with linear salt gradients, and assayed for AS and PRT activities. The two activities co-eluted and the ratio of the two activities in each fraction was constant (Fig. 4). The two activities are thus inseparable on the basis of molecular size or charge properties, at least under the conditions employed here.

In the type 1 organisms the phosphoribosylanthranilate isomerase (PRAI) and indoleglycerol phosphate synthetase (InGPs) co-eluted from a G-200 at a probable molecular weight of 47,000.

The distribution of the tryptophan enzymes in *Aeromonas formicans* were also examined. This was done previously for this strain by Crawford and co-workers (10), but the previous study was done under conditions where AS was unstable and competition for the substrate chorismic acid was noted. Under those conditions, with Sephadex G-150 used for the molecular weight determination, the small amount of AS activity recovered was found in the excluded volume. Under the conditions used her, AS was stable and there was no observable competition for chorismate; however, when the cells were grown and treated as described by Crawford et al. (10), their observations were corroborated. The AS in these experiments was found to elute

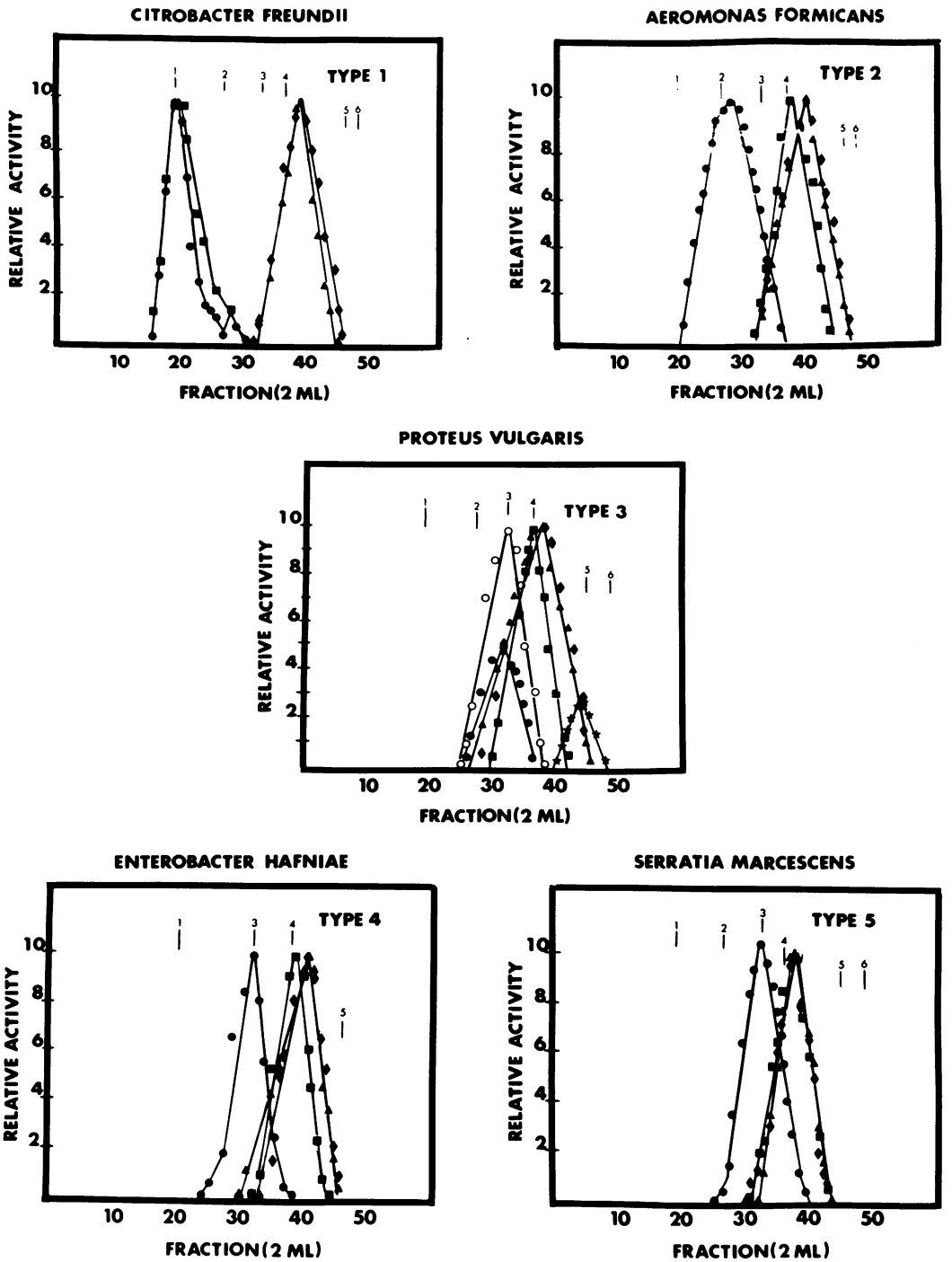

FIG. 2. *G-200 Sephadex elution profiles. Cell-free extracts of the various bacteria were applied to the column as described in Materials and Methods. Blue dextran 2000 was mixed with the applied sample as a measure of the excluded volume of the column. Activities are represented relative to the peak fraction for each activity, to which a value of 10 has been assigned. In the case of the P. vulgaris extract, where ammonia-dependent and glutamine-dependent AS activity as well as glutamine amidotransferase complementing activity have been measured, the ammonia-dependent AS has been used as a reference for all three activities so that they might be compared directly. Also, for P. vulgaris the PRT was run on a separate column equilibrated in buffer + 30% (vol/vol) glycerol and has been superimposed on an elution profile for the other activities as described in Results. Total recovery of the applied activity was usually greater than 70% under the conditions employed. Symbols: ●, AS glutamine-dependent; ■, PRT; ◆, PRAI; ▲, InGPs; ○, AS ammonia-dependent; ★, AS glutamine-dependent of fraction #39 with other fractions added to measure their GAT complementing activity. The molecular weight markers used in the calibration of the column are indicated in the graph by bars with numerals above them: (1) blue dextran, 2 × 10^6; (2) bovine catalase, 247,500; (3) yeast alcohol dehydrogenase, 142,000; (4) bovine serum albumin, fraction V, 67,000; (5) chymotrypsin, 23,000; and (6) cytochrome c, 12,500.*

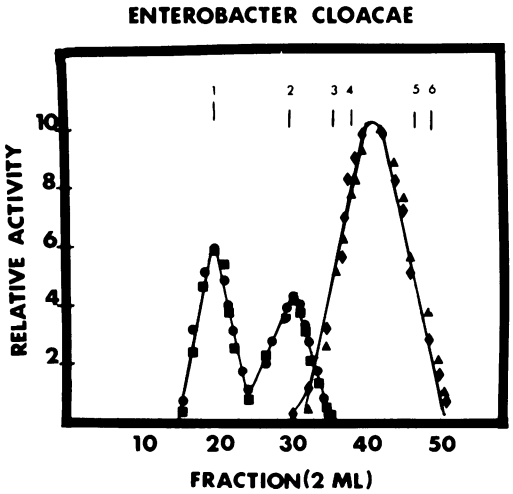

FIG. 3. *G-200 Sephadex elution profile for E. cloacae. All methods and symbols are as described for Fig. 2. Activities in the peak fractions (units/ml) are: AS, 50; PRT, 39; PRAI, 33; and InGPs, 3.*

in a relatively broad peak at 220,000 molecular weight. The PRT was eluted at 67,000 molecular weight whereas the PRAI and InGPs coeluted at a position characteristic of 45,000 daltons. These results are in substantial agreement with those previously reported (10).

In the two *Proteus* species examined, *morganii* and *vulgaris*, the AS dissociated into two peaks. The first peak, which eluted at a position characteristic of 140,000 daltons, possessed both ammonia-dependent and glutamine-dependent AS activity. One of the fractions at the edge of this peak (#39) possessed low levels of ammonia-dependent activity and no detectable glutamine-dependent activity.

Fraction #39 was used to measure the glutamine-stimulating activity (ability to confer glutamine-dependent AS activity on a molecular species which has only NH_3-dependent activity) of the lower-molecular-weight fractions. Since none of these lower-molecular-weight fractions possess glutamine-dependent AS activity on

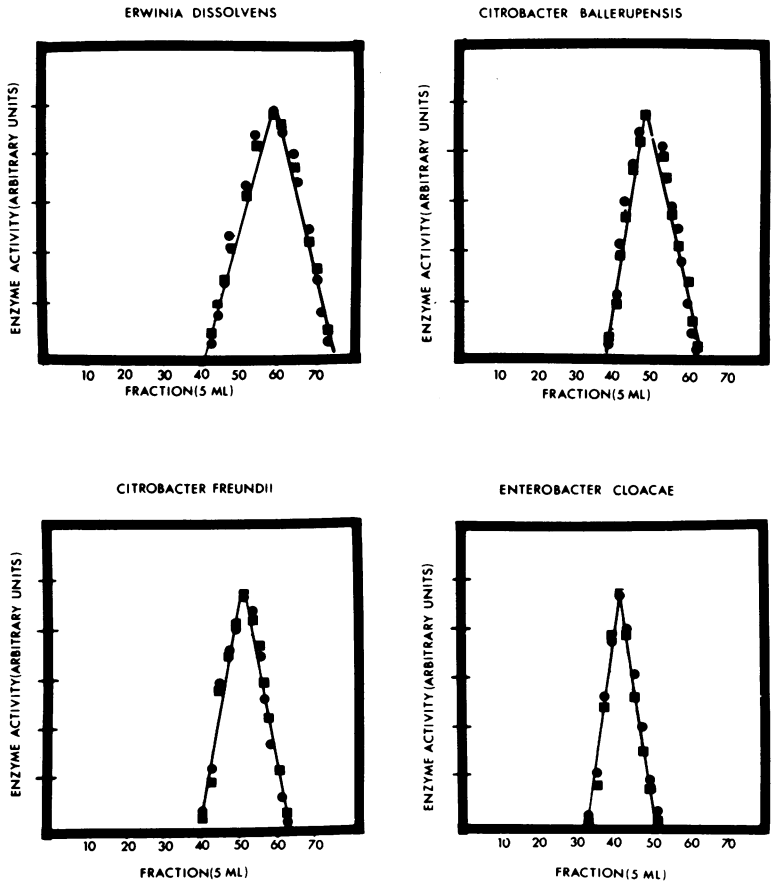

FIG. 4. *DEAE-cellulose elution profiles of AS and PRT. The techniques are described in Materials and Methods. Symbols: ○, AS; ■, PRT. Activities in the peak fractions (units per milliliter) are: E. dissolvens AS (46), PRT (20); for C. ballerupensis AS (51), PRT (40); for C. freundii AS (130), PRT (80); and for E. cloacae AS (39), PRT (28).*

their own, the activity measured is presumably a measure of complementation between the *Proteus* component I (Co I) and glutamine subunit (GAT), components of AS. The glutamine subunit molecular weight is approximately 20,000. Although there is about a two-fold excess of NH_3-dependent activity over glutamine-dependent activity in the peak if the fractions are assayed immediately upon elution, the ratio of the two activities quickly approaches 1 in a matter of hours after elution of the column. It would appear that "free" Co I is labile under the conditions employed for elution of the column, and that the excess of NH_3-dependent activity is due to an equilibrium situation in which Co I is labile when dissociated but stable in association with GAT. At this time we are unable to make any statement concerning the molecular weight of *Proteus* AS Co I. We have also found that the *Proteus* GAT will complement with *E. coli* Co I. In all of the other organisms studied, we assayed for NH_3-dependent AS and found it to be coincident with AS glutamine-dependent activity. In no case was there GAT complementation between the low-molecular-weight fractions from G-200 and an *E. coli* Co I preparation. This indicates that no stable dissociable GAT subunit is generated by the experimental procedure. Thus, among the organisms studied here, the two *Proteus* species appear to be unique in the partial dissociation of AS upon elution from G-200. The PRT activity for *Proteus* was found at approximately 65,000 daltons. Since the PRT is labile in the two proteus species in the absence of glycerol, the columns were eluted with PED buffer supplemented with 30% (vol/vol) glycerol. In Fig. 2 the PRT profile from such a column was superimposed on a elution profile for the other activities run in the absence of glycerol. Both columns were calibrated and the two were combined for purpose of comparison with the other organisms. The PRAI and InGPs co-eluted in a broad peak at a position corresponding to 53,000 molecular weight.

The type 4 organization was characteristic of *E. carotavora* and *E. hafniae* (formerly *Hafnia alvei*). These organisms produce AS activity of molecular weight 140,000 which is not associated with PRT. The free PRT in this group has a molecular weight of 67,000, with PRAI and InGPs co-eluting at a molecular weight of 48,000.

The type 5 pattern has been described previously (19). This pattern differs from type 4 only in that the PRT activity has a lower molecular weight and co-elutes with PRAI and InGPs at a molecular weight of 45,000. This pattern was confirmed for *Serratia marcescens* and also observed for the marine bacterium *Serratia marinorubra* and for *Enterobacter liquefaciens*. The PRT activity in these organisms was found to be rather unstable, but instead of using glycerol-supplemented PED to stabilize the enzyme, we found that either PRPP or anthranilic acid added to PED could stabilize the PRT of both *S. marcescens* and *S. marinorubra*. In *E. liquefaciens* only PRPP was effective in stabilizing the enzyme. The type 5 pattern for *E. liquefaciens* was obtained in the presence of PRPP (2 μM) since otherwise PRT activity was lost. In both the serratias, however, the situation was quite different. For these organisms the type 5 pattern was observed only in the presence of anthranilic acid (1 mM) in the elution buffer. If the extract was prepared in and eluted from the column in PED buffer which was supplemented with PRPP rather than anthranilic acid, a dramatic shift in the molecular weight of PRT occurred. Figure 5 shows that PRT shifts

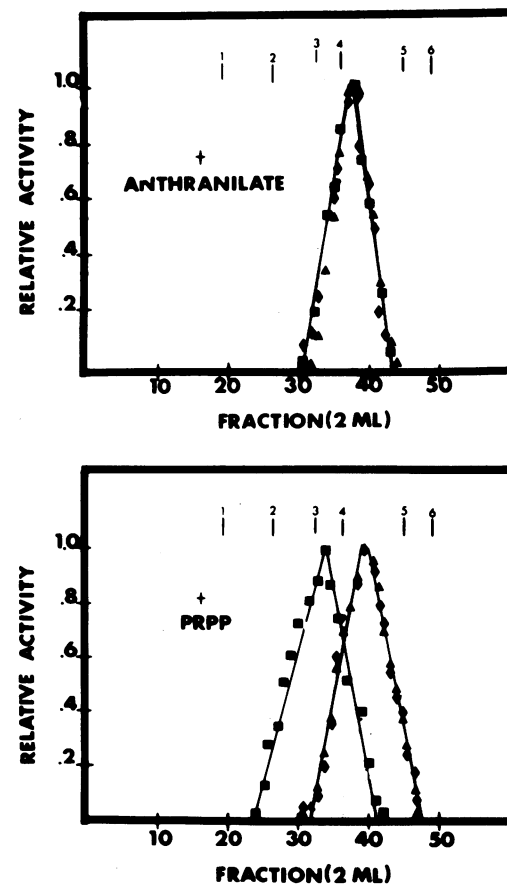

FIG. 5. *G-200 Sephadex elution of PRT from Serratia marcescens. Methods, molecular weight markers, and symbols are as in Fig. 2. Anthranilic acid (1 mM) or PRPP (0.5 μM) were added to the PED buffer for sonic treatment and elution buffers.*

from a position characteristic of 45,000 daltons to one corresponding to 84,000 daltons. It may be that *E. liquefaciens* PRT would shift to a higher molecular weight with higher concentrations of PRPP, but this experiment was not performed. The shift of the *Serratia* PRT from 45,000 daltons to 84,000 daltons in the presence of PRPP is consistent with the dimerization of a monomeric species. This raises the interesting possibility that the active species may be of molecular weight 84,000 rather than 45,000. Consistent with this hypothesis is the observation that when the PRT assay of *Serratia* extracts is initiated with PRPP rather than anthranilic acid, there is a short lag before the disappearance of anthranilic acid begins (M. A. Hutchinson and W. L. Belser, unpublished experiments).

In Fig. 2 it can be seen that in all cases the PRAI and InGPs co-elute from Sephadex G-200 columns. To ascertain whether the PRAI and InGPs activities were contained in a single polypeptide as had been shown for several other enteric bacteria (32, 35), crude extracts of all the organisms were subjected to DEAE-cellulose chromatography and the fractions were assayed for both PRAI and InGPs. In all cases the two activities were perfectly coincident with the ratio of the two activities constant in each fraction. An elution profile for the two activities in a representative organism from each type class is presented in Fig. 6.

A summary of the type patterns and the organisms represented in each class is presented in Table 2.

DISCUSSION

Although there is no system of genetic exchange available at the present time in most of the organisms studied here, it is possible to make some deductions concerning the organization of the genes responsible for synthesizing the tryptophan enzymes. It has been shown elsewhere (M. Largen, Ph.D. thesis, University of California, Riverside, 1972) that, in *E. liquefaciens*, *E. cloacae*, *E. hafniae*, *E. carotovora*, *C. ballerupensis*, and *P. vulgaris*, the tryptophan enzymes are derepressed coordinately as evidenced by linear Ames-Garry plots (1). Although we can make no definitive statement about operon structure with these data, it should be noted that in almost all cases of enzymes being synthesized coordinately, operon structure has been confirmed by genetic mapping. A notable exception to this generalization is the genes and their corresponding products for pyrimidine biosynthesis in *E. coli* (4). After this work was completed, genetic mapping revealed clustering of the tryptophan genes in *P. vulgaris* (8) and *S. marcescens* (H. Matsumoto, personal communication). The possibility should be considered that operon structure, if it does indeed exist for all the enteric bacteria, may represent an example of evolutionary convergence rather than derivation from a common ancestral stock. An argument against evolutionary convergence is the data we have presented previously (28) suggesting the presence of a second promoter in these organisms. If this is a case of evolutionary convergence, not only the gene cluster but also the second promoter would have had to have been selected. It would appear that the bacteria studied here were possibly derived from a common ancestor which possessed a tryptophan operon. Based upon the co-elution of PRAI and InGPs from both DEAE-cellulose and G-200 Sephadex, these two activities are probably contained in a single polypeptide in the organisms studied here. Thus we would hypothesize that the enteric common ancestor possessed within a tryptophan operon a single cistron coding for the two activities.

The various distribution patterns seen here are defined by differences in the AS and PRT of these organisms. The evolutionary event needed to evolve from an organism like *S. marcescens* with a free AS and PRT to an organism like *S. typhimurium* with an AS-PRT complex has already been proposed by several workers (16, 29, 44). What they propose is a simple gene fusion event which fuses the 20,000-dalton glutamine amidotransferase to the amino terminus of the *trpD* polypeptide. This could be accomplished if the terminating signal between two possibly adjacent cistrons were destroyed. The hypothesis has been strengthened by the amino acid sequences for the amino terminal portions of *S. marcescens* glutamine amidotransferase and *E. coli* component II, which show that more than 75% of the residues are conserved among the first 61 (29). Another possibility which exists for the evolution of these two molecules would be the introduction of a termination signal in the early part of the *trpD* cistron of an organism like *E. coli* which would produce an AS of the *Serratia* type, and the introduction of a translation reinitiation signal would provide a functional PRT. This is simply a reversal of the direction of evolution as proposed by other workers.

The relationship between organisms of types 3 and 4 and organisms of type 5 poses an interesting problem. Although they all have a free AS of 140,000 molecular weight, the PRT of types 3 and 4 is clearly 20,000 to 25,000 daltons larger than the type 5 enzyme. There are several possibilities for this difference. The PRT from type 5 organisms might be subject to proteolytic

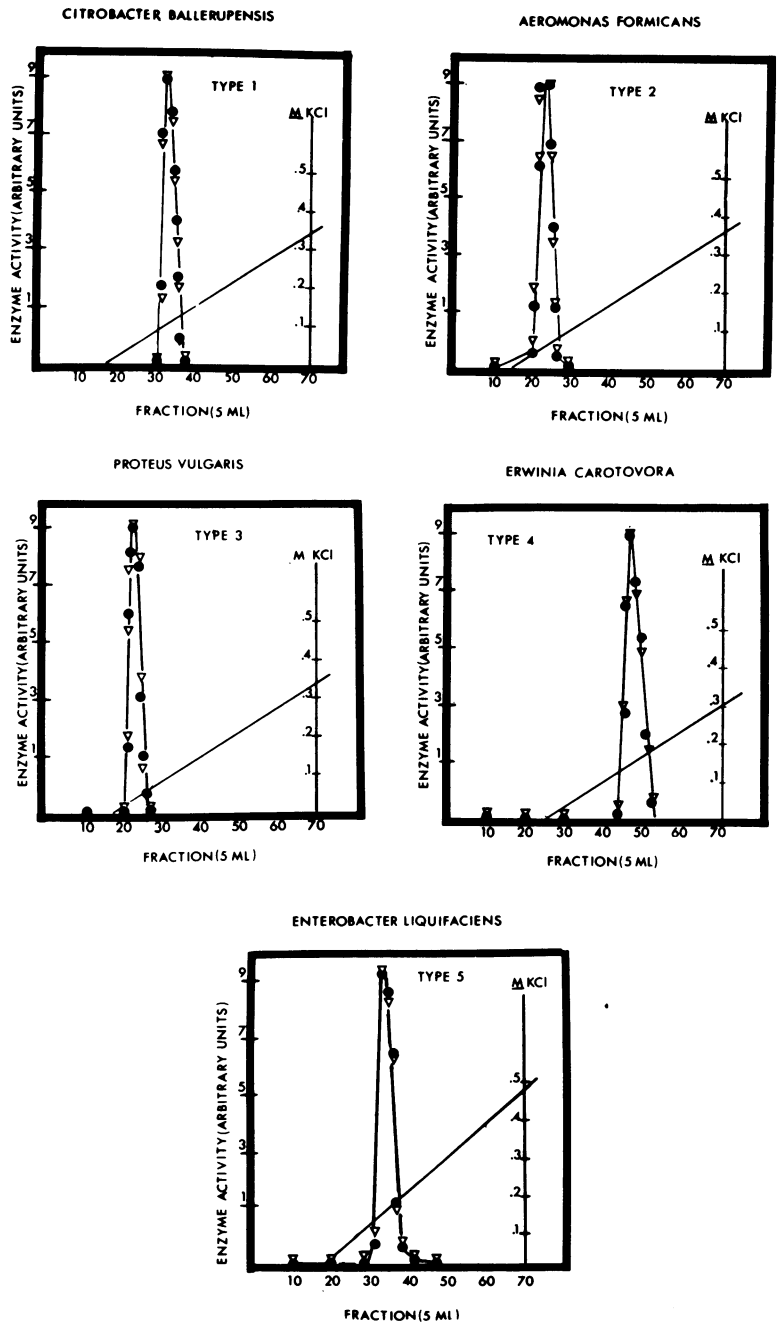

FIG. 6. *DEAE-cellulose profiles of PRAI and InGPs. Techniques are described in Materials and Methods. Symbols: ●, PRAI; ▽, InGPs. Activities in peak fractions (units per milliliter) are for C. ballerupensis PRAI (162), InGPs (9.1); A. formicans PRAI (40), InGPs (5); E. carotavora PRAI (63), InGPs (2.9); and E. liquefaciens PRAI (49), InGPs (11).*

attack under the extraction procedures used here. According to this hypothesis the in vivo PRT would actually be a molecule of 67,000 daltons, which might or might not be aggregated with AS, but then a specific clip would produce an enzyme of 45,000 daltons. There are two arguments against this hypothesis. Extracts prepared and chromatographed in the presence of the protease inhibitor phenylmethane sulfonyl fluoride (1 mM) also indicate a molecular

TABLE 2. *Summary of molecular distribution patterns of the tryptophan enzymes*[a]

Pattern no.	Organism	Mol wt × 10^3 daltons				
		AS (Gln)	AS (NH_3)	AS (Gln stim; GAT)	PRT	PRAI-InGPs
1	*E. cloacae* *C. ballerupensis* *C. freundii* *E. dissolvens*	Void and 250	Void and 250		Void and 250	47
2	*A. formicans*	220	220		67	45
3	*P. vulgaris* *P. morganii*	140	140	20	67	53
4	*E. carotovora* *E. hafniae*	140	140		67	48
5	*S. marcescens* *S. marinorubra* *E. liquifaciens*	140	140		45	45

[a] These data were obtained as described in the legend to Fig. 1. The word "void" in the table above indicates that a particular activity was found in the excluded volume upon elution from G-200 Sephadex; the probable molecular weight and molecular configuration of these activities are discussed in the text.

weight of 45,000 for the PRT. The sharp symmetrical peaks obtained in the absence of protease inhibitor also argue against this possibility. Another possibility which exists is that the common ancestor of the enteric bacteria may have had a 67,000 molecular weight PRT and the divergence came about as *Serratia* lost a portion of the genetic material corresponding to some portion of the PRT molecule. According to this scheme, *Serratia* diverged from the enteric common stock after the organisms of types 3 and 4. There is, of course, also the possibility that the enteric progenitor had a PRT of 45,000 daltons and the other organisms diverged with an insertion of genetic material into the *trpD* cistron. One other intriguing possibility is that the 67,000-dalton PRT has an amino terminal portion which has an ancestral relationship with the glutamine amidotransferase subunit of AS. By this scheme the glutamine subunit evolved in these organisms by a gene duplication of the amino terminal portion of PRT, thus allowing the nucleotide sequence for the amino terminus of PRT to diverge. Nonetheless, according to this hypothesis there should still be homology between the amino terminus of PRT and the glutamine subunit in these organisms.

In the above discussion of the evolution of PRT, the AS of groups 3 and 4 were lumped together. Although it is true that the AS of both types is 140,000 daltons, there is a rather fundamental difference since the *Proteus* AS dissociates to some extent during gel filtration. This behavior is reminiscent of the AS of various pseudomonad bacteria (36) and various members of the genus *Bacillus* (18, 27). A further characterization of the *Proteus* AS will be presented in a subsequent publication (M. Largen and W. L. Belser, manuscript in preparation).

It is difficult at this time to know where to place *A. formicans* in this evolutionary scheme. It possesses a 67,000-dalton PRT and presumably the PRAI-InGPs polypeptide, but the AS appears different from group 4 organisms. In five independent G-200 gel filtration experiments, the AS eluted at a position characteristic of 220,000 molecular weight. This may be a 140,000 dalton type AS capable of dimerization or it may be a reflection of an association with some other enzymatic activity. This molecular weight is the largest reported for an AS not associated with any other activity of the tryptophan pathway.

Our results have some relevance to the current classification schemes of the enteric bacteria. With the exception of *E. dissolvens*, all the type 1 bacteria have generally been considered to form a cluster of closely related organisms. It is interesting that DNA-DNA hybridization data obtained by Brenner and his colleagues (6) indicate that all type 1 organisms show at least 35% cross-hybridization with *E. coli* as a reference strain.

The type 3 organization, characteristic of *P. morganii* and *P. vulgaris*, was conserved within the genus even though the guanine-cytosine content of their DNA differs by 11% (39% G + C for *P. vulgaris* to 50% G + C for *P. morganii*). This conservation of the molecular organization of the tryptophan enzymes within a genus is also evident in the other type organizations. There are several exceptions to this generalization which are notable. *Erwinia* species are represented in types 1 and 4. Other workers have suggested that *E. dissolvens* should be

reclassified within the genus *Enterobacter* (7). Our data show that the molecular organization for the tryptophan enzymes for *E. dissolvens* is the same as that for *E. aerogenes* and *E. cloacae*. Unfortunately not all the *Enterobacter* species are represented by the type 1 organization. *E. hafniae*, previously known as *Hafnia alvei*, exhibits the type 4 pattern. Bascomb and co-workers (2), in a numerical taxonomical study of the tribe *Klebsiella*, suggested that *E. hafniae* was sufficiently distinct from other members of *Enterobacter* to merit reclassification as *Hafnia alvei*. *E. liquifaciens* is another anomaly in that the enzymes appear to be organized in the type 5 pattern characteristic of *Serratia* species. It has been known for some time that there is extensive cross-reaction between bacteriocins and lytic phages of *Serratia* species and *E. liquifaciens* (17). Bascomb et al. (2), in the above-mentioned study, concluded that the phenotypic similarities between *Serratia* and *E. liquifaciens* were statistically significant to the degree that *E. liquifaciens* should be reclassified as *Serratia liquifaciens*. Recently this conclusion was also reached by a different group of workers (14).

It should be emphasized that, because this study was conducted with crude extracts, the possibility exists that some of the patterns observed were due to the interaction of the tryptophan enzymes with proteins in crude extracts. Nonetheless, studies such as this provide a framework for a detailed examination of evolution at the molecular level. Furthermore, the techniques utilized provide an additional means for the resolution of taxonomic controversies.

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