BACTERIOLOGICAL REVIEWS, June 1975, p. 87-120
Vol. 39, No. 2

Printed in U.S.A.

Gene Rearrangements in the Evolution of the Tryptophan Synthetic Pathway

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INTRODUCTION

Knowledge of the natural relationships of the major bacterial families is clearly still in its infancy (119). It is not clear what effect, if any, the more recent molecular taxonomic approaches, exemplified in higher organisms by sequence comparison of proteins like cytochrome *c* (144), may have in the maturation of this subject. Since bacteria are predominately haploid and have a short generation time, it is not at all obvious that a meaningful outline of bacterial evolution will result from application of strategies that have been successfully employed with eukaryotic organisms.

This review is an attempt to summarize still fragmentary data on the comparative biochemistry and genetics of the tryptophan synthetic pathway, emphasizing work with various bacteria but not excluding eukaryotes. From these results one may develop a case for common ancestry for several of the major gram-negative and gram-positive bacterial taxa. This in turn suggests that the rate of accumulation of mutational substitutions and chromosomal rearrangements during evolution of this pathway has not been too rapid to obscure the natural affinities of the main bacterial families.

DESCRIPTION OF THE PATHWAY

Reactions

The de novo synthesis of tryptophan proceeds from chorismic acid (73), the last intermediate common to the aromatic amino acids and vitamins, through a series of reactions that has not varied in those prokaryotes and eukaryotes studied (Fig. 1). The first reaction is an enol-pyruvyl elimination accompanied by a glutamine amidotransfer and is catalyzed by the enzyme anthranilate synthase (AS; EC 4.1.3.27). In all organisms so far examined this complex enzyme contains at least two nonidentical subunits (225). As with other glutamine amidotransferases, under certain conditions ammonia can replace glutamine in the reaction giving anthranilate and pyruvate as products. This version of the reaction can usually be catalyzed by a single subunit of the enzyme and will be referred to as the chorismate "amination" reaction.

The second step in tryptophan synthesis is the addition of the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate to the 3-position of anthranilate, catalyzed by anthranilate phosphoribosyltransferase (PRT; EC 2.4.2.18). Although certain members of the *Enterobacteriaceae* have the enzymes for the first and second steps associated in an enzyme complex (11, 61, 106), this situation is not seen outside the enteric family and is not true even for some species within the family (96, 125a, 225).

The third step is an Amadori rearrangement of phosphoribosylanthranilate catalyzed by phosphoribosylanthranilate isomerase (PRAI), and the fourth step is a decarboxylation and ring closure catalyzed by indoleglycerol phosphate synthase (InGPS; EC 4.1.1.48). In all the enteric bacteria studied to date, the activities for both these steps are associated with a single polypeptide chain (125a, 139). In other bacteria two smaller proteins coded by separate genes carry out these functions.

The final step is the removal of the glycerolphosphate side-chain from indoleglycerol phosphate (InGP) and its replacement by the alanyl moiety of L-serine, catalyzed by the complex enzyme, tryptophan synthase (TS; EC 4.2.1.20) (216). The earliest known microbial multicomponent enzyme, this protein has been extensively studied, especially in *Escherichia coli*, and often reviewed (41, 118, 216). In enteric bacteria, each molecule consists of four noncovalently bound polypeptides, two α-chains primarily concerned with InGP aldolysis (producing indole and glyceraldehyde-3-phosphate), and two β-chains, each binding a pyridoxal-5'-phosphate cofactor molecule and primarily concerned with the synthesis of tryptophan from indole and serine. The enzyme complex normally functions without releasing indole into solution (long arrow in Fig. 1), but given the proper conditions and choice of substrates, the half-reactions of aldolysis (TS-A,

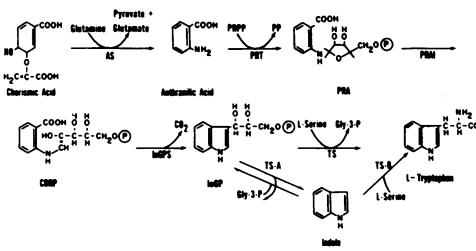

FIG. 1. *Biosynthetic pathway for tryptophan. Abbreviations: AS, anthranilate synthase (amidotransferase reaction is shown); PRPP, 5-phosphoribosyl pyrophosphate; PP, pyrophosphate; PRT, anthranilate phosphoribosyltransferase; PRA, N-phosphoribosylanthranilate; PRAI, phosphoribosylanthranilate isomerase; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose phosphate; InGPS, indoleglycerol phosphate synthase; InGP, indoleglycerol phosphate; Gly-3-P,* D-*glyceraldehyde-3-phosphate; TS, tryptophan synthase; TS-A and TS-B, see text.*

reversible arrows in Fig. 1) or tryptophan synthesis from indole (TS-B, short arrow in Fig. 1) can be observed at excellent rates. With minor variations, these general characteristics are shared by all bacterial tryptophan synthases studied.

One unusual feature of the tryptophan pathway is that it contains both phosphorylated and non-phosphorylated intermediates. Two of the latter, anthranilate and indole, are freely permeable to most bacterial and fungal cells. Thus, tryptophan auxotrophs lacking only the first enzyme of the pathway can grow on anthranilate or indole in place of tryptophan. Similarly, auxotrophs with a normal TS, or even an abnormal one lacking TS-A activity but retaining TS-B activity, can grow on indole. (It may be noted here that although indole is normally an enzyme-bound intermediate, TS either in vitro or in vivo utilizes it in preference to InGP [46, 50], probably effecting significant metabolic economies thereby).

Auxotrophs can often be characterized by the accumulation of compounds antecedent to their defective enzyme. TS mutants may accumulate InGP, indole-glycerol, indole, or a mixture of these, depending on their ability to perform the TS-A half-reaction; InGPS mutants accumulate the dephosphorylated form of the substrate of this reaction along with some anthranilate. Only mutants in steps 2 and 3, the PRT and PRAI reactions, cannot easily be distinguished from each other without an enzyme assay, for the lability of phosphoribosyl anthranilate causes mutants in either step to accumulate only anthranilate.

Regulation of Metabolite Flow

Feedback inhibition. Tryptophan may participate with other substances in the feedback regulation of chorismic acid formation, by inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthesis (71), and glutamine formation, by influencing glutamine synthase (213), but the major point of feedback control for the amino acid is on the enzyme catalyzing the first tryptophan-specific reaction, AS(165a, 196b). As this topic has been reviewed recently (71, 146, 225), little will be said about it here except to note that notwithstanding the diversity in molecular architecture of this enzyme observed throughout the prokaryotic and eukaryotic kingdoms, all forms of the enzyme have in common an effective feedback inhibition mechanism that operates equally well on the amidotransferase and amination reactions. Therefore, the allosteric binding site for tryptophan must be on the large, chorismate-binding subunit (196b), and it has been evolutionarily well conserved, all the way up to higher plants (14).

Repression of enzyme synthesis. Very early in the study of the tryptophan pathway in enteric bacteria, it was found that exogenous tryptophan represses the synthesis of TS (155). On the other hand, auxotrophs grown to tryptophan limitation are derepressed more than 10-fold above the wild-type level in the synthesis of any tryptophan pathway enzyme not ablated by the mutation (214, 215). Together, repression and derepression allow the specific activity of tryptophan enzymes in enteric bacterial extracts to vary over two orders of magnitude. In these bacteria the structural genes for the tryptophan pathway enzymes are clustered in one operon. The five gene products vary coordinately (105), and corresponding changes in the level of the polycistronic *trp* messenger ribonucleic acid (mRNA) are seen (101). *TrpR*, the unlinked structural gene for the tryptophan repressor, has been well characterized (38, 159), and it is clear that tryptophan and not tryptophanyl-transfer RNA (tRNA) is the effector in this negative transcriptional control system (160, 197). Not all bacterial groups employ this regulatory system, however. Some, such as the pseudomonads, have different controls for different parts of the pathway (42). Others, such as *Chromobacterium violaceum*, seem completely unable to alter the rate of synthesis of the *trp* enzymes in response to end product scarcity or excess (204). Repression of enzyme synthesis is, as we will see, a variable that is related to chromosomal location of the structural genes. It

thus seems a better source of information on natural relationships than the study of control by feedback inhibition, at least as far as concerns the tryptophan pathway.

Early Evolutionary Speculations

Soon after it was determined that the TS of enteric bacteria dissociates into dissimilar subunits whereas that of the ascomycete, *Neurospora crassa*, does not (51), Bonner et al. (17), impressed by the enzymological and genetic similarities shown by the two types of TS, speculated that gene fusion may have taken place at some time during the evolution of the fungi from their presumed prokaryotic progenitor. They recognized that there were discordant aspects to the situation; even then it was known that the green alga, *Chlorella ellipsoidea*, has a dissociable TS, and that the gene-enzyme relationships of the early enzymes of the tryptophan pathway in *Neurospora* were not, and still are not, wholly explainable on the basis of an evolutionary trend towards fusion of genetic elements of related function in eukaryotes. Nevertheless, their speculations concerning gene fusion now seem prescient in view of the results of the subsequent ten years. As we will see, gene fusions have certainly occurred during evolution of the tryptophan pathway, though the trend of evolution from prokaryotes to the higher eukaryotes has not necessarily always favored the condensation of genes.

ENTEROBACTERIACEAE

Operon Structure

Genetic studies. All members of the enteric bacterial family studied so far seem to have the genes for the tryptophan pathway clustered in one operon on the chromosome. The genetic evidence for this in *E. coli* (221) and *Salmonella typhimurium* (49) was first obtained through study of deletions and by co-transduction of structural gene point mutations with each other and with neighboring *cysB* mutants. Less direct evidence for gene clustering is available for *Serratia marcescens* (96), *Aeromonas formicans* (43), and several species of the genera *Citrobacter*, *Enterobacter* (*Aerobacter*), *Erwinia*, and *Proteus* (125, 125a). Most of this indirect evidence rests on coordinate regulation of the tryptophan enzymes, evidence of polarity and antipolarity (see below), and indications of the same low-level, operon-internal promoter found in the *E. coli* and *S. typhimurium* operons (10, 157). Although the number of enteric bacterial species examined so far is still small, the sample seems representative, including as it does species from each of the three major taxonomic subdivisions, *Escherichia-Salmonella-Shigella-Citrobacter* (G+C content, 50 to 52%), *Enterobacter-Serratia-Klebsiella-Erwina* (G+C content, 54 to 59%) and *Proteus-Providencia* (G+C content, 39 to 50%), as well as the taxonomically controversial *Aeromonas*. Direct genetic confirmation of *trp* gene clustering should be available for many of these species soon, for techniques of genetic exchange in all the enteric bacteria seem to be developing rapidly.

The *trp* gene order appears to be identical in all enteric bacterial *trp* operons. This is illustrated in the top two lines of Fig. 2, by using a "universal" nomenclature (187) for the *trp* genes rather than the varied ones that were proposed originally for different organisms. *TrpE* is closest to the promoter-operator region, is transcribed first, and encodes the large subunit of AS. Auxotrophic mutants in *trpE* are unable to perform either the glutamine amidotransferase version or the amination version of the AS reaction. *TrpD* is a complex gene in both *E. coli* and *S. typhimurium*, having its proximal third devoted to the glutamine amidotransferase AS function and its distal two thirds to the

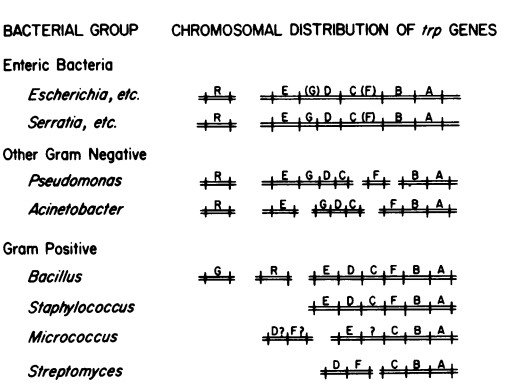

FIG. 2. *Tryptophan gene distributions in bacteria. A universal nomenclature (187) is used; analogous genes have the same letter designation in each organism. The trp prefix for all gene symbols has been omitted for simplicity. Gene-enzyme key: trpA, TS α-chain; trpB, TS β-chain; trpC, InGPS (except in enteric bacteria where the bifunctional PRAI-InGPS is produced); trpD, PRT (except in enteric bacteria of the Escherichia type where this activity and the glutamine amidotransferase function for AS are combined); trpE, AS large subunit; trpF, PRAI; trpG, AS small, glutamine amido-transferase subunit; trpR, tryptophan repressor. Not shown: trpS, tryptophanyl-tRNA synthase. In Acinetobacter the gene order in the three-gene clusters is not certain; the most probable order is shown. A question mark following a gene designation indicates doubt concerning its true location.*

PRT reaction. There is reason to believe (see "Subunit Structure" below) that these two segments of the *trpD* gene are two separate cistrons in *Serratia* and *Aeromonas*, so a more precise designation for the fused element in *Escherichia* and *Salmonella* might be *trp(G)D*. *TrpC* in all enteric bacteria is also a complex cistron, encoding in its proximal half the InGPS function and in its distal half the PRAI function. Missense mutants normally lose only one of the two activities, depending on their location (195). Other bacteria have separate cistrons for these two activities. We have called the gene for PRAI in other organisms *trpF*, so an unambiguous designation for *trpC* in enteric bacteria might be *trpC(F)*. *TrpB*, coding for the β-chain of TS, and *trpA*, coding for its α-chain, complete the operon. There are apparently no other structural elements associated with the enteric *trp* operon, for the messenger RNA size corresponds fairly closely to the length of deoxyribonucleic acid (DNA) required to encode these five polypeptide chains (180). There is, however, a transcribed "leader" region of some 160 base pairs lying between the operator and *trpE* that appears to be of regulatory significance (21a, 110).

The *trp* operon has opposite orientations in *E. coli* and *S. typhimurium* (185), being transcribed in a counterclockwise direction in the former organism and clockwise in the latter, as the map is usually drawn. This difference results from its being situated within an inversion now estimated to involve about 10% of the chromosome (31). There is no evidence that this inversion affects the transcription or translation of the *trp* genes in any significant way. Other members of the enteric bacterial group have not yet been examined for the presence of this inversion, which extends from 24 to 34 min on the *E. coli* map (31).

mRNA studies. The isolation of specific transducing lambdoid phages carrying part or all of the *E. coli* tryptophan operon on their chromosome has permitted detailed study of the mRNA transcribed in vivo or in vitro from this gene cluster (101, 179). Regulation of the level of the tryptophan enzymes in the cell is accomplished primarily by varying the number of mRNA transcripts of the operon. The details of synthesis and degradation of this typical polycistronic mRNA molecule have been recently reviewed (100) and are beyond the scope of this article. These details are not known to differ in any fundamental way in the several enteric bacterial species that have been studied. Of some interest is the occurrence of a second, low-level promoter within the distal third of the *trpD* structural gene (10, 108, 157). The activity of this second promoter is revealed when the operon is repressed and transcription beginning at the normal promoter is inhibited. Under these conditions (but not normally, see reference 105), enzyme production is not coordinate, and the gene products of the last three genes are produced at five times the basal rate of the first two genes. Whether this is of selective advantage to the organisms is unknown, but it is common to all enteric bacterial species in which it has been sought (125). Other, high efficiency promoters can be created within several of the structural genes of the operon (24, 138, 158, 207), frequently at the expense of the functional integrity of the polypeptide normally encoded there (24, 138, 158).

The hybrid phage-bacterial chromosomes used as molecular probes in detection and measurement of mRNA molecules in *E. coli* can be employed with other species as well. When these *E. coli* probes are used with RNA from five members of the enteric group (55), mRNA molecules of similar size but diminished hybridizing efficiency are found. Their weakened affinity for *E. coli* DNA, obviously due to base-pair mismatching, can be quantitated and compared with the amino acid sequence differences known for one region of the operon, the gene for the TS α-chain. When these measurements are made (132) the results show, as expected, an inverse relationship between the strength of hybridization and the number of amino acid differences. By calculation it appears, however, that at least twice as many base changes have occurred and been "fixed" as would be required to elicit the amino acid substitutions. Obviously "silent" base changes to synonymous codons can be evoked in explanation, and this illustrates how certain bacterial relatives having a similar chromosome organization and closely related amino acid sequences (see below) may yet have quite different DNA base ratios. It also explains why nucleic acid hybridization methods, useful though they may be in assigning various strains to certain taxonomic groups, are often too sensitive to be effective in measuring the relatedness of different bacterial families. Denney and Yanofsky (55) could see no significant hybridization between *E. coli trp* DNA and mRNA from *Bacillus subtilis*, for example, though it is easy to recognize a relationship in the amino acid sequence of their TS α-chains (134).

Regulatory mutations. Selection for resistance to tryptophan analogs such as 5-methyltryptophan very often results in the appearance of constitutive or partially constitutive strains

that coordinately overproduce all the enzymes of the tryptophan pathway (8, 38, 84, 159). These mutants are usually found either in the *trpR* locus near *thr* at 90 min on the *E. coli* map or in the operator region (*trpO*) of the tryptophan operon at 27 min (8, 84). As expected for the structural locus for a repressor protein, mutations in *trpR* conferring constitutivity may be either missense or nonsense and are recessive to the wild-type form of the gene (159). Similarly, as one would predict, constitutive mutants located in the operator region of the *trp* operon are cis-dominant in merodiploids (8, 84). Although it was first estimated by recombinational mapping that the operator region was rather large (84), more recent results suggest that it consists of a normal-sized repressor recognition region plus the "leader" segment mentioned earlier (21a, 110). Nucleic acid base sequence studies are under way in this region and should be available in the near future.

The protein product of the *trpR* gene has a molecular weight of about 58,000 (194, 197), does not appear to require interaction with tryptophanyl-tRNA or tryptophanyl-tRNA synthase for function (160, 197), and in the absence of its effector, tryptophan, binds to at least one other operator site in addition to *trpO*, the one affecting the tryptophan-sensitive isozyme of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase at 32 min on the *E. coli* chromosome (71).

Mutations in *trpS*, the gene for tryptophanyl-tRNA synthase at 66 min on the *E. coli* chromosome, indirectly produce a repressing effect on the tryptophan operon because mutants that permit some growth and protein synthesis usually accumulate a large intracellular pool of tryptophan (56, 71). A suggestion that the *trpS* gene product interacts with the *trpR* repressor (107) has not been confirmed in in vitro studies (197).

In addition to yielding constitutive mutations at the *trpR* and *trpO* sites, selection for resistance to 5-methyltryptophan in *E. coli* may produce mutants having fairly normal repression and derepression behavior; these mutants are linked to *argG* at 61 min and have been designated *mtr* for methyltryptophan resistance (85). The mechanism of their resistance remains unknown, though the three most interesting sites of interaction of tryptophan analogs with the tryptophan synthetic pathway, false feedback repression of AS, false repression of the *trp* operon, and inhibition of tryptophanyl-tRNA synthetase, are clearly unaffected in these mutants. In addition to the classes of tryptophan analog-resistant mutants described above, *S. typhimurium* exhibits yet another class of constitutive mutants unlinked to either the *trp* operon, the *trpR* gene, the *trpS* gene, *argG*, or the aromatic amino acid permease (8). These mutants have been tentatively designated *trpT*, although their map position is unknown and they may not even define a single locus.

Recently a new tool for study of the regulation of tryptophan genes in enteric bacteria has been reported by Hershfield et al. (83). They were able to study the function of a normal *E. coli trp* operon inserted artificially into the DNA of the Col E1 plasmid. In this way they ascertained that the single *trpR* gene normally produces sufficient repressor molecules to inactivate about 30 *trp* operators. It will obviously be of interest to study the function of such "extrachromosomal" *trp* operons in the presence of some of the regulatory mutations described above and to transfer operons from one species to another by using this technique.

Polarity and antipolarity. As very little is known on this subject outside the *Enterobacteriaceae*, the molecular mechanism is still in doubt (100), and as the subject has been competently reviewed by Margolin both as a phenomenon (145) and specifically with respect to the *trp* operon (146), there would seem little necessity for an extensive coverage of polarity here. It is a dramatic regulatory event, however, and can serve to establish the direction of transcription and translation as well as the extent of an operon. As such it could be useful in characterizing units functioning as operons when genes of the pathway are disassociated, as is the case in most bacteria outside the enteric group. The occurrence of polarity suppressor mutations, such as *suA* in *E. coli* (189), having the effect of abolishing the polarity phenomenon anywhere on the chromosome, opens up the possibility that this phenomenon may not be universal in different bacterial taxa, however. More information on this point would be very valuable.

Early studies of nonsense mutations in the *E. coli trp* operon showed that when these are located proximally in a structural gene (*i.e.*, at the end nearest the *trp* operator) they have a depressing effect on the amount of protein produced from all the more distal genes of the operon (105, 150, 219). The polarity "gradient," the intensity of this effect as a function of position of the mutation within the gene, has been accurately determined for all of the relevant genes of the tryptophan operon, *trpE,D,C*, and *B* (218). Similar but less extensive data exist for the *trp* operon of *S. typhimurium* (11, 16). In each case polarity is more drastic with *trpE* nonsense mutants than with mutants in

other genes. Frameshift or deletion mutants that result in premature termination of translation followed by an appreciable length of untranslated message are as polar as analogous nonsense mutations, and polarity is associated with absence (100) or hyperlability (86) of that portion of the polycistronic mRNA distal to the site of the nonsense mutation. The precise mechanism of polarity and polarity suppression remains controversial, however (100, 120).

Early studies of polarity in the *trp* operon also revealed an apparent decrease in function of the gene immediately proximal to that occupied by the nonsense mutant as well as distal ones. Unlike the distal polarity effects, this was usually a moderate diminution, and it never extended back more than one or at most two genes (7, 105, 220). It was termed a "short range antipolar" effect, and was noticed with both point mutants and deletions. The degree of antipolarity seen with nonsense mutants in *trpA* was proportional to their location in the gene (220), just as is the case with polar mutants in the rest of the operon (218). More recent work has failed to demonstrate significant antipolarity from nonsense mutants in any gene but *trpA*, the last gene of the operon (218). When sought in the second and third genes of the nine-gene histidine operon of *S. typhimurium*, no antipolarity was found (68). Thus, the status of antipolarity as a regulatory phenomenon is anything but clear. Ito (104a) showed that during tryptophan starvation genes nearest the operator are preferentially expressed. Varied results obtained in different studies may reflect variations in strains, growth conditions, and techniques used for derepression. Factors such as these have also been evoked as reasons for discrepancies in studies of polarity and mRNA production (100).

Although the mechanisms bringing about polar and antipolar effects are still in doubt, it should not be assumed that these phenomena are inconsequential. In the enteric bacteria a polar mutation may drastically impair the ability of a strain to use anthranilate or indole as a precursor of tryptophan, though the genes for the necessary enzyme are intact and can be released from their functional stranglehold by any of several direct or indirect genetic events (7, 24, 100, 120, 158, 207).

Deviations from Coordinacy

Several deviations from coordinate production of the tryptophan operon gene products have already been mentioned. Though the five gene products are produced in approximate equimolarity under conditions of moderate or extreme derepression, the last three enzymes are produced at about five times the basal rate of the first two during full repression (10, 157) because of the second, low-level promoter in *trpD*. Mutational events within the operon may abolish coordinacy, as when a new, high-level promoter is created in an internal gene (138) or a polar nonsense mutation occurs (16, 101, 105, 218, 219). An interesting additional example of noncoordinate synthesis is seen when an ordinary auxotroph such as a missense or nonpolar nonsense mutant is starved for tryptophan (104a, 196a, 219). Under these circumstances the *trpE* and *trpA* products, and to a lesser extent the *trpD* product, continue to be synthesized (albeit slowly) after the total cell mass stops increasing due to tryptophan limitation, but the amount of *trpC* and *trpB* gene products increases little if at all. The reason for this lack of coordinacy is not known with certainty, but it is possible that the few molecules of tryptophan that do become available under these circumstances (from proteolysis, for example) are preferentially used by ribosomes translating the proximal genes. Since the α-chain of TS lacks tryptophan, ribosomes beginning at the *trpA* initiation site can synthesize the entire gene product, however, Some support for this hypothesis comes from the work of Brammer (21), who showed that α-chains containing tryptophan at residue 212 as a result of mutation are not synthesized at all during tryptophan starvation.

Subunit Structure of Enzymes and Amino Acid Sequences

Primary sequence analysis of the α-chain of TS has been accomplished for three enteric bacterial species (76, 136, 137), and partial, amino-terminal sequences are available for two others (133, 135). These data are summarized in Fig. 3. It is apparent that *S. typhimurium* and *Enterobacter aerogenes* both differ from *E. coli* in about 15% of their residues, and they differ from each other to a similar extent. *S. dysenteriae* differs from *E. coli* in only one of the first 50 residues analysed, whereas *S. marcescens* is somewhat more distant from *E. coli* than *S. typhimurium* and *E. aerogenes*, with nine changes in the first 28 residues. It is worth noting in passing that none of these amino acid substitutions affect the nine sites within the polypeptide chain where inactivating missense mutations have been found in *E. coli* (217). There is a noticeable clustering of evolutionary substitutions between residue 243 and

A.

Residue	2	6	12	13	15	24	30	39	42	43	52	56	66	68	91	97	104	108
E. coli	Gln	Ser	Lys	Glu	Lys	Thr	Ile	Thr	Glu	Ala	Ile	Asp	Asn	Thr	Lys	Ile	Asn	Asn
S. typhimurium	Glu	Asn	Asn	Asp	Arg	↓	↓	↓	Asp	↓	Val	Asn	↓	Asn	Asn	Val	Lys	Ser
E. aerogenes	Gln	Thr	Lys	Lys	↓	Ile	Thr	Ala	Glu	Gly	Ile	↓	Gly	Ala	↓	Ile	↓	↓

109	113	114	117	119	120	134	144	148	166	180	189	194	197	198	201	204	208	216	218
Lys	Glu	Phe	Gln	Glu	Lys	Gln	Leu	Val	Ile	Ala	Ala	Asn	Val	Ala	Lys	Asn	Pro	Ala	Asp
Pro	↓	Leu	Arg	↓	Gln	↓	↓	Ile	Val	Ser	Gly	His	Ile	Glu	↓	His	Ala	Ser	Glu
↓	Ala	Phe	Gln	Ala	Arg	Glu	Met	↓	Ile	Ala	Ala	↓	Val	↓	Ala	↓	Pro	Ala	↓

221	225	243	244	245	246	247	249	250	253	254	256	257	260	261	262	266	267	268	269
Lys	Asp	Gln	His	Asn	Ile	Glu	Glu	Lys	Ala	Ala	Lys	Val	Gln	Pro	Met	Thr	Arg	Ser	Δ
Val	Arg	Lys	Asn	Leu	Ala	Ser	Lys	Gln	↓	Glu	Arg	Ser	Ser	Ala	↓	Ser	↓	Ala	Δ
Ser	Asp	Arg	His	↓	Asp	Glu	Gln	Thr	Asp	↓	Lys	Ala	Gln	Ser	Leu	Thr	Lys	Thr	Ala

B.

Residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
E. coli	Met	Gln	Arg	Tyr	Glu	Ser	Leu	Phe	Ala	Gln	Leu	Lys	Glu	Arg	Lys	Glu	Gly	Ala	Phe	Val	Pro	Phe	Val	Thr	Leu	Gly	Asp	Pro
S. dysenteriae	↓	Glu	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
S. marcescens	↓	Glu	↓	↓	Gln	Gln	↓	↓	Lys	Arg	↓	Glu	Ser	Asn	Lys	↓	↓	↓	↓	↓	↓	↓	↓	↓	Ile	↓	↓	↓
P. putida	Δ	Ser	↓	Leu	Glu	↓	Arg	↓	Ala	Glu	↓	Lys	Ala	Glu	Gly	Arg	Ser	↓	Leu	↓	Ile	↓	↓	↓	Ala	↓	↓	↓
B. subtilis	Δ	Δ	Δ	Δ	Δ	Δ	Met	↓	Lys	Leu	Asp	Leu	Gln	Ala	Ser	Glu	Lys	Leu	Phe	Ile	Pro	↓	Ile	↓	↓	↓	↓	↓

Residue	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
E. coli	Gly	Ile	Glu	Gln	Ser	Leu	Lys	Ile	Ile	Asp	Thr	Leu	Ile	Glu	Ala	Gly	Ala	Asp	Ala	Leu	Glu	Leu	Gly	Ile
S. dysenteriae	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	X	X
P. putida	↓	Tyr	Asp	Ala	↓	↓	Gln	↓	Leu	Lys	Gly	↓	Pro	Ala	↓	↓	↓	↓	Val	Ile	↓	↓	Gly	X
B. subtilis	Ser	Pro	Glu	Val	↓	Val	Lys	Leu	Ala	Pro	Ser	↓	Glu	Val	↓	↓	Val	Thr	Lys	Leu	↓	↓	↓	Val

FIG. 3. *Amino acid sequences for the α-chain of tryptophan synthase. (A) Positions of divergence in the total sequences of E. coli (268 residues), Salmonella typhimurium (268 residues), and Enterobacter aerogenes (269 residues). (B) Positions of divergence in the N-terminal portions of E. coli (52 residues shown), Shigella dysenteriae (50 residues known), S. marcescens (28 residues known), Pseudomonas putida (50 residues known), and B. subtilis (46 residues known). Symbols: ×, unknown amino acid; Δ, absence of an amino acid; and ↓, identical with the residue above.*

the end of the chain, suggesting a lesser degree of structural constraint near the carboxy-terminus of the protein.

As yet only a portion of the primary structure of the β-chain of *E. coli* TS is known, and no sequences from other enteric bacteria are available for comparison. It has been known for a long time that α and β chains from *E. coli*, *S. typhimurium*, and *S. marcescens* are interchangeable and active in any combination (6), though the affinity of the *S. marcescens* enzyme is somewhat less for a complementary subunit from *E. coli* or *S. typhimurium* than for its own homologous one (178). An immunological approach to measuring the evolutionary divergence of various enteric bacterial TS molecules gave results in accord with the sequences of the α-chain (161, 178).

Although sequences are not yet available for the *trpC* gene product, a single polypeptide of 45,000 molecular weight with both PRAI and InGPS activities, the proteins from *E. coli*, *S. typhimurium*, and *E. aerogenes* have been purified to near homogeneity (139). Trypsin plus chymotrypsin peptide patterns of these three molecules were examined and found to be about as similar in appearance as corresponding maps of the TS α-chains from the same species (139). The enzyme from *S. marcescens* is also very like the above in its activities and molecular weight (96).

As described earlier, the AS and PRT activities reside in a single, large complex enzyme in *E. coli*, *Salmonella sp.*, *E. aerogenes*, and *Erwinia dissolvens*, whereas they are separate in *Proteus sp.*, *Erwinia carotovora*, *Erwinia hafniae*, *Serratia sp.*, *Enterobacter liquefaciens* and *A. formicans* (125a). From genetic and biochemical evidence, several authors (75, 226; reviewed in reference 225) have proposed that the *E. coli* type of enzyme has resulted from fusion of two polypeptides that are separate in the other species, a small one of 20,000 molecular weight that binds to and provides the glutamine amidotransferase function for AS, and the 45,000 molecular weight PRT enzyme. Direct evidence for this hypothesis was provided by Li et al. (133a), who sequenced the amino-terminal regions of the fused protein (*trpD* gene product) from *E. coli* and *S. typhimurium* and the small AS subunit from *S. marcescens*. The former two were very similar, as expected, differing only in three of the first 53 residues, but homology was quite apparent in the latter also, which differed from the *E. coli* sequence in only 14 of the first 61 residues. This work provides documentation

for the occurrence at least once during the evolution of the *Enterobacteriaceae* of a gene fusion similar in result to that created in the laboratory by the fusion of two *his* genes by Yourno et al. (223).

As we will see later, the *trpC* gene product also appears to be a fusion product of two smaller genes that are separate in other bacterial taxa. Since this fused, bifunctional polypeptide is invariant in all the *Enterobacteriaceae* studied to date, fusion seems to have taken place in a precursor to many or all of the surviving forms assigned to the group. We will also see later that gene fusion has apparently not been confined to the enteric bacterial group, but can also be deduced to have occurred during the evolution of fungi.

Organisms Unable to Synthesize Tryptophan

For over forty years it has been known that most or all strains of *Salmonella typhosa* require tryptophan for growth but can give rise to tryptophan-independent variants (67). The same is apparently true for some strains of *Salmonella cholerasuis* and *Salmonella paratyphi* A (126). In all three *Salmonella* species, this tryptophan requirement is often associated with auxotrophy for the sulfur-containing amino acids cysteine or methionine (126). Many, perhaps most, strains of *S. dysenteriae* also require tryptophan, cysteine, and sometimes other amino acids (115). It was the observation of Fildes (66) that indole could replace tryptophan as a growth supplement for *S. typhosa* that led to the first use of tryptophan auxotrophs in various microbes as subjects for gene-enzyme investigations.

The only relatively recent investigation of a naturally occurring defect in the tryptophan synthetic machinery of an enteric bacterium is that of Eisenstein and Yanofsky (62) using the avirulent *S. dysenteriae* strain Sh/S. They were able to show a partial requirement (bradytrophy) for tryptophan traceable to the first gene of the operon; this requirement was satisfied equally well, of course, by anthranilate or indole. This laboratory line of *Shigella* quite obviously has undergone some adaptations. Like many of the auxotrophic pathogens mentioned above, however, it gives rise to tryptophan prototrophs by reversion. When its bradytrophic character was transduced into *E. coli*, it resulted in derepression during growth on minimal medium of TS and perhaps the *trpD* and *trpC* gene products as well (62). In the parental *S. dysenteriae* strain carrying this block, no derepression of these late pathway enzymes was seen. When one considers the great similarity in the genes and enzymes of *S. dysenteriae* and *E. coli* (135), this unexpected finding begs for an explanation. Other interesting, unanswered questions attract attention also. Do the auxotrophic mutations in the *Salmonella* species also reside in *trpE*, and are they polar? Do commensal enteric bacteria show an appreciable frequency of auxotrophy or is this restricted to pathogens? Does auxotrophy ever result from a deletion of all or part of the *trp* operon? These and other questions should not have to wait much longer before molecular biologists interested in pathogens decide to study them.

OTHER GRAM-NEGATIVE BACTERIA

Pseudomonadaceae

Multiple operon trp gene arrangement. *Pseudomonas* strains were the first bacterial exceptions to the single operon *trp* gene configuration studied in any detail. In their pioneering efforts, Fargie and Holloway (65), studying mutants of *Pseudomonas aeruginosa*, noted that certain genes which are clustered in the enteric bacteria, notably those for the histidine, leucine, and tryptophan biosynthetic pathways, are separate on the *Pseudomonas* chromosome. In these early studies by the Australian group not all the *trp* loci were represented or distinguished; nevertheless it was clear that there must exist at least three *trp* gene locations unlinked to each other in transduction experiments. Studies in a closely related fluorescent pseudomonad, *P. putida*, soon established (77) the disposition of the *trp* genes shown in Fig. 2. (The assignment of *trpG* to the position shown is still somewhat conjectural, for no mutants affecting the small AS subunit have been obtained; however, strictly coordinate synthesis of both AS subunits has been reported [173]).

A general description of the genetics of the fluorescent pseudomonads is beyond the scope of this review. The topic has been reviewed by Holloway (93), and a general chromosome map has been formulated. At present there is no discernable similarity between the maps of the enteric species and the pseudomonads. The three *trp* gene clusters are assigned locations 15 to 20 min apart on the approximately 70-min *Pseudomonas* chromosome (93).

The genetic structure differs and, similarly the patterns of regulation of enzyme synthesis in *Pseudomonas* (e.g., in the pyrimidine [103] and isoleucine-valine [147] pathways), show little similarity to those seen in enteric bacteria. Considerable effort has been devoted to study of the genetics and regulation of the extensive pathways of catabolism of diverse carbon and

energy sources in strains of *Pseudomonas*. In contrast to biosynthetic pathways, clustering of functionally related dissimilatory genes is common (127, 181), even though different members of the gene cluster may show independent regulation. Genes for initiating the degradation of certain classes of organic compounds, including camphor, salicylate, and naphthalene, are found on transmissible plasmids rather than in chromosomal clusters (35, 60, 175). In the case of the camphor pathway, it appears that certain enzymes concerned with isobutyrate utilization late in the pathway may be duplicated, having both a chromosomal and plasmid-borne version (78). It has not escaped the attention of workers in this area that clustering of the genes for degradative functions may be advantageous for the pseudomonads, allowing an enormous repertory of dissimilatory enzymes to be maintained in one or another fraction of the population, to be amplified by plasmid replication or genetic transmission when conditions favor it (127, 175). The nearly ubiquitous ability of members of this genus to grow on mineral salts when supplied with an oxidizable carbon and energy source suggests that an adequate biosynthetic apparatus is independently maintained. It is probably significant that all of the chromosomal dissimilatory pathway gene clusters appear confined to one segment representing only 10 to 15% of the total chromosome (127).

Although the chromosomal disposition of the genes of several biosynthetic pathways appears similar in *P. aeruginosa* and *P. putida* (93), it can not yet be assumed that their genomes are as uniform overall as those of many members of the enteric group, for the genetic analysis of *Pseudomonas* is at a rather primitive level. What follows concerning subunit composition and regulation of the tryptophan enzymes has proved fairly uniform in the few species studied, but does not justify the conviction that there is a monotonous genetic structure underlying this highly varied taxon (166).

Subunit structure of enzymes and amino acid sequences. These aspects have been better examined in *P. putida* than in other species. This was the first organism found to have discrete enzymes for the third and fourth steps of the pathway. The sum of their sizes (molecular weight of 39,000 for PRAI and 32,000 for InGPS; [63]) is greater than the size of the bifunctional enteric bacterial protein (molecular weight 45,000). Amino acid sequences are not yet available to determine their exact homologies. Assuming that the *Pseudomonas* PRAI and InGPS will be homologous with the amino-terminal and carboxy-terminal halves, respectively, of the enteric bacterial protein, it will be interesting to see the nature and location of the extra amino acids in *Pseudomonas*. These may give a clue to whether the two *Pseudomonas* proteins arose by a duplication followed by differentiation of an *E. coli*-like gene, or whether, as seems more likely a priori, the enteric bacterial gene arose as a fusion of the completely independent *Pseudomonas* genes, with loss of any unnecessary segments.

P. putida was also the first bacterium found to have a small, dissociable glutamine amidotransferase AS subunit (172). Both the large (*trpE*, molecular weight 63,400) and small (*trpG*, molecular weight 18,000) subunits have been obtained in pure form from *P. putida* (173); less purified preparations from *P. aeruginosa*, *P. acidovorans*, *P. stuzeri*, *P. multivorans*, and *P. testosteroni* have also been studied. All six *Pseudomonas* species form active hybrid AS molecules, but the native complex from the first two has a molecular weight of 79,000, whereas that of *P. acidovorans* and *P. testosteroni* is above 150,000, suggesting $\alpha\beta$ and $\alpha_2\beta_2$ structures, respectively.

At the moment there are two equally unsupported and diametrically opposite reasons advanced for the failure to find tryptophan auxotrophs lacking the small AS subunit. The first is that this gene product is also used as a glutamine amidotransferase in other pathways, so multiple metabolic defects would result from its loss; in support of this possibility, the sharing of a single small subunit for both *p*-aminobenzoate and anthranilate synthesis in *Acinetobacter calcoaceticus* and *B. subtilis* (see below) may be adduced. The other possibility is that activity in the chorismate amination reaction catalyzed by the large AS subunit alone is sufficient to permit growth of a small subunit mutant on minimal medium without tryptophan. It has been found (72) that whole cells of an *E. coli* glutamine auxotroph can use ammonia as the nitrogen source for tryptophan synthesis. Whether either of these hypotheses is correct remains for future study.

The possibility that a simple fusion of the *trpEGDC* segment to one end of *trpF* and *trpBA* segment to the other, creating a single operon of the enteric type, is a fair approximation of an event that actually occurred in bacterial evolution may someday be testable; some indirect support for it might come from amino acid sequence analyses showing homology between the small AS subunit of *Pseudomonas* and that of *Serratia*, known (133a) to be homologous to the amino-terminal portion of the *E. coli trpD* gene product, or between the small *Pseudo-*

monas InGPS and PRAI enzymes and the two halves of the enteric *trpC* gene product. The feasibility of such sequence studies is underscored by the similarity shown between the *TS* α-chains of *P. putida* and the enteric bacteria (Fig. 3) (44). Their sequence homology extends from residue 2 (the N-terminal methionine residue present in all enteric bacterial α-chains is absent in *P. putida*, probably because of an intracellular aminopeptidase capable of cleaving a methionylserine bond rather than a fundamental genetic nonhomology) through residue 50, the end of the known sequence, without deletions or insertions. The sequence seems best conserved in residues 21 to 28 and 42 to 50, segments surrounding the two residues known to mutate to give inactive missense proteins in *E. coli* and suspected, therefore, to be important for the active site (41, 216).

Thirteen of the 24 amino acid differences in the *P. putida* and *E. coli* α-chains require more than a single-base change in the codon. Analysis of the sequence data shows that the *P. putida* sequence differs more from the known enteric bacterial sequences than any enteric organism's differs from another (135), but suggests that all must have evolved from a common ancestral gene, despite the fact that the chromosomal organization of the *trp* genes in *Pseudomonas* and the *Enterobacteriaceae* is so different.

Regulation of enzyme synthesis. Three distinct and apparently unrelated regulatory situations prevail in the tryptophan pathway of *P. putida* (42) and *P. aeruginosa* (23). Each chromosomal location seems associated with a unique regulatory mechanism. The products of the *trpE*, *G*, *D*, and *C* genes respond to repression by tryptophan, whether exogenously supplied or endogenously synthesized, much as the entire enteric bacterial operon does. Mutations at an unlinked regulatory gene can be selected through their resistance to 5-fluoroindole or 5-fluorotryptophan (152). These mutations result in constitutive overproduction of the two AS subunits, PRT and InGPS, with no discernible influence on enzymes in the remainder of the pathway. In general these tryptophan analog-resistant mutants of *Pseudomonas* resemble *trpR* mutants of *E. coli* and *S. typhimurium*; whether in fact they affect a tryptophan repressor protein interacting with an operator region for the *trpEGDC* cluster remains to be proved. The most meticulous study of regulation of this segment of the pathway was performed by Queener et al. (173), who followed derepression during tryptophan starvation of a *trpF* mutant of *P. putida*. The two AS subunits increase coordinately with each other but not in perfect unison with the *trpD* and *C* gene products, for at the end of 3 h the former two polypeptides had increased more than ninefold over the repressed value while the latter two had increased only half as much. The fine structure map of the mutants in this area shows an appreciable gap between the *trpE* and *trpD* mutants (77), but whether this space is real or artifactual and whether regulatory as well as structural gene (*trpG*) elements might occupy it remains to be seen.

The solitary *trpF* gene of *P. putida* and *P. aeruginosa* does not respond to tryptophan scarcity or excess, nor is it affected by regulatory mutations affecting the remainder of the pathway (23, 42, 152). That is not to say that all strains and species have identical enzyme levels, but whatever level a given strain may have, it seems fixed and unable to respond to scarcity or excess of the end product. In this respect *trpF* resembles all the enzymes of the pyrimidine pathway in *P. aeruginosa* (103) and all the tryptophan enzymes of *C. violaceum* (204).

Probably the most interesting regulatory situation in the *Pseudomonas* tryptophan pathway, one that is unique so far among the bacteria, is the inducibility of tryptophan synthase by its substrate InGP (42). Both the α- and β-chains of this enzyme remain at a low, basal level in *P. putida* cells undergoing tryptophan starvation if InGP is absent, and tryptophan even in large excess will not prevent induction of the enzyme if InGP is present. Similar results were found for *P. aeruginosa* (23). Mutations leading to constitutivity of the *trpA* and *B* genes always lie in close proximity to this gene pair and frequently result in loss of the enzyme activity associated with the *trpA* gene product (42, 77). This has led to the hypothesis (167a) that the *trpAB* gene pair exemplifies the phenomenon of autogenous regulation (74), with the α-chain forming at least part of the repressor protein. Proof or disproof of this hypothesis should be readily obtainable in the near future as improvements in genetic methodology become available for the *Pseudomonas* group.

Acinetobacter

Multiple operon arrangement of genes. Members of the genus *Acinetobacter* are gram-negative, oxidase-negative, nonflagellate, short rods or diplobacilli found ubiquitously in soil. Modern taxonomy assigns them to the *Neisseriaceae*, a group of diplococci and diplobacilli quite distinct from the pseudomonads or the enteric bacteria (81). Among their rela-

tives are the oxidase-positive *Moraxella* and some organisms originally called *Neisseria*, especially *Neisseria catarrhalis*, *N. ovis* and *N. caviae* (12, 13, 81). All members of the *Acinetobacter* genus can serve as DNA donors for transformation of certain receptor strains of *A. calcoaceticus* (113); transduction also occurs in this genus (82). Transformation is also frequent among *Moraxella* and *Neisseria* strains (20, 34). Though Bövre (18) and Catlin (33) demonstrated frequent "intergeneric" transformation between certain *Moraxella* species and the *N. catarrhalis* group, *Acinetobacter* DNA seemed capable of transforming only one trait, streptomycin resistance, into oxidase-positive species, and that only at low frequencies (19). Juni (112) confirmed this feeble exchange of the streptomycin resistance marker between oxidase-positive *Moraxella* and oxidase-negative *Acinetobacter* in the reciprocal direction, but he was unable to show a similar transformability of auxotrophic markers in the tryptophan and isoleucine-valine pathways. The present conclusion (81) is that *Acinetobacter*, represented by a single species, *A. calcoaceticus*, is a distant relative of the moraxellas and the "false" neisserias (*N. catarrhalis*, *N. ovis* and *N. caviae*), and still more distantly related to *Neisseria gonorrhoeae* and *N. meningitidis*.

The relationships just described in the *Acinetobacter-Moraxella-Neisseria* group discerned through conventional taxonomic (12, 13) and genetic (19, 112) means are in agreement with the results of nucleic acid homology studies (114).

Although the general chromosome organization in *Acinetobacter* and *Moraxella* is still unknown, it is known that the six *trp* genes of *A. calcoaceticus* are dispersed in three regions, no two of which can be carried on the same transforming DNA molecule (187). The assignment of the genes to each location is indicated in Fig. 2. Even though exact gene order within clusters has not been determined, it is clear from the distribution that the *trp* gene arrangement is quite unlike that in *Pseudomonas*.

The gene for the large AS subunit is alone, those for the small AS subunit, PRT and InGPS are linked, and the gene for PRAI is linked to the pair encoding the TS subunits. Mutations in *trpG*, the gene for the small AS subunit, are remarkable for conferring an absolute requirement for *p*-aminobenzoate (or folate) in addition to tryptophan (188). It is interesting that both in *A. calcoaceticus* and in *B. subtilis*, where the small AS subunit has also been implicated in *p*-aminobenzoate synthesis (116), the genes for the two AS subunits are unlinked.

If the *trp* genes of *A. calcoaceticus* are in the order shown in Fig. 2, two translocations, with the correct fusion of *trpC* and *trpF* and the loss of unnecessary control regions, would be capable of creating the enteric bacterial *trp* operon. To evolve from the *Pseudomonas* to the *Acinetobacter* gene order also involves a minimum of two translocations, so Occam's razor does not suggest any one pathway of evolution of the gram-negative bacterial families studied so far.

Investigation of the tryptophan genes and enzymes in *Moraxella osloensis* has just begun. Preliminary results suggest that the chromosomal *trp* gene disposition there is the same as in *A. calcoaceticus* (P. A. Buckingham and E. Juni, personal communication).

Subunit structure of enzymes. Twarog and Liggins (202), in the first study of the tryptophan enzymes of *A. calcoaceticus*, determined that the five enzyme activities are readily separable. The PRAI and InGPS enzymes appear to be smaller than 30,000 in molecular weight by gel filtration and zonal centrifugation in sucrose gradients; PRT is larger at 42,500 to 51,000 and AS larger still at 80,000 or more. Subsequent to their work it was shown that the AS molecule can be dissociated into a large (*trpE*) subunit of 70,000 molecular weight and a small one (*trpG*) of 14,000 (188); the molecular weight of the AS complex in 30% glycerol was estimated to be 86,000, suggesting an $\alpha\beta$ structure. *Acinetobacter* TS has not received much attention so far. The little that has been learned is compatible with the occurrence of a readily dissociating $\alpha_2\beta_2$ molecule (187, 202).

In *N. crassa* and several other fungi, the enzymes of both the tryptophan and the common aromatic amino acid synthetic pathways have associations quite unlike those of bacteria. *A. calcoaceticus* possesses, like *N. crassa*, a hydroaromatic degradation pathway leading from quinic or shikimic acid to protocatechuic acid (200), and also possesses, as does *N. crassa*, two dehydroquinases, one inducible in the degradative pathway, the other constitutive and used for biosynthesis. Although the constitutive type occurs in a five-enzyme complex in *Neurospora*, these same enzymes are separate in *A. calcoaceticus*, as is typical for all other bacteria (15).

Regulation of enzyme synthesis. In view of the unusual diversity of regulatory mechanisms for the three *trp* gene clusters in *Pseudomonas*, it is obviously of considerable interest to know

how the *trp* genes are regulated in *Acinetobacter* and *Moraxella*. The first investigations along this line led to the conclusion that the AS of *A. calcoaceticus* does respond to tryptophan excess or scarcity, being derepressible as much as 20-fold when certain auxotrophs are starved for tryptophan, but that the other enzymes of the pathway are largely unresponsive (202). More recent work has forced a revision of this interpretation.

As in *B. subtilis*, certain of the tryptophan synthetic enzymes in *A. calcoaceticus* are unstable in extracts but can be stabilized by adding glycerol. This is particularly true for AS and PRT. When all gene products are assayed in stabilized extracts of *trp* auxotrophs blocked at various points along the pathway, measurable derepression occurs for all seven polypeptides (39). The increase found for the first five gene products in the pathway ranges from 5- to 15-fold, whereas that found for the *trpA* and *trpB* gene products is only 1.5- to 3-fold. In experiments similar to those that easily demonstrated the inducibility of TS by InGP in *Pseudomonas*, no obvious inducibility of any of the gene products by any of the pathway intermediates was seen.

The operators for *trpE* and the *trpGDC* cluster appear to respond similarly to regulatory perturbations, resulting in a similar level of expression for all four of these early gene products. The situation in the *trpFAB* cluster is more complex, however. When wild-type *A. calcoaceticus* is shifted from minimal medium without tryptophan to tryptophan excess, only the *trpF* gene responds by a decreased level of expression (39). Apparently *trpF* is the only *trp* gene that is not maintained near its maximally repressed level when the cell is growing "normally" and making its own tryptophan. Moreover, when an auxotroph is starved for tryptophan, the *trpF* gene product increases three- to sixfold, whereas the expression of *trpA* and *trpB* is augmented only 1.5- to 3-fold (39). This is suggestive evidence for the existence either of two operators for the *trpFBA* cluster or for some other more complex regulatory situation.

Among the tryptophan analog-resistant mutants obtained to date, two types are noteworthy. One has constitutive, derepressed levels for all the enzymes of the pathway (39). It thus resembles *trpR* mutants in the enteric bacteria and differs from the *trpR* mutants of *P. putida*, which affect only the gene cluster for the early enzymes (152). Another, showing resistance to 5-methylindole in the presence of anthranilate and a mixture of the other major compounds derived from chorismate (phenylalanine, tyrosine, *p*-aminobenzoate, *p*-hydroxybenzoate, 2,4-dihydroxybenzoate, and vitamin K), has constitutive derepressed levels for only the products of the *trpGDC* cluster. Whether or not there is in fact a single repressor molecule (recognized at three or more spatially distinct and functionally independent regions of the chromosome) for all of the tryptophan genes in *Acinetobacter* is certainly open to further investigation. The hypothesis is a reasonable speculation at present.

GRAM-POSITIVE BACTERIA

Bacillus and Clostridium

Chromosomal arrangement. Notwithstanding a long history of investigation of the tryptophan genes and enzymes in the sporeforming bacteria, only recently has a reasonably complete picture become evident. The first studies were done with *B. subtilis* 168 shortly after it was learned that this strain is capable of DNA-mediated transformation (4). This strain carries an auxotrophic requirement for tryptophan that had been introduced earlier (22); the mutation proved to be in the *trpC* gene. Other tryptophan auxotrophs blocked in various parts of the pathway were obtained, and all were found to be closely linked to each other by transformation (4). Later a quantitative recombinational map of mutations affecting all the steps in the pathway was obtained and found to be remarkably similar to the map of the *trp* operon in enteric bacteria (Fig. 2) (5, 25). As better enzymological data appeared (89), it became clear that mutations affecting PRAI and InGPS occur in independent but adjacent genes. And finally, when it was shown that PRT and AS are unassociated in *B. subtilis* (89) and that AS has a large and small subunit (117), it was obvious that another *trp* gene must exist. Kane et al. (116) found a mutation in the gene affecting the small subunit, and showed that the mutation produces a defect in both anthranilate and *p*-aminobenzoate synthesis and that it is unlinked to the original *trp* gene cluster (Fig. 2). Surprisingly, this mutant did not show an absolute requirement for either folate or tryptophan when grown in minimal medium. It was isolated in a strain with low chorismate mutase and hence high chorismate levels; when transferred to a strain with normal chorismate mutase levels, this mutation does confer a requirement for both tryptophan and *p*-aminobenzoate (J. F. Kane, personal communication). Recently Kane (personal communication) has

shown that *trpG* lies close to another gene for *p*-aminobenzoate synthesis (*pabA*) on the *B. subtilis* chromosome, and that the *pabA* protein serves the same function in *p*-aminobenzoate synthesis that the *trpE* product does in anthranilate synthesis. This suggests that in *B. subtilis trpG* was originally a component of *p*-aminobenzoate synthase that was recruited into service in the tryptophan pathway at some time during evolution. The protein produced by *trpD* (estimated molecular weight of 57,000; [89]) is sufficiently large to support speculation that a vestigial but nonfunctional glutamine amidotransferase segment could remain appended to the PRT portion.

Subunit structure of enzymes and amino acid sequences. Not only *B. subtilis*, but *Bacillus pumilis*, *B. licheniformis*, *B. coagulans*, *B. macerans*, and *B. alvei* all have low-molecular-weight AS molecules which dissociate into large and small subunits (90, 171). *Clostridium butyricum* AS is similar with a molecular weight near 89,000 (9); it also has been inferred to have a large and small subunit. Although the PRT of *C. butyricum* was not detected in extracts (9), that of *B. pumilus* was found at 45,000 molecular weight and that of *B. alvei* at 54,000 (90), separate from AS in both cases, of course. Though the AS molecules of the six *Bacillus* and one *Clostridium* species all show similar molecular weights (9, 32, 90, 117, 171), they differ considerably in their subunit affinities, the conditions permitting dissociation of subunits and their stabilization, the ratio of amidotransferase to amination activities of the complex, and the apparent molecular weight of the large subunit (90, 171). In a group of bacteria as large and diverse as the sporeformers, it would indeed be surprising if all the enzymes of the pathway were uniform structurally. In a later section, I will consider the use of subunit interchange to assess evolutionary relatedness. Functional hybrid complexes can be formed with all six *Bacillus* species (171). One surprising result published recently is that hybrid AS molecules containing one subunit from *B. subtilis* and another from *P. aeruginosa* show appreciable activity in the glutamine-dependent AS reaction (170).

The sequence of the first 46 amino acids of the α-chain of *B. subtilis* TS has been determined (134) and is shown in Fig. 3 along with the analogous regions for *E. coli* and *P. putida*. Although only 20 of the 47 residues are identical with the *E. coli* sequence, the homology is obvious, and it appears that the first six residues of the gram-negative chain (assuming only a single methionine is cleaved from the *P. putida* version) are missing from the *B. subtilis* version. Fifteen of the 27 amino acid substitutions between *E. coli* and *B. subtilis* require a two-base change in the codon. As with the *Pseudomonas-Enterobacteriaceae* comparison, two regions of the known sequence seem better conserved than the rest, residues 19 to 28 and 48 to 52 (*E. coli* numbering). These two conserved areas include the only two positions in the first 52 residues of the *E. coli* α-chain known to give rise to inactive molecules by missense mutations, phenylalanine at position 22 and glutamic acid at position 49 (216, 217). These two residues are among those conserved in all bacterial TS α-chains so far studied (44, 134, 135). The molecular weight of the *B. subtilis* TS α-chain is about 10% less than that of *E. coli* (87). Thus the *B. subtilis* α-chain probably will have additional segments deleted somewhere in its amino acid sequence.

Regulation of enzyme synthesis. Despite an earlier report to the contrary (208), upon satisfactory stabilization of all the enzyme activities, production of the proteins of the *trp* operon in *B. subtilis* was seen to be coordinate (89). The product of the unlinked *trpG* gene is also controlled by tryptophan and appears to respond to the same repressor molecule as the *trp* operon (116), though apparently it cannot be derepressed to the same extent as the rest of the *trp* gene proteins. This fact explains a break in the Ames-Garry plot for glutamine-dependent AS activity at high levels of derepression (89). The location of the *B. subtilis mtr* gene (named for methyltryptophan resistance), the putative structural gene for the *trp* repressor, is known with some precision. It is carried on the same transforming DNA molecule as the *trp* operon, but is not immediately adjacent to it (88a, 91, 165, 208). Mutants lacking an effective *trp* repressor also show some derepression of a *his* and *tyr* gene located distal to *trpA* beyond the *trp* operon, as though some read-through were occurring (182).

Mutants in the *mtr* locus may or may not excrete appreciable amounts of tryptophan. This difference was found to depend partly on the chorismate mutase level (91). Lorence and Nester (131) showed that *B. subtilis* strains may have a chorismate mutase with low activity, one with high activity, or both. Strains with low chorismate mutase activity accumulate significant amounts of chorismate; this overcomes the feedback inhibition of AS by tryptophan and allows a considerable overproduction and excretion of tryptophan (91). Interestingly enough, this excess tryptophan then exerts an inhibitory effect on the phenylalanine synthetic enzyme,

prephenate dehydratase (174), resulting in the paradoxical situation that some 5-methyltryptophan-resistant *mtr* mutants exhibit a partial requirement for phenylalanine. In addition, these mutants show unexplained growth responses to tyrosine, histidine, arginine, and proline (88a, 91).

One mutant bearing a temperature-sensitive tryptophanyl-tRNA synthase has been examined for regulatory anomalies at permissive and elevated temperatures (197a). Although the author concluded that the *trpS* gene product interacts in some way with the regulatory mechanism, it should be noted that similar claims have been made for the *trpS* gene product of *E. coli* (107). Judgment may be suspended until suitable in vitro regulatory assays are available in *B. subtilis* to subject the hypothesis to a critical test.

Staphylococcus and Micrococcus

Chromosomal arrangement. Proctor and Kloos (168, 169) used transduction to study a group of *Staphylococcus aureus* tryptophan auxotrophs blocked at various points in the pathway. All their mutants were linked, and by quantitative recombination experiments they determined the gene order to be *trpE, D, C, F, B,* and *A* (Fig. 2). If a *trpG* locus exists, its position is unknown, for no mutants have been obtained to define it. In an analogous study from the same laboratory, Kloos and Rose (118a) used transformation to study a set of tryptophan auxotrophs of *Micrococcus luteus.* Here close linkage was demonstrated for *trpE, C, B,* and *A* mutants, and both quantitative mapping experiments and three-point tests indicated the genes in the cluster to be arranged in that order (Fig. 2). Ten mutants were deduced by growth and accumulation tests to lack either PRT or PRAI, hence, they must have been either *trpD* or *trpF* mutants. None of the ten was linked by transformation to the *trpECBA* cluster. Unfortunately it cannot be stated at present whether only one or both of these genes, *trpD* and *trpF,* are unlinked to the main cluster; moreover, the existence and location of a *trpG* gene will remain speculative until mutants defective in a glutamine amidotransferase AS subunit are found. Additional genetic and biochemical studies with these gram-positive cocci may be awaited with interest.

Subunit structure and regulation of enzymes. In *S. aureus* the PRAI and InGPS enzymes are separable by gel filtration. Both have molecular weights below 30,000, indicating that *trpC* and *trpF* are separate loci, not two parts of a single gene (169). AS is not found in a complex with PRT or any other enzyme of the pathway. Its estimated molecular weight of 65,000 seems quite low, but not amazingly so in view of the values obtained with some of the bacilli, such as *B. alvei,* under certain conditions (32, 90, 171); this enzyme too is probably a complex of a large and small subunit, similar to the ones found in all other bacteria except the *Escherichia-Salmonella-Enterobacter* group.

PRT is small in *S. aureus* at molecular weight 30,000. TS activities were labile in extracts, so that enzyme has not yet received a careful characterization in *Staphylococcus.* Little is known of the subunit structure and molecular weights of the *Micrococcus* enzymes.

Proctor and Kloos (169) studied the tryptophan enzyme levels in *trpA* and *trpE* auxotrophs of *S. aureus* grown under conditions of tryptophan excess or limitation. They found that all five enzymes (both the A and B reactions of TS were studied, so six specific activities were measured) were derepressed concurrently and coordinately upon tryptophan starvation. The highest level of derepression observed was 100-fold greater than the repressed level. Thus, the mode of regulation of this pathway in *Staphylococcus* seems quite analogous to that in the enteric bacteria and the bacilli, but unlike that in *Pseudomonas* or *Acinetobacter.* It will be interesting to learn how the enzyme levels are controlled in *Micrococcus* where there are at least two *trp* gene clusters.

Streptomyces

Chromosomal arrangement. A large body of genetic work has been performed by using conjugation in *Streptomyces coelicolor* and some closely related actinomycetes (for a review see reference 94). These prokaryotes grow as a mycelium and make spores asexually. Their genetic map shows a single circular chromosome. Tryptophan auxotrophs are found at two different sites on the map (64, 94, 195a). Eight mutants lacking either the A or the B reaction of TS have been mapped at one site near two mutants lacking InGPS; one mutant lacking PRAI and 17 lacking PRT map at another site (195a). So far no mutants lacking AS have been found, so the number of *trp* gene clusters may be more than two. There have been no reports of enzyme associations in this group of organisms, but there are indications that *S. coelicolor* regulates the level of all the enzymes of the pathway except InGPS (195a).

BACTERIA STUDIED NON-GENETICALLY

Chromobacterium, Lactobacillus, and Blue-Green Bacteria

Subunit structure and regulation of enzymes. This section summarizes some enzymological work done in organisms where a method of genetic exchange has either not yet been found or has not yet been employed to study the chromosomal location of the *trp* genes. Bacteria in the genus *Chromobacterium* are gram-negative rods characterized by the production of an insoluble, dark blue pigment, violacein. This pigment is a rather simple molecule formed enzymatically from two molecules of tryptophan (53). Mutants unable to synthesize tryptophan make colonies that are colorless on a medium with limiting tryptophan but bright blue in tryptophan excess (204). This property makes the selection of tryptophan auxotrophs rather easy. Mutants blocked in each step of the pathway have been studied physiologically and enzymatically (204). In their molecular weights and absence of aggregation, the enzymes of the pathway resemble those of *B. subtilis*. *Chromobacterium* is quite different, however, in the apparent lack of a mechanism of regulation of tryptophan enzyme synthesis. Feedback inhibition of AS by tryptophan is very efficient in this organism (204), and as far as can be determined, it is this mechanism alone that keeps the flow of metabolites down the tryptophan pathway in balance with the other uses of chorismate, yet adequate both for protein synthesis and pigment formation. Mutants resistant to 5-methyltryptophan were isolated and studied (J. W. Hirst and I. P. Crawford, unpublished data). None were found to have elevated tryptophan enzyme levels.

In the earliest investigation showing that anthranilate can be used in place of tryptophan, Snell (196) used several species of lactic acid bacteria having a natural tryptophan requirement. Among these, *Lactobacillus casei* and *Lactobacillus arabinosis* 17-5 would accept either anthranilate or indole in place of tryptophan, whereas three other species would not take either intermediate. The similarity of this situation to that in enteric bacteria showing a natural tryptophan requirement is further emphasized by more recent work with *L. casei* (156) showing that spontaneous and mutagen-induced reversion of a lesion near the end of the pathway can occur. In this case no reversion of the natural AS block was seen. Clearly the lactobacilli are candidates for more detailed investigation of enzyme size and aggregation, subunit interchange with other gram-positive and even gram-negative forms, and mode of regulation of enzyme synthesis.

Studies of the tryptophan pathway in the blue-green bacteria (formerly called blue-green algae) are at an early stage. In one species, *Anabaena variabilis*, association of the enzymes has been studied. AS and PRT sediment independently, while PRAI and InGPS cosediment at about 5.5*S* (99); the authors note that this pattern resembles that of the oomycetous fungi. Sakaguchi (184), on the other hand, found that the TS of the same organism dissociates into α and β_2 subunits like bacterial versions of the enzyme and unlike the known fungal ones. In a different blue-green bacterial species, *Agmenellum quadruplicatum*, a tryptophan auxotroph blocked in the TS-A reaction has been obtained and used in studies of the regulation of enzyme levels (102). All the enzymes of the pathway increase in response to tryptophan deprivation, but the increase of the first four activities of the pathway is only two- to threefold while TS-B activity increases 20-fold. AS amination activity was shown to be fully inhibited by 10 μM L-tryptophan; no AS glutamine amidotransfer activity was detected in extracts (102). Lest it be thought from the above that the blue-green bacteria are very close relatives of other bacterial taxa, it should be noted that they synthesize tyrosine by a novel pathway involving transamination of prephenate prior to aromatization, whereas all other groups of prokaryotic and eukaryotic organisms perform these steps in reverse order (198).

EUKARYOTIC ORGANISMS

Fungi

Chromosomal arrangement. This survey of the genetics of the tryptophan pathway in eukaryotes will be very cursory, primarily because the topic has been adequately covered elsewhere (51, 97). Some of the evolutionary mechanisms that have been observed in bacteria, including gene fusion, appear to have occurred in eukaryotic organisms as well, especially among the fungi, necessitating a brief review of the topic, however.

Study of the fungal tryptophan genes and their products has a long history, beginning with the pioneering efforts of Tatum and Bonner in 1944 (199). *N. crassa* devotes four unlinked genes to the enzymes of the tryptophan pathway (Fig. 4). All mutations affecting TS are found at a single locus, *tryp-3*. Mutations affecting PRT map at *tryp-4*. At the *tryp-1* and *tryp-2* loci are found mutations having diverse pheno-

types affecting AS, PRAI, and InGPS (51, 97). The *tryp-1* and *tryp-2* gene products combine to form a multimeric enzyme catalyzing these three reactions, as will be described in the next section.

There is a fifth locus in *N. crassa* capable of mutating to give rise to tryptophan auxotrophs (3), but this has been shown to be the structural gene for tryptophanyl-tRNA synthase (163) so it is not, properly speaking, a component of the tryptophan synthetic pathway.

The *trp* genes are also well characterized in several other fungi. The gene-enzyme relationships in *Aspergillus nidulans* are certainly very similar to *Neurospora* (98, 177), and *Coprinus radiatus* is probably also similar (80). In the yeast *Saccharomyces cerevisiae* there are five *trp* pathway structural genes instead of four, all unlinked to each other (51, 57). The gene-enzyme assignments are similar to *Neurospora* except for PRAI, which is encoded by a separate gene in yeast (Fig. 4). Work has recently begun on the tryptophan gene-enzyme relationships in the fission yeast *Schizosaccharomyces pombe* (193). Some details remain to be elucidated, but the authors suggest that the PRAI and InGPS loci may be genetically distinct though adjacent on the chromosome, which is a situation intermediate between *Saccharomyces* and the filamentous fungi.

Subunit structure of enzymes. Two multimeric proteins are known in the tryptophan pathway of *N. crassa*. The most recent evidence suggests that one of them, TS, is a dimer of molecular weight 150,000 having two identical subunits (149, 200a). Earlier findings that this molecule had an $\alpha_2\beta_2$ structure, with α and β chains both about 35,000 daltons in size (26), may have resulted from endogenous proteolytic attack. In both *N. crassa* (224) and yeast (142, 183) there are inactivating proteases that copurify with TS, as well as an inhibitor of this protease that may be removed from the preparation at certain steps. Genetic evidence strongly supports the homodimer model for the TS enzyme (17). There is a localization of mutant phenotypes within the gene both in *N. crassa* (17, 121) and *S. cerevisiae* (143); mutations affecting one of the two TS half reactions map together at one end of the gene, separate from mutations affecting the other half-reaction. In both organisms mutations affecting only one of the two TS half-reactions are in the minority. Some of the mutations leading to total inactivity are missense, termed "profound" in *N. crassa* (17, 121), whereas some are nonsense. In yeast the effect of nonsense mutations in one small segment of the gene is particularly interesting, for the nonsense fragments produced retain activity in the TS-A reaction, though their molecular weight determined from gel filtration is only 35,000, about one-fourth of that of the native enzyme (141). The most reasonable interpretation of all the data available for the tryptophan synthase locus in *N. crassa* and *S. cerevisiae* is that it encodes a single long polypeptide chain having the activity of the bacterial TS-α chain at one end and the β-chain at the other. The orientation of the two segments of the locus is such that the α-chain portion is translated first.

Organism	Enzyme					
	AS	InGPS	PRAI	PRT	TS	TTS
Neurospora crassa	tryp-2 ⟷	tryp-1		tryp-4	tryp-3	tryp-5
Linkage group	VI	III		IV	II	V
Aspergillus nidulans	trypA ⟷	trypC		trypD	trypB	trypE
Linkage group	II	VIII		II	I	VI
Saccharomyces cerevisiae	trp-2 ⟷	trp-3	trp-1	trp-4	trp-5	-
Linkage group	V	XI	IV	IV	VII	-

FIG. 4. *Tryptophan gene-enzyme relationships in three fungal genera. The nomenclature is that in use currently (51, 177) in each organism. Enzyme abbreviations are as in Fig. 1 and the text; TTS, tryptophanyl-tRNA synthase. Linkage groups are those specified for each organism and are not uniform. The two trp genes on linkage group IV of S. cerevisiae are widely separated. Products of the genes connected by the double arrow (⟷) associate to form a dependent enzyme complex. All other gene products appear to be unassociated.*

The second multimeric protein in the fungal tryptophan synthetic pathway has three activities in *Neurospora* (AS, PRAI, and InGPS) and two in *Saccharomyces* (AS and InGPS) (51, 57). The most recent work suggests that in *Neurospora* it is a 310,000 molecular weight protein with two 94,000 and two 70,000 molecular weight subunits (95). This is in good agreement with the genetic evidence that two unlinked loci control this enzyme aggregate (51). Apparently one of the polypeptide chains is concerned primarily with AS (*tryp-2* product), the other with PRAI and InGPS (*tryp-1* product), but both must be present for appreciable AS activity (54, 69). Earlier evidence suggesting a subunit molecular weight as low as 40,000 for this protein (69) may have been due to proteolysis (95) as suggested above for TS. It is conceivable that the *tryp-2* gene product in *Neurospora* represents a fusion peptide containing the activities of the bacterial *trpE* and *trpG* gene products, and the *tryp-1* gene produce represents a fusion of the bacterial *trpC* and *trpF* functions.

The AS-InGPS complex of *S. cerevisiae* has

received less study than the *Neurospora* trienzymic complex just described, but both in its genetics and enzymology it resembles the latter without the PRAI segment of the *tryp-1* product (51, 57). In yeast, PRAI is a separate protein, smaller than the PRAI-InGPS bifunctional enzyme in *E. coli* (51). Recently H. Henke and R. Hütter (personal communication) have found that in some basidiomycetes, such as *Coprinus lagopus* and *Thanatephorus cucumeris*, all the activities of the pathway except TS can appear in a single, large enzyme complex. Additional studies in this group will be awaited with interest.

Hütter and DeMoss (99) performed an extensive survey of the size and association of enzymes of the tryptophan pathway in 22 conventional fungal species plus the myxomycete *Physarum polycephalum*. The patterns found in the filamentous ascomycetes and some phycomycetes conform reasonably closely to the pattern just described for *Neurospora*. Two of the yeast genera, *Dipodascus* and *Endomyces*, share the *Saccharomyces* pattern described above; *Schizosaccharomyces* is now known to be quite similar also. One group of phycomycetes, the *Oomycetes* group, including *Saprolegnia* and *Pythium*, differs markedly from all other fungi in having its AS unassociated with other enzymes of the tryptophan pathway. In this group the PRT is also an independent molecule, but the PRAI and InGPS cosediment at 3.5 *S* in sucrose gradients (99). Whether these two activities are associated on a single polypeptide chain or not remains undetermined. The unique taxonomic position of the *Oomycetes* group vis-a-vis the rest of the fungi is reflected in other characters as well. They have cellulose instead of chitin as the main cell wall constituent, and they synthesize lysine by the diaminopimelic acid pathway as do bacteria, algae, and vascular plants, rather than by the α-amino-adipate route employed by ascomycetes, basidiomycetes, chytrids, and euglenoids (203). It would be highly desirable to perform a genetic analysis of the *trp* loci in a typical oomycete, and a study of the TS in this group to see if it conforms to the bacterial model would also be very interesting.

Regulation of enzyme synthesis. This subject has been intensively studied in fungi, principally *Neurospora*, and has recently been reviewed (97, 154). The degree of derepression attainable differs for different enzymes, but in general is less than is seen in comparable experiments with enteric bacteria, pseudomonads, or bacilli (57, 163). Since tryptophan is an effective feed-back inhibitor of both AS and PRT (52, 57), the increased enzyme synthesis may result either from induction by accumulated intermediates or from derepression by decreased levels of tryptophan or charged tryptophanyl-tRNA. In fact, at least two signals are involved in the regulatory response of the tryptophan enzymes in *Neurospora*, for the accumulation of InGP has an inducing effect on TS synthesis (201), but even mutants unable to form this intermediate respond to tryptophan deprivation (128, 129). Study of the *N. crassa* mutant deficient in tryptophanyl-tRNA synthetase suggests that it is the charged tRNA rather than free tryptophan that is the effector in the latter case (163). The increase in TS levels brought about by indoleacrylic acid is due to both these mechanisms, for Matchett has shown that this compound effectively inhibits TS-B activity, resulting in the accumulation of InGP, the inducer, and a decrease of charged tryptophanyl-tRNA, the repressor (148a).

The regulatory interrelation of the histidine and tryptophan synthetic pathways suggested long ago by Hogness and Mitchell (92) has been documented and amplified recently. Imidazoleglycerol phosphate, an intermediate in the histidine pathway structurally similar to InGP, has an inductive effect on TS similar to InGP (201). This effect is specific for the *tryp-3* gene product and is not counteracted by exogenous tryptophan. But Carsiotis and his co-workers have shown that this is not the only point of interaction between these pathways, for histidine auxotrophs of all types, even those blocked before imadazoleglycerol phosphate formation, increase the level of all the tryptophan enzymes when starved for histidine (28, 30). Moreover, exogenous tryptophan does not reverse the histidine starvation effect (28). This effect is reciprocal, for tryptophan starvation also results in an increased synthesis of enzymes of the histidine pathway (27). Two other amino acids have recently been included in this regulatory interrelationship in *Neurospora*, arginine (27, 29) and lysine (206). The present interpretation of this situation is that regulatory signals, probably related to the degree of charging of specific tRNA molecules with these four amino acids, are reflected in the rate of formation of the enzymes for the synthetic pathways and that considerable cross-pathway response to these signals occurs (130, 205). The mechanism and full significance of these relationships are still not entirely clear, but it is apparent that similar interrelationships of the histidine, tryptophan, and arginine pathways occur in *S. cerevisiae* (191), and at least the histidine and tryptophan pathways are interrelated in *Euglena* (123).

Algae, Euglenoids, and Plants

Subunit structure of enzymes. Genetic analysis of the tryptophan genes in these eukaryotic organisms has not yet been accomplished, but biochemical experiments have uncovered some properties of the enzymes of the pathway. The TS of the conventional, unicellular green alga *Chlorella ellipsoidea* is a two-component, dissociating enzyme like that of the prokaryotes rather than a single-component one like that of the fungi (184). Sakaguchi reports that this organism's B (= β_2?) subunit, as well as that of the blue-green bacterium *A. variabilis*, is weakly complemented by α-chains from *E. coli* or *S. typhimurium* TS. The *Chlorella* B component, but not the *Anabaena* one, weakly cross-reacts immunologically with antiserum to *N. crassa* TS (184). Confirmation of these results with more purified enzyme and some amino acid sequence information would be extremely interesting.

The eukaryote *Euglena gracilis* is unique among organisms studied so far in having all the enzymes of the pathway beyond AS combined in a single aggregate of about 234,000 molecular weight (122). The AS of this organism has a molecular weight of 80,000 (C. Hankins and S. E. Mills, manuscript in preparation). The subunit structure of AS as well as that of the large *trp* enzyme complex in *E. gracilis* remains a subject for future study. Even with the present knowledge, however, it is clear that there are major differences in the tryptophan enzymes of euglenoids and the higher fungi, despite the fact that these two groups share the α-aminoadipic acid pathway of lysine formation (203).

And finally, the tryptophan synthetic pathway of higher plants has been the subject of several enzymological investigations. It is clear that the pathway and intermediates are the same as in microorganisms (48, 211). TS from several genera of higher plants is dissociable into two unlike components (36, 47, 162). The molecular weight of TS is about 140,000, close to that of the microbial forms (36, 162). The early enzymes of the pathway all appear to reside on separate molecules; the molecular weights of these enzymes as found in corn and peas are: AS, 90,000; PRT, 80,000; PRAI, 26,000; and InGPS, 50,000 (C. Hankins and S. E. Mills, manuscript in preparation). The InGPS is probably a dimer, for active material at half that molecular weight is also present.

Regulation of enzyme synthesis. Little is known of the regulatory mechanisms employed by algae and euglenoids, and the subject is certainly not closed in plants either. It is quite clear that in plants feedback inhibition of AS plays an important role in the regulation of the flow of metabolites down the tryptophan pathway (14). Widholm has demonstrated that the mechanism of growth inhibition by tryptophan analogs such as 4- and 5-methyltryptophan and 5- and 6-fluorotryptophan is false feedback inhibition at this site (209). Mutants resistant to these analogs have a modified AS resistant to feedback inhibition by either the normal amino acid or its analogs (210). Whether, as in most prokaryotes and fungi, this feedback inhibition mechanism is ever supplemented by a modulation of enzyme synthesis remains an open question at present.

GENERAL CONCLUSIONS ABOUT EVOLUTION OF THE PATHWAY

Conservation of Reaction Mechanisms

It is always dangerous to pontificate, but it can be safely stated that if a pathway of biosynthesis of tryptophan different from that shown in Fig. 1 exists in living things, rather wide-ranging investigations have so far failed to uncover it. At least seven widely divergent prokaryotic groups and five eukaryotic ones have been surveyed, and although representatives from groups with very different mechanisms of tyrosine (198) and lysine (203) synthesis are present in the assemblage, no variation in the route of tryptophan synthesis was found.

Even more remarkable, perhaps, is that certain nuances of the reaction mechanisms also seem to have been retained during evolution. The ability of AS to use either glutamine as an amino group donor at physiological pH or molecular ammonia at a slightly higher pH has been found wherever sought, from bacteria (225) through fungi to plants (210). Similarly, TS from all groups retains the ability to utilize indole in place of InGP despite the fact that indole is a "captive" intermediate in the physiological reaction (118, 216) and must rarely be encountered in the natural environment of some of the organisms studied.

If the reactions of the pathway present a monotonous uniformity, the associations between the enzymes catalyzing them show a bewildering variety. Discounting the aggregates that are covalently bound as a result of fused genetic elements, it is still a puzzle to understand the occurrence of AS, PRAI, and InGPS in a complex in most fungi and the last four enzymes of the pathway in a complex in *Euglena*. Some of the puzzle may be resolved as more structural information becomes available. It now seems reasonable that as a result of the fusion of the genes for the small AS subunit and PRT in the *Escherichia-Enterobacter-Sal-*

monella group of the enteric bacteria, AS and PRT exist in a relatively firm complex, whereas they show no aggregation in *Serratia* where this fusion has not occurred (225). Similar explanations must be sought in the other cases, but it might also be remembered that certain advantages accrue to assembling the enzymes of one pathway into a single complex. Some examples of catalytic "facilitation" by such molecular "organelles" in the tryptophan and the multiple aromatic pathways of *Neurospora* (70) and *Euglena* (14a) have been adduced. Perhaps many organisms have their tryptophan pathway enzymes associated intracellularly through noncovalent interactions of varied strength. When cell-free extracts are made only the strongest of these interactions may be manifest in residual enzyme complexes.

Variation in Chromosomal Disposition of Genes

Translocation and gene fusion. Nearly every major bacterial group studied has a distinctive chromosomal arrangement of the genes concerned with tryptophan synthesis (Fig. 2). Although less information is available from eukaryotes, among the fungi also there is variety in the number and location of the *trp* genes (Fig. 4). Differences in regulatory response seem correlated with chromosomal location. It seems rather obvious that the structural genes for the tryptophan enzymes, many of them having the same evolutionary origin, as indicated by residual sequence similarities (Fig. 3), have been translocated to various positions on the chromosome during evolution and are associated with different *trp* gene partners, even responding to different regulatory stimuli. All this has probably resulted from ordinary mechanisms of chromosomal rearrangement (i.e., reciprocal translocations, insertions, inversions, *etc.*), coupled with a considerable plasticity of regulatory elements capable of responding to selection to maximize cellular economy under the prevailing conditions.

An unexpected finding in the study of the *trp* genes is the number of examples of fusion of genes encoding two polypeptides in the same pathway. In discussing fusions it will be assumed that the original state consisted of six quite independent genes, *trpE* and *trpG* for AS, *trpD* for PRT, *trpF* for PRAI, *trpC* for InGPS, and *trpA* and *trpB* for TS. Fusion must have occurred twice in the lineage of the enteric bacteria, once combining *trpC* and *trpF* to form a single bifunctional peptide, and later a fusion of *trpG* to *trpD*. The first may have occurred in a strain ancestral to the entire taxon, the second in one common to only the *Escherichia-Salmonella-Enterobacter* group. In both cases there is suggestive evidence that the bifunctional polypeptide assumes a conformation consisting of two "primal" domains connected by a linking segment (45, 75, 124). In one case, at least, the linkage appears to be protease sensitive (75).

In the fungi the prediction of Bonner et al. (17) concerning TS appears to have been borne out; the fungal enzyme consists of a fusion of α- and β-chain elements seen in all other organisms (149 200a). Kaplan et al. have presented immunochemical evidence for two independent polypeptide "domains" in the *Neurospora* protein (117a). It will be interesting to see if the site(s) most sensitive to protease attack in this enzyme are in a linkage region between two independently folding α and β domains. Though this fusion may have occurred ancestrally to all the higher fungi, fused *trpC* and *trpF* functions are found only in the filamentous ascomycetes and the basidomycetes, not the yeasts (99). Whether in fact a fusion of the *trpG* and *trpE* functions has also occurred or whether *trpG* may be fused to *trpC* in this interesting complex will probably become clear with additional study.

It is easy to note that all the examples of gene fusion just described are reflected in enzyme aggregations (considering TS-A and TS-B to be two activities). In those organisms where genetic analysis has not been performed, the existence of multienzyme complexes might betoken still more examples of gene fusion. A prime candidate for such an occurrence would be *Euglena* with its large complex embodying all the reactions of the pathway beyond AS (122).

Regulatory mechanisms. Among the prokaryotic species studied an almost bewildering range of regulatory variations has been described. Where units of function occur they seem to reflect chromosomal organization, and the regulatory signal seems usually to be the amino acid itself or a pathway intermediate rather than the charged tRNA. Few other generalizations are possible, however, for segments of the pathway may be controlled by either induction or repression over a wide or very narrow range. There are even instances where the two polypeptide chains of a given enzyme are not under fully coordinate regulation, as in *B. subtilis* AS (89, 116).

With the bewildering variety of bacterial examples, it is perhaps no surprise that the fungi should have developed something quite different for their regulation. Though more work

remains to be done, the fungal mechanism appears to recognize charged tRNAs as effectors, and a given signal may influence the synthesis of enzymes in several amino acid pathways. It is far too early to assume that these regulatory differences embody a major eukaryote-prokaryote dichotomy, however, The impression at present is that regulatory mechanisms can be altered during evolution at least as easily as chromosomal gene location and much more easily than the amino acid sequence of an enzymatically active polypeptide.

Enzymes Shared with Other Pathways

The sole example so far uncovered of a tryptophan pathway element shared with another pathway is the use of the same glutamine amidotransferase subunit for both AS and *p*-aminobenzoate synthesis in *B. subtilis* (116) and *A. calcoaceticus* (187). The obvious similarity of these two reactions, having the same substrates and a product differing only in *ortho* or *para* placement of the amino group, leads one to suspect that one enzyme may have evolved from the other. Gene duplication followed by active site modification is an evolutionary mechanism favored by theoreticians, and it may well have occurred in this case.

Rather than postulate duplication of the large AS subunit gene without a corresponding duplication of the small subunit in the ancestry of *B. subtilis* and *A. calcoaceticus*, however, I will propose a different possibility which recognizes the fact that the "*trpG*" of *A. calcoaceticus* is linked to other *trp* genes, whereas that of *B. subtilis* is linked to a *p*-aminobenzoate pathway gene. If one assumes that both organisms originally possessed complete, two-subunit enzymes, but that through deletion, insertion of extraneous DNA, or mutation within the gene, *A. calcoaceticus* lost its amidotransferase subunit for *p*-aminobenzoate synthase, while *B. subtilis* lost the one for AS, then each organism might have become dependent on the small subunit from the counterpart enzyme. This situation, which is not very bizarre either genetically or biochemically, proposes independent occurrence in separate lineages of "mirror-image" inactivating events. Subsequent modification of the regulatory region for the *p*-aminobenzoate enzyme in *B. subtilis*, to bring it under tryptophan control, would produce the present situation, as far as is known.

Whether the hypothesis just stated is approximately true or merely fanciful, it certainly behooves investigators to be alert to the possibility of other elements of similar function that could be shared between distinct pathways in specific organisms. Not only amidotransferases, but many other transferases and isomerases are obvious candidates. Such features may be as much a fixture of certain taxa as an alternate pathway, multiple enzymes for one reaction, or fused genes.

NATURAL RELATIONSHIPS REVEALED BY COMPARATIVE STUDIES

Amino Acid Sequences

Predictably, I begin this section on determination of natural relationships by pointing out that pitfalls exist in any one method, so a conclusion based on many different lines of evidence is more likely to stand the test of time than one resting on a single support, however clear-cut and logical. Many ways exist to determine relatedness by the degree of similarity shown within a large, natural group such as the enteric bacteria or the pseudomonads. When applied with intelligence, most of these methods seem to give nearly identical answers. It is in the determination of natural relationships among different large groups that many existing taxonomic methods fall short when applied to prokaryotes. My preference for this purpose lies with the comparison of amino acid sequences in homologous proteins, preferably those of major importance to cellular economy. This preference is based on the experience obtained so far with enzymes of the tryptophan synthetic pathway.

Much of the relevant, admittedly limited data is summarized in Fig. 3. There is a comforting conservation of sequences in the several enteric bacteria and a recognizable but more distant relationship between these and the *Pseudomonas* and *Bacillus* sequences. Somewhat similar results involving other prokaryotic taxa have been obtained for the iron-sulfur proteins (222).

Though it seems obvious that amino acid sequence comparisons will provide highly reliable evidence of relatedness between large taxons, and considerable experience with a wide range of eukaryotes seems to bear this out, there is a pecularity of prokaryotic genetics that might lead to misleading results. Plasmid transfer has been observed between bacteria of different groups, specifically between *Pseudomonas*, *Vibrio*, and enteric bacteria (79). Doubtless this range will soon be extended. Plasmids may on occasion bring chromosomal elements with them; these, if analyzed, could lead to false estimates of similarity. Perhaps the best defense against such errors would be to choose proteins of basic, nearly indispensable path-

ways, to study their genes whenever possible to assure a conventional chromosomal location, and above all not to depend on the analysis of a single polypeptide.

Nucleotide Sequences and Nucleic Acid Hybridization

Protein sequence similarities are reflected in similarities in sequence of the corresponding mRNA and DNA molecules. The development of sensitive DNA-DNA and DNA-RNA hybridization methods has provided a methodology for assessing sequence similarities in nucleic acids without knowing the actual nucleotide sequence. Careful measurements of the stability of RNA-DNA hybrids corresponding to the TS α-chain in three enteric bacteria led Li et al. (132) to the conclusion that nucleotide sequence has diverged almost twice as rapidly as amino acid sequence, i.e., that as many base changes to synonymous codons have been fixed in evolution as base changes leading to amino acid substitutions. This fact limits the usefulness of nucleic acid hybridization techniques to relatively closely related species, a conclusion borne out by the inability of Denney and Yanofsky (55) to demonstrate hybridization between the mRNA of *B. subtilis* and the *trp* DNA of *E. coli* despite the recognizable conservation of the amino acid sequence of their TS α-chains (134). The above is not meant to denigrate the usefulness of hybridization techniques for determination of natural relationships within bacterial families, or to deny that certain nucleotide sequences such as those concerned with ribosomes may be conserved better than the *trp* ones (37, 58, 59, 213a). Nevertheless, the techniques of protein purification and automatic Edman degradation have now progressed to the point where wide surveys of the prokaryotic kingdom are quite feasible, and would be expected to orient on a larger canvas the detailed relationships revealed by numerical taxonomy and nucleic acid hybridization (for reviews see references 40 and 140).

Subunit Interchange

Purification of proteins and determination of their amino acid sequence has not always in the past proved a simple or infallible procedure; therefore many schemes have been concocted to circumvent it. Two of the more obvious ones will be dealt with briefly in this and the next section. Where there are multimeric enzymes with readily dissociable, unlike subunits, it is logical to assume that the affinity of these subunits obtained from different organisms might closely approximate the degree of sequence similarity, and hence the evolutionary relatedness of the sources. In the context of the tryptophan genes and enzymes of bacteria, this expectation has not been fully borne out.

There are two multimeric enzymes in the bacterial tryptophan pathway suitable for subunit exchange experiments, AS, when glutamine rather than ammonia is the amino group donor, and TS, where the low activity of the dissociated subunits versus the complex allows activity in any reaction to serve as a yardstick for measuring complex formation.

It has been shown that the *trpE* product of *E. coli* will function with the *trpD* proteins of *S. typhimurium* and *E. aerogenes* quite readily (104), and can even accept the much smaller *trpG* product from *S. marcescens* for glutamine utilization (176). A similar complementation with the small *trpG* subunit of *E. hafniae* has been reported (124), but there are no indications that the large and small subunits of *S. typhimurium* and *P. putida* can function cooperatively (173). Functional hybrid AS molecules involving six different species of *Bacillus* have been observed (171). More surprisingly, the large *B. subtilis* subunit complements well with the small *P. putida* one; the reverse combination is somewhat less catalytically effective though not entirely inert (170). Large *A. calcoaceticus* AS subunits do not form very effect complexes with the small subunits of *B. subtilis* or *P. putida*, and no activity at all was found with the small *S. marcescens* subunit (188).

TS complexes formed with the α and β_2 subunits from different enteric bacterial species are quite active (6), though the affinity between *S. marcescens* β_2 subunit and *E. coli* α-chains is somewhat less than that of the *Shigella*, *Salmonella*, and *Enterobacter* β_2 subunits (178). The reverse is not true, as α-chains from all four of these organisms have excellent affinity for the *E. coli* β_2 subunit (161). *P. putida* α and β_2 subunits give little or no activity with *E. coli* ones, and in fact no physical binding of *P. putida* β_2 and *E. coli* α subunits is seen (151). *B. subtilis* β_2 subunits are stimulated in the TS-B reaction by both *E. coli* and *P. putida* α subunits to 30% of the activity of the homologous complex, however (88). This interaction is not reciprocal, for *B. subtilis* α-chains have no effect on *E. coli* or *P. putida* β_2 subunits (87). Within the *Bacillus* genus, *B. pumilus* α-chains complement *B. subtilis* β_2 subunits perfectly, but *B. alvei* α-chains do not complement them at all (90). Weak stimulation by the B (= β_2?) subunits of *Anabaena* and *Chlorella* of the

α-chains of *E. coli* and *S. typhimurium* in the conversion of indole to InGP was reported (184).

The general conclusion to be drawn from these admittedly scattered observations is that subunit interchange has not lived up to its promise as a means of ascertaining intermediate and weak natural relationships. Perhaps this is not really surprising, for the two requirements for establishing an enzymatically effective hybrid molecule, heterologous subunit binding, and effective active site formation or facilitation, may both be drastically influenced by the change of a few critical amino acid residues, yet may be largely unaffected by many of the substitutions occurring elsewhere in the molecule during evolution. When it is further realized that most of these experiments are performed by mixing crude extracts of mutants with lesions that may be missense or nonsense and whose content of proteases or other inhibitors for the foreign subunits is unknown, it is no surprise that the results seem somewhat irregular.

Immunological Cross-Reactivity

There is a body of work (167, 186) with several proteins from eukaryotes showing a correlation between strength of immunological cross-reaction and amino acid sequence resemblence, therefore evolutionary distance. This approach to the determination of prokaryotic natural relationships would appear to be less time consuming than the determination of many amino acid sequences. Once proteins from several different organisms have been purified and used to generate antisera, many other organisms can be tested against these reference sera for the extent of cross-reaction of the homologous protein in their crude extracts. As yet no systematic interfamily survey of any of the tryptophan enzymes has been done, but scattered observations have been made. These will be reviewed briefly, for they do lead to certain conclusions.

The immunological cross-reactivities of the α and β_2 subunits of TS in the enteric bacteria have been studied with some care (161, 178). No antigenic determinants are shared by the *trpA* and *trpB* gene products. When the homologous reactions are considered, it can be seen that, with respect to the α-chains where the sequence is known, the degree of cross-reaction demonstrated by complement fixation is in agreement with sequence divergence and also agrees with immunological studies of the unrelated protein alkaline phosphatase from the same species (212). Enzyme neutralization tests performed with the same sera showed a stronger cross-reaction than microcomplement fixation, suggesting that this might be a more sensitive test for detecting distant evolutionary relationships (161). When the same enteric bacterial species were studied systematically for the immunological relatedness of their TS β_2 subunits, the somewhat surprising result was that the differences found, while in proportion to those of the α-chains, were much less (178). Enzyme neutralization results were rather similar for the two subunits, however. These results are summarized in Table 1.

The other immunological results available with this system represent more sporadic efforts to compare tryptophan synthases from different taxa. Weak neutralization and precipitation reactions were found when the *P. putida* β_2 subunit was tested with antiserum prepared to the *E. coli* β_2 subunit (151); no reciprocal neutralization of *E. coli* enzyme was found with *P. putida* antiserum, but this result deserves reinvestigation. *B. subtilis* β_2 subunits are weakly neutralized by antisera prepared to either *E. coli* or *P. putida* β_2 subunits (88). The respective neutralization titers are only 1.5 and 3.5% that of the homologous system, however. It has also been reported that the B (= β_2?) subunit of *Anabaena* is neutralized by *S. typhimurium* antiserum at 2.9% of the homologous titer, whereas the *Chlorella* enzyme does not cross-react with the enteric bacterial system at all (184). The significance of the extremely weak (0.05%) cross-reaction between the B (= β_2?) subunit of the *Chlorella* two-component TS and antiserum prepared to the *N. crassa* single

TABLE 1. *Immunological cross-reactivity of the tryptophan synthetase α and β_2 subunits from five enteric bacteria*

Organism	Index of dissimilarity[a]		Neutralization efficiency[b]	
	α	β_2	α	β_2
E. coli	1.0	1.0	1.0	1.0
S. dysenteriae	1.1	1.0	1.0	1.02
S. typhimurium	2.2	1.4	1.25	1.49
E. aerogenes	2.8	1.2	1.39	1.64
S. marcescens	8.4	1.8	1.89	2.38

[a] Ratio of antiserum concentrations required with homologous (*E. coli*) and cross-reacting antigens to give identical levels of complement fixation. Data for α subunits from (161) and for β_2 subunits from reference 178.

[b] Ratio (homologous/heterologous antigen) of the number of enzyme units neutralized by a given amount of antiserum made to the *E. coli* subunit. Data for α subunits from (161) and for β_2 subunits from reference 178.

component one (184) remains problematical, but is remiscent of the weak neutralization of *B. subtilis* β_2 subunits by *N. crassa* antiserum reported by Hoch (88).

The conclusion to be drawn from these immunological studies is that the easily quantifiable methods of complement fixation and precipitation which have proven most useful in taxonomic studies of higher organisms (167, 186) may be too sensitive for determining the natural relationships between the major bacterial groups. In this respect they resemble nucleic acid hybridization techniques; like them they will be of considerable use in assignment of individual organisms to positions within their group, however. The use of enzyme activity neutralization appears to hold promise for ascertaining group relationships, but must be applied on a broader scale and more systematically than has been done heretofore. Relationships determined in this way should also be confirmed by amino acid sequencing, at least in some cases, for the possibility exists that low-level cross-neutralization may occur when the two enzymes in question really have very little overall sequence similarity but possess a similar orientation of cofactors and essential amino acid side chains at the active site. In this connection it is worth noting that there are two unrelated families of proteases sharing the "active serine" type of active site; though the coupled amino acid side chains that these enzymes use in catalysis are present in identical orientations, the remainder of the molecule shows no sequence homology (148). With only limited data available, there is no evidence so far for any similar convergent lines of evolution for any of the tryptophan pathway enzymes, though the circumstances surrounding the small, glutamine amidotransferase subunits of AS and *p*-aminobenzoate synthase described earlier deserve closer study.

Regulatory Mechanisms

As with immunological and subunit interchange experiments, investigators have hoped to avoid purifying and sequencing many proteins in ascertaining relationships by studying regulatory phenomena. To a certain extent this has been successful. As described earlier, nearly every major taxonomic group investigated differs from the others in some aspect of the mechanism by which it controls, or does not control, the synthesis of the enzymes of the tryptophan pathway. In a like fashion, the means by which the various prokaryotes and fungi regulate the activity and synthesis of the first enzyme of the common aromatic pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, has proved to be characteristic and quite varied (71, 111, 164). Although it may prove possible to correlate these patterns with genetic and physiological differences and to assign controversial organisms to one or another family by their use — as in the case of *Aeromonas*, whose tryptophan enzymes are those of a typical enteric bacterium rather than a pseudomonad (43, 124) — it is not generally possible to determine the natural relationships of different families in this way. Moreover, elucidation of the regulatory mechanisms used by a certain taxon usually requires a time-consuming study, surveying under varied growth conditions the levels of all the enzymes in auxotrophic and regulatory mutants as well as the wild type. A similar amount of effort devoted to the isolation of a protein and determination of its amino acid sequence is likely to produce information of greater taxonomic value.

EVOLUTIONARY TRENDS

Fused versus Separate Genetic Elements

In their paper published in 1965, Bonner et al. (17), extrapolating from the information available about the tryptophan enzymes of enteric bacteria, *Neurospora*, and a few other organisms, suggested a progression in evolutionary development from independent proteins through dependent, multimeric enzymes to fused genetic elements. In the ensuing decade, even though the examples of fused genetic elements in the tryptophan pathway have multiplied, the direction of evolution no longer seems so obvious.

The examples of gene fusion pointed out in the preceding sections are: the TS of yeasts and filamentous fungi, where the α- and β-chains of the dependent multimeric bacterial enzyme have fused; the PRAI-InGPS fused polypeptide of the enteric bacteria and *Neurospora*, though other organisms appear to have independent enzymes; the small AS subunit-PRT fusion in certain enteric bacteria, though in all other organisms these two elements are independent; and a few other less definite but suggestive cases such as the large and small subunits of AS in *Neurospora* and yeast. The occurrence of interdependent complexes such as the AS-PRAI-InGPS in *Neurospora* and the AS-InGPS of yeast, and possibly the large complex performing all the reactions beyond AS in *Euglena*, also deserves to be mentioned. The evolutionary advantages supposedly accruing at successive stages in this progression would be conservation

of diffusible intermediates in the case of dependent complexes and increased stability and regulatory simplicity in the case of fused genes (17). It is apparent that these factors may have been effective in a few instances, but organisms thought to be most highly evolved, such as sporeforming bacilli and blue green bacteria among the prokaryotes and plants among the eukaryotes, have not responded to their pressure.

Gene fusion has taken place at various times during evolution of the tryptophan pathway, probably by the same mechanism observed in the laboratory in the case of two adjacent histidine genes (223), deletion of the translation stop and start codons. However, if these events confer a decisive advantage, one wonders why, in the course of evolution, some particular form of the fusions found has not replaced the separate genes in all subsequent organisms. Perhaps there is also an advantage, from time to time at least, in the flexibility conferred by separate genes, because they may more easily interact with other metabolic pathways in the cell and may be controlled independently.

Dependent Enzyme Aggregates versus Independent Proteins

If in fact there was once an independent form of AS, where glutamine was deamidated by one enzyme and the ammonia formed was used by another, or of TS, where indole formed in the A reaction by one enzyme diffused freely to another enzyme to be converted to tryptophan, the organisms bearing them have not survived or still remain to be found. In these instances aggregation, by associating two unlike active sites in spatial proximity, may have triumphed early over the more primitive state. This dependency could just as well have been extended to other reactions in the pathway. In the higher fungi the apparent dependence of AS on association with another polypeptide coding for activities further down the pathway has been noted, and other instances will doubtless come to light in the future. Still it is fair to note that in most of the bacteria and in the higher plants no increased dependence on aggregation is seen. In fact, it has been suggested that the TS of *Anabaena* and *Chlorella* (184) as well as that of higher plants (36, 47, 162) is less aggregation dependent than that of bacterial because its B ($= \beta_2$?) subunit catalyzes the TS-B reaction equally well in the presence or absence of the A ($= \alpha$?) component, though the latter must be present for the conversion of InGP to tryptophan. A word of caution must be injected here, however, for the same claim was made earlier for the *B. subtilis* enzyme (153, 192). Upon stabilizing and purifying the latter enzyme to homogeneity, Hoch (87, 88) found little residual TS-B activity catalyzed by the pure β_2 subunit, though there was marked stimulation of this activity upon combination with the α subunit. Probably the earlier conclusions resulted from some α-chains remaining bound to the impure "B" fraction. Whatever the reason, these studies underscore the universal preservation in the TS complex of the ability to utilize indole in place of InGP, even in these highly evolved organisms. Similarly, the AS of all organisms appears to have preserved the ability to utilize ammonia in place of glutamine, albeit under somewhat unphysiological conditions. In the first case, at least, a case can be made for the usefulness of this activity as a "salvage" operation in cases where indole molecules become available.

One Operon or Several

There seem to be few organisms having all their tryptophan genes in a single operon. Outside the *Enterobacteriaceae*, only *Staphylococcus* appears to have a good chance to enjoy the single operon arrangement; even there, information on the small AS subunit is lacking and so the *B. subtilis* situation may prevail. It is not clear why having all the genes for this rather uncomplicated pathway in a single operon is not the evolutionarily preferred solution. Two facts seem obvious, however. Translocation of single genes or multigenic portions of a *trp* gene cluster are rather common events; recently Jackson and Yanofsky (109) described a selective system in *E. coli* in which duplications and translocations of multigenic segments of the *trp* operon to another part of the chromosome could be sought and their frequency could be determined. These events were found to be surprisingly frequent. Secondly, once genes have been translocated to a new region of the chromosome an organism seems to have little difficulty in designing or converting regulatory elements for their control. This may account for the regulatory variety seen up to now primarily among gram-negative bacteria, but probably to be expected throughout the prokaryotic and eukaryotic kingdoms.

Predictions Concerning Future Studies

Two sets of predictions can be made, one concerning genetic and biochemical studies desirable to increase our understanding of the tryptophan synthetic apparatus, and the other concerning the use of the tryptophan genes and

enzymes in determining taxonomic relationships.

In the former case, the dependent enzyme aggregates in this pathway, both in prokaryotes and eukaryotes, seem sure to be the objects of continued study. Among the features making them attractive to students of enzyme mechanics are their relative simplicity and low molecular weight, the fact that organisms can be genetically tricked into synthesizing them in large amounts, the availability of many mutant forms of them, and the opportunity to construct functioning complexes with subunits derived from different organisms. The TS from *E. coli* can be crystallized in the $\alpha_2\beta_2$ complex (1) as well as in individual subunit forms (2, 190), leading one to hope for eventual three-dimensional structural analysis of this multimeric enzyme. Among its many attractive features are the variety of amino acid substitutions available at certain sites in the α chain. At position 211, for example, 15 different amino acids have been inserted by mutation or suppression, eight of which are functional and seven are not (160a). One totally unanswered question concerning TS is the rationale for its occurrence as an $\alpha_2\beta_2$ tetramer instead of an $\alpha\beta$ dimer. No cooperative interactions between the two identical active sites have been detected, though these have been sought by several techniques (41, 46, 50, 118, 216).

The cross-pathway relationships described for the glutamine amidotransferases of the tryptophan and folate pathways in *Bacillus* and *Acinetobacter* seem destined to be studied in some detail as examples of metabolic interdependence. They may be just an example of many similar events involving enzymes of related function in different pathways. Similarly, the study of regulatory variations found in the tryptophan pathway of various organisms should be extended in breath and depth to clarify the possibilities for control of gene expression after translocation or duplication of structural gene elements.

In the second case the future is less easily predicted. I have tried to make a case for the use of amino acid sequences in determining the path of evolution in the prokaryotes, using the fragmentary sequence information presently available from the tryptophan enzymes as my vehicle. It seems clear to me that the results obtained so far encourage additional studies, and the wide variety of organisms with tryptophan enzymes that have not been studied suggests that some amplification of effort is needed. Even organisms with a natural requirement for tryptophan may have preserved portions of the pathway that can be studied. Quite frankly, it must be admitted that other proteins may become more important than the tryptophan pathway enzymes in this type of endeavor in the future, but this pathway should find a place among the pioneer systems used for the study of prokaryotic evolution, just as it has among the early models for the study of enzyme aggregates.

ACKNOWLEDGEMENTS

I am indebted to the National Science Foundation (grants GB-4267, GB-6841, and GB-16388) and the National Institutes of Health (grant AM-13224 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, and grant GM-19098 from the National Institute of General Medical Sciences) for finding portions of our work worthy of grant support.

For that portion of this work emanating from my laboratory, I am indebted to the students and collaborators who shared in its joys and disappointments. Their identity will be obvious from perusal of the reference list. My colleagues Sallie O. Hoch, Junetsu Ito, and Stanley E. Mills gave generously of their time to read and criticize the manuscript. Lastly, I wish to take this opportunity to express here my gratitude to two superb teachers, C. B. van Niel, who introduced me to the microbes, and Charles Yanofsky, who has helped me to discern the mysteries of their methods for synthesizing tryptophan.

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