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Regulation of Catabolic Pathways in *Pseudomonas*

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INTRODUCTION

Induction of catabolic enzymes in bacteria has not suffered from lack of attention in the past decade. In 1961, Jacob and Monod (83, 84) proposed that the lactose operon of *Escherichia coli* was governed by a macromolecular repressor which, in the absence of inducer, prevented the synthesis of the messenger ribonucleic acid (RNA) that encoded β-galactosidase. Since then a series of elegant genetic and biochemical experiments have led to the characterization of the messenger RNA as well as to the isolation of crystalline β-galactosidase (155, 159), a homogeneous preparation of the repressor (55, 124), and ultimately the deoxyribonucleic acid (DNA) of the *lac* operon itself (135). In view of these magnificent achievements, it is remarkable that one of the most ambiguous components of the total system is a relatively simple biochemical, the inducer. Burstein et al. (13) showed that the natural substrate of β-galactosidase, lactose, does not elicit the synthesis of the enzyme; rather, a β-galactosyl alcohol formed from lactose by the action of β-galactosidase appears to be the true inducer. This indirect mechanism of "by-product induction" appears curious, but there is little theory concerning the nature of inducers for it either to confirm or to contradict.

The highly developed genetic systems of the coliform bacteria have given a unique insight into

the macromolecular mechanisms governing enzyme synthesis, but the limited metabolism of this bacterial group restricts the information that it can provide about the evolutionary forces that endow metabolic intermediates with inductive function. In contrast to the coliform bacteria, most *Pseudomonas* species possess extraordinary nutritional versatility: one strain of *P. multivorans*, for an extreme example, can utilize up to 108 of 146 diverse organic compounds proferred as growth substrates (147). Hence, a wealth of catabolic pathways of various degrees of complexity is available for examination within a single *Pseudomonas* strain.

Further advantages accrue from the choice of pseudomonads for the analysis of the inductive controls governing catabolic enzymes. The ubiquity of these bacteria attests to their ability to adapt readily to various environments; this metabolic flexibility suggests that their enzymes are subject to efficient inductive control. The ability of pseudomonads to form inducibly large quantities of enzymes catalyzing unique reactions has attracted the attention of talented chemists and biochemists. These investigators have elucidated hitherto unknown pathways and have sped the development of simple assay procedures for the participating enzymes. The value of many separately conducted biochemical investigations of the pseudomonads was enhanced by the comprehensive study of Stanier et al. (147), who united many different strains within a single taxonomic scheme. As a result of these studies, it became possible to compare observations that had been made in different laboratories with independently isolated representatives of the same species.

Prospects for *Pseudomonas* genetics are bright (74). Systems have been developed for conjugation within *P. aeruginosa* (73, 103, 104, 149) and *P. putida* (25), and for transduction within *P. aeruginosa* (76, 102) and *P. putida* (24, 28, 156). Contiguous structural genes have been identified, and linkage maps have been established in these species. Should techniques for forming merogenotes within the pseudomonads be developed, they may be used to test theories of molecular genetics derived from the analysis of the coliform bacteria in organisms of widely divergent evolutionary origin.

For the foregoing reasons, a general survey of the inductive controls governing catabolic pathways in the pseudomonads appears warranted. This article opens with a brief history of the development of present concepts; a description of the analytical methods that have been employed to characterize the inductive patterns operative within specific pathways follows. Pathways in two different stages of analysis will be described. Some have not been completely characterized, but have yielded valuable information about the evolutionary pressures that dictated the selection of specific metabolites as inducers. In other catabolic systems, both the inducers and the enzymes that they govern have been identified unambiguously. The interaction of independent evolutionary forces in the development of specific regulatory mechanisms is discussed in the final section.

EARLY DEVELOPMENTS

Simultaneous Adaptation

The first physiological studies of catabolic pathways in bacteria produced a striking conclusion: growth at the expense of a single substrate endowed cells with the ability to oxidize specifically that compound and catabolites thereof (140). As a rule, chemically related substances that were utilized via metabolically distinct pathways were not oxidized immediately. This generalization, expressed as the principle of *simultaneous adaptation*, was proposed independently by Stanier (139), by Karlsson and Barker (88), and by Suda et al. (151) as the basis of a new technique for the analysis of catabolic sequences.

Both Stanier and Suda applied the method to elucidate the broad outlines of the pathways used for the utilization of aromatic acids by fluorescent pseudomonads. After growth at the expense of a benzenoid substrate, cells were exposed to chemicals with structures that rendered them likely intermediates in the dissimilatory pathway. Immediate consumption of oxygen by the bacteria was taken as presumptive evidence that the chemical was a catabolic intermediate; compounds that were not oxidized were tentatively excluded from the pathway. An obvious limitation was introduced by permeability barriers: regardless of their enzymic composition, bacteria cannot oxidize substances that do not permeate the cell membrane. Often this difficulty was circumvented by examining cell extracts to determine which enzymes were induced by growth with a given carbon source (65); the technique of simultaneous adaptation still rested upon the guiding postulate that inducible enzymes are synthesized at high rates only when they serve a necessary function during growth.

Sequential Induction

To account for the generally accepted specificity of the inductive response, Stanier proposed a precise, testable hypothesis, the theory of *sequential induction* (139). In its extreme form, the theory states that each enzyme mediating a catabolic reaction is induced by its substrate: thus, when cells

are exposed to a potential growth substrate, the chemical elicits the synthesis of only the enzyme that converts it to the first catabolite in the pathway. The newly formed compound induces solely the enzyme that catalyzes its conversion to the subsequent intermediate. Repetition of the process results in the specific induction of all of the enzymes directly associated with utilization of the growth substrate. Strict sequential inductive control offers an economy of protein synthesis that gives an obvious selective advantage to cells that employ it: since the control prohibits the synthesis of inducible enzymes in the absence of their substrates, amino acids and energy may be efficiently used for the synthesis of essential cellular components. Since no other selective forces had been implicated in the evolution of regulatory mechanisms and because no contradictory evidence was at hand, the concept of rigorous sequential induction gained wide acceptance shortly after its formulation.

The first evidence indicating that inductive events in bacteria were not necessarily independent emerged from genetic studies of carbohydrate catabolism in *E. coli*. In the process of defining the *lac* operon, Jacob et al. (85) demonstrated coordinate expression of contiguous structural genes coding for three separate proteins: β-galactosidase, lactose permease, and galactoside transacetylase. Since only one of the three is an enzyme playing a known role in lactose utilization, this result in itself did not contradict the interpretation of strict sequential induction. Buttin and Englesberg, however, swiftly extended the operon concept to the galactose and arabinose pathways, respectively. Buttin (14) demonstrated that D-fucose, a chemical analogue of D-galactose, was not metabolized by *E. coli* but did induce the synthesis of all three enzymes that convert D-galactose to glucose-1-phosphate. Englesberg (45) showed that L-arabinose directly elicited the synthesis of the three enzymes that convert it to D-xylulose-5-phosphate in *E. coli*. Each author (14, 46) isolated constitutive mutant strains that formed high levels of the three enzymes that he studied in the absence of any exogenous inducer. These results, which clearly demonstrated the absence of sequential inductive steps in two catabolic pathways, reopened the question of the extent to which sequential induction is operative in the metabolically versatile pseudomonads. Catabolic pathways that had been characterized biochemically in these organisms were analyzed to determine the points at which sequential inductive steps are interposed; efforts were made to identify both inducers and the enzymes whose synthesis they govern.

DEFINITIONS

Little is known about the molecular mechanisms that govern enzyme synthesis in the pseudomonads. To protect this developing area of research from potentially misleading interpretations, the limitations of physiological definitions must be emphasized. Partially diploid *Pseudomonas* strains have not been characterized. Consequently, no genetic information regarding the positive or negative control of genetic transcription within these bacteria is available. Most of the following physiological definitions have been described in detail by Cohn (33).

As first pointed out by Cohn et al. (34), the term "enzymatic adaption" is ambiguous because it could describe either a phenotypic or a genotypic change. They suggested substitution of the expression "enzyme induction" to describe "a relative increase in the rate of synthesis of a specific apoenzyme resulting from exposure to a chemical substance." The *inducer* of an enzyme is the metabolite which most directly elicits its synthesis. *Repression* may be defined physiologically as the opposite of induction: a relative decrease in the rate of synthesis of a specific apoenzyme resulting from exposure to a chemical substance. Use of the term "metabolite repressor" (106) to describe the intermediate that most directly represses the synthesis of an enzyme avoids confusion with the well-known *lac* (55) and lambda (119) repressor proteins.

A sequential induction is characterized by a shift in the chemical nature of the inducer (115). Hence, regulatory units that undergo sequential induction are always controlled independently. Enzymes that are induced by the same metabolite are governed by *coincident induction* (107). The synthesis of such enzymes may be controlled either independently or by a more tightly united regulatory control, *coordinate induction* (84). The ratio of the rates of synthesis of enzymes that are subject to coordinate control remains constant despite extreme fluctuations in their absolute rates of synthesis. Enzymes whose syntheses are subject to coordinate control are members of a *regulatory unit*.

METHODS OF APPROACH

Biochemical Background

Most of the time spent in the analysis of inductive control is devoted to the measurement of enzyme activities in crude extracts; both the efficiency and the reliability of these operations are enhanced greatly by a thorough characterization of every enzyme under investigation. The pathway itself cannot be assumed, since divergent catabolic routes have been observed frequently in the pseu-

domonads (6, 144). Indeed, some individual strains possess two independent biochemical mechanisms for the dissimilation of a single compound (42, 52). Furthermore, regulatory linkage sometimes causes the gratuitous induction of enzymes that do not play a role in the utilization of the growth substrate (115). Consequently, convincing proof that a particular catabolic sequence is indeed employed by the organism under investigation requires considerable documentation.

As is the case with biosynthetic pathways, the outlines of an entire dissimulatory sequence may be revealed by investigation of mutant strains that have lost the ability to catalyze component reactions. Although such organisms are unable to utilize the primary growth substrate, they may accumulate an identifiable intermediate in its presence. If the mutant strain, unlike the wild type, lacks an enzyme that attacks the accumulated compound, then there is good reason to believe that the enzyme plays an essential role in the sequence. Reversion of the mutant organism to growth with the original carbon source should be accompanied by the reappearance of the deleted enzyme activity.

Measurement of the influence of *p*H, buffering ion, ionic strength, and temperature on the stability and activity of an enzyme usually permits establishment of readily reproducible assay conditions. Once the enzyme has been purified and characterized, crude extracts may be examined for activators or inhibitors. Constant specific activities in extracts of different cultures of the same strain grown under identical conditions indicate reproducible methods of extraction and measurement.

Induction or Activation?

The isolation of pure enzymes from extracts of mandelate-grown *P. putida* has revealed that from 5 to 10% of the protein in an appropriately induced culture may be in the form of enzymes that catalyze a single strictly inducible catabolic pathway (64, 69, 113, 114). Since the bacteria may possess up to a hundred such metabolic sequences (147), only a small fraction of their catabolic capacity can be expressed at any one time. These observations strongly support the view that most observed increases in the activity of catabolic enzymes in these bacteria are due to de novo protein synthesis rather than to the activation of macromolecular precursors. Further support derives from the observation that the formation of inducible enzymes in pseudomonads is strongly inhibited by ultraviolet light and by chemical inhibitors of protein synthesis (130, 141). Immunological data derived from two pure enzymes of the benzoate pathway of *P. putida* has provided direct evidence for the absence of serologically related precursors of these proteins in uninduced cells (142, 148). Nethertheless, the substrate-dependent activation of a protein precursor into an active enzyme has been demonstrated in bacteria (23), and the possibility that it may occur in the pseudomonads should not be overlooked.

Identification of Inducers

Under ordinary circumstances, the inducer of an enzyme is a member of a series of continuously interconverted metabolites. Hence, the intermediate which most directly elicits the synthesis of an enzyme cannot be identified before it has been rendered nonmetabolizable. Identification has been achieved by three methods: chemical modification of the inducer, genetic alteration of the bacterial strain, and physiological restriction of the metabolism of the cell.

Inducer analogues. A general property shared by many nonmetabolizable inducer analogues is a chemical modification that removes the susceptibility of the compound to enzymic attack without introducing a major steric variation. Four analogues that have been employed successfully in investigations of the pseudomonads are depicted in Fig. 1. L-Kynurenine, the inducer of the first three enzymes of the L-tryptophan pathway of a fluorescent pseudomonad, strain Tr-23, is shown in Fig. 1. By reducing the γ-ketone of this compound to an alcohol, Palleroni and Stanier (118) prevented its in vivo enzymatic hydrolysis (to L-alanine and anthranilate) without destroying its ability to act as an inducer. Similarly, Lessie and Neidhardt (100) showed that reduction of urocanate to imidazolepropionate totally prevents its enzymic conversion to the next intermediate in the pathway without destroying its ability to induce enzymes of the L-histidine catabolic pathway in *P. aeruginosa*. The same effect had been observed previously by Schlesinger and Magasanik (132) in cultures of *Aerobacter aerogenes*. A more striking chemical modification was introduced by Hegeman (66) who demonstrated that phenoxyacetate, which is sterically similar to inducers of the mandelate enzymes (Fig. 1), is an effective inducer analogue in *P. putida*. Despite the steric similarity, the radical structural variation in the side chain of phenoxyacetate completely prevents its metabolism by the bacteria. Radical steric modification of a compound need not remove its effectiveness as an inducer. Kelly and Clarke (90) have shown that secondary substitution on the nitrogen of acetamide (Fig. 1) prevents enzymic hydrolysis of the compound but does not impair its ability to induce amidase in *P. aeruginosa*.

Mutant strains. In addition to permitting the elucidation of catabolic pathways, mutant strains

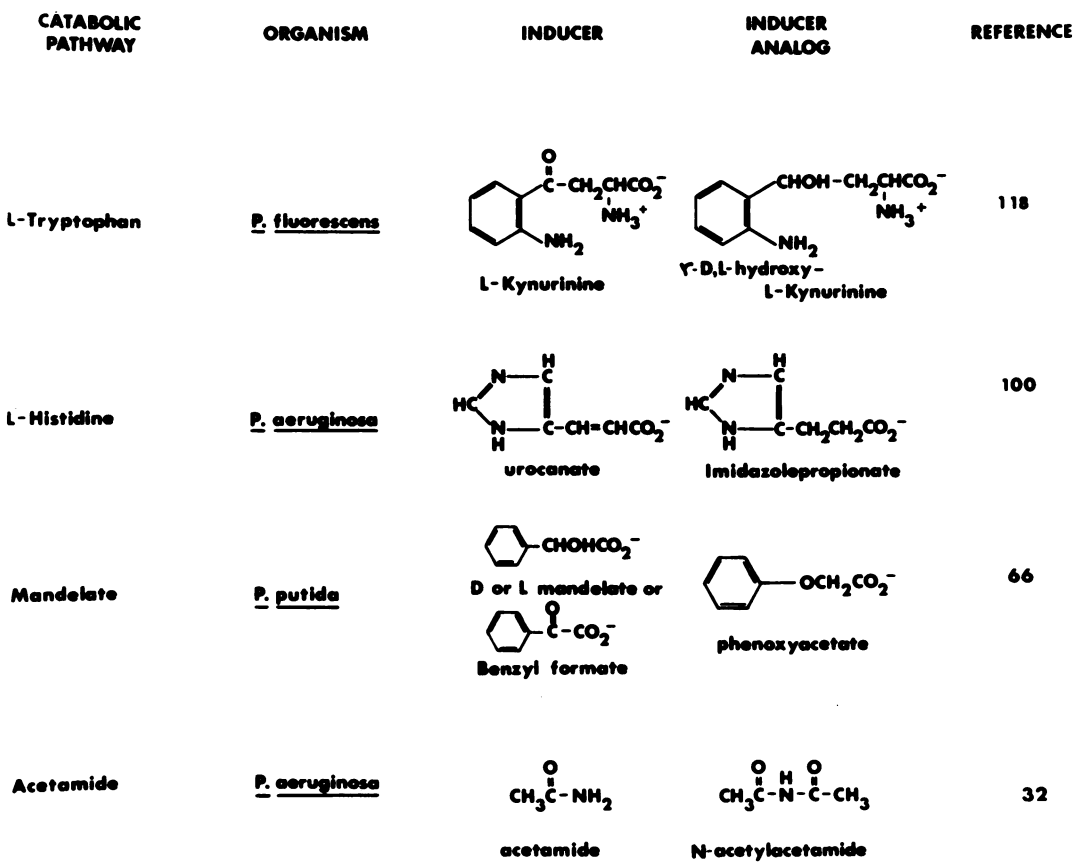

FIG. 1. *Chemical analogues of inducers that elicit the synthesis of catabolic enzymes in fluorescent pseudomonads.*

have played a major role in the analysis of regulatory control. Blocked mutants, unable to complete catabolic pathways due to enzymatic deficiencies, have permitted the unambiguous identification of inducers. Palleroni and Stanier (118), for example, were able to demonstrate that L-kynurenine (Fig. 1) is the inducer of the enzymes that catabolize L-tryptophan in a fluorescent pseudomonad by studying two mutant strains, one blocked in the catabolic step that gives rise to L-kynurenine and other unable to hydrolyze it to alanine and anthranilate. Metabolic precursors of L-kynurenine did not elicit induction in the former mutant strain, but did in the latter. Hence, the endogenous synthesis of L-kynurenine is essential for enzyme induction, and L-kynurenine need not be catabolized to cause enzyme synthesis. These results clearly define L-kynurenine as the catabolite which most directly elicits the synthesis of the L-tryptophan enzymes.

The regulatory linkage of enzymes subject to coincident control has, in some instances, been confirmed by the isolation of constitutive mutant strains. Hegeman (68) employed two generally applicable methods to achieve this goal in his study of the five enzymes that mediate the conversion of D-mandelate to benzoate in *P. putida*. The first technique was the repeated transfer of cultures between growth medium containing mandelate as the only carbon source and medium containing succinate as the sole growth substrate. The mandelate enzymes were diluted out in wild-type cells during growth with succinate; consequently, the constitutive mutant strains possessed a distinct selective advantage when the culture was exposed to mandelate. The second technique employed by Hegeman was the continuous growth of a culture in a chemostat with the concentration of mandelate severely limiting the rate of growth. Under these conditions, the intracellular concentration of inducer is very low and mutant cells that form high levels of the mandelate enzymes appear to enjoy a selective advantage. During growth with noninducing carbon sources, the constitutive strains isolated by Hegeman formed induced levels of all five mandelate enzymes, but synthe-

sized none of the enzymes catalyzing subsequent steps in the pathway. Hence, the constitutive formation of the mandelate enzymes could not be attributed to internal induction due to the endogenous production of mandelate.

Physiological restriction of metabolism. A physiological restriction was used to facilitate the characterization of the inductive mechanism governing the catechol branch of the β-ketoadipate pathway in fluorescent pseudomonads (Fig. 2). Growth of either *P. putida* or *P. aeruginosa* with *cis,cis*-muconate as carbon source elicits the synthesis of catechol oxygenase and of the regulatory unit of two enzymes that converts muconate to β-ketoadipate enol-lactone (115). Since the oxygenation of catechol cannot be reversed, muconate or a subsequent catabolite must be the inducer of the oxygenase. Growth with metabolic precursors of muconate elicits the synthesis of catechol oxygenase and muconolactone isomerase (but not enol-lactone hydrolase) in mutant strains that lack muconate lactonizing enzyme. Hence, a catabolite derived from muconate cannot be an inducer and the inducer must be muconate itself.

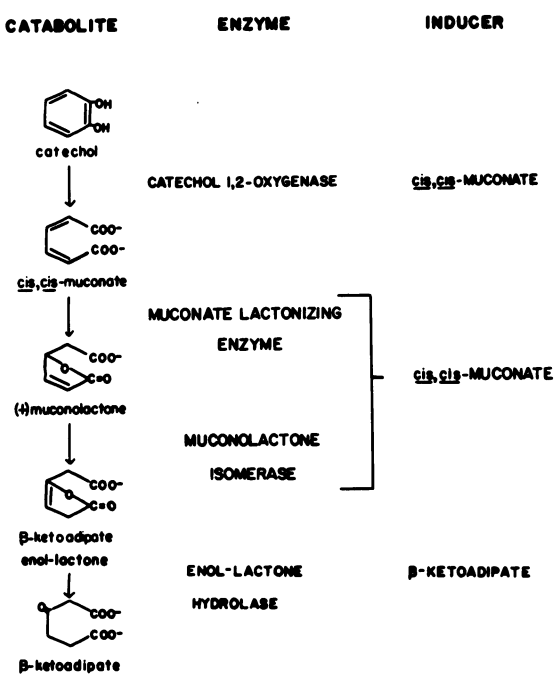

FIG. 2. *Regulation of the catechol branch of the β-ketoadipate pathway in P. putida. The bracket indicates that the synthesis of muconate lactonizing enzyme and of muconolactone isomerase are under coordinate control; the synthesis of catechol oxygenase is regulated independently, but all three enzymes are induced by cis,cis-muconate.*

That catechol is not an inducer was established by Kemp and Hegeman (91) and by Bird and Cain (7) who exploited the ability of *P. aeruginosa* to use nitrate as a terminal electron acceptor for respiration. Since oxygen is a substrate of catechol oxygenase, catechol cannot be cleaved by the bacteria in its absence. Cultures of *P. aeruginosa* were grown at the expense of lactate in the presence of catechol with nitrate as the sole terminal electron acceptor. In the absence of oxygen, the enzymes of the β-ketoadipate pathway were not induced; admission of oxygen to the culture permitted the cleavage of catechol to muconate by the low levels of catechol oxygenase in uninduced cells and thus triggered induction of the enzymes. These results clearly showed that catechol does not directly elicit the synthesis of the enzymes of the β-ketoadipate pathway.

Demonstration of Coordinate Induction

Coordinate inductive control can best be demonstrated with cultures in which the absolute rate of induced enzyme synthesis is varied over a wide range. Partial induction may be achieved either by limiting the amount of inducer or by adding carbon sources that repress the synthesis of induced enzymes. The former approach was employed by Palleroni and Stainier (118), who examined the three enzymes that initiate the catabolism of L-tryptophan in a fluorescent pseudomonad. All three enzymes are subject to coincident control and are induced by the nonmetabolizable analogue γ-hydroxy-L-kynurenine (Fig. 1). Palleroni and Stanier grew cultures in the presence of various concentrations of the analogue and found that the relative activities of two enzymes, L-tryptophan pyrollase and *N*-formylkynurenine formamidase, remained constant over a 50-fold range of induction. Hence, the regulatory sites governing their synthesis bear the same affinity for the inducer and, by this criterion, are subject to coordinate control. The same test established the independent control of kynureninase, which requires a relatively high concentration of inducer for full induction.

The enzymes of the β-ketoadipate pathway are sensitive to catabolite repression, a property that permits examination of the degree to which they are under coordinate control. For example, the three enzymes that convert catechol to β-ketoadipate enol-lactone (Fig. 2) are all subject to coincident induction by *cis,cis*-muconate in *P. putida* (115). The synthesis of catechol oxygenase, however, is far more sensitive to catabolite repression than is the synthesis of the other two enzymes which are repressed as a regulatory unit (115).

The Value of Kinetic Experiments

The degree of induction in a bacterial culture can be varied by adding an inducing growth substrate to cells growing at the expense of a noninducing carbon source; the kinetics of induction for different enzymes may then be observed. Such studies may provide firm evidence for the independent regulation of induced enzymes. For example, two sequential inductive steps intervene between the synthesis of the mandelate enzymes by mandelate and the induction of the catechol enzymes by *cis,cis*-muconate in *P. putida* (Fig. 2). The separation of inductive steps is reflected in a temporal lag between the appearance of the mandelate enzymes and the appearance of the catechol enzymes in a succinate-growth culture (Fig. 3; 66, 146). The lag of 40 min convincingly demonstrates the independent regulation of the two groups of enzymes.

Kinetic experiments can give misleading evidence that suggests that the synthesis of independently governed enzymes is subject to coordinate control. The danger inherent in drawing conclusions from such experiments was shown by Hosakawa (79), who followed the rate of appearance of the enzymes of *p*-hydroxybenzoate catabolism in cultures of *P. putida*. Studies with mutant strains had revealed that *p*-hydroxybenzoate hydroxylase, the first enzyme in the *p*-hydroxybenzoate pathway, was induced by its substrate and that five metabolic conversions and two sequential inductive steps are required before the synthesis of enol-lactone hydrolase, an enzyme catalyzing a subsequent step in the pathway, is triggered in cells exposed to *p*-hydroxybenzoate (79, 115). Nevertheless, after Hosakawa added *p*-hydroxybenzoate to cultures of *P. putida* that were growing at the expense of glucose, the two enzymes appeared simultaneously (Fig. 4). These results clearly demonstrate that the sequential induction of enzymes, which is defined as a shift in the *chemical* nature of the inducer, need not be reflected as a discernable difference in the time of their initial appearance after the addition of a metabolizable inducing substrate to a growing culture.

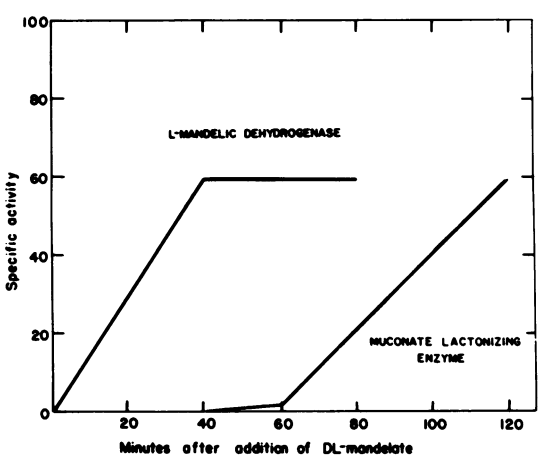

FIG. 3. *The kinetics of induction of catabolic enzymes by mandelate in P. putida under semigratuitous conditions. After the addition of* D,L*-mandelate to a culture that was growing at the expense of succinate, a lag of 40 min (almost one mass-doubling time) elapsed between the appearance of enzymes of the mandelate regulatory unit and the sequential induction of muconate lactonizing enzyme (66, 146).*

ISOLATED PATHWAYS

Hexoses

Most common hexoses appear to be metabolized via the Entner-Doudoroff pathway (47; Fig. 5) in *Pseudomonas* (44, 48, 99). The two reactions that are uniquely associated with this biochemical sequence are catalyzed by 6-phos-

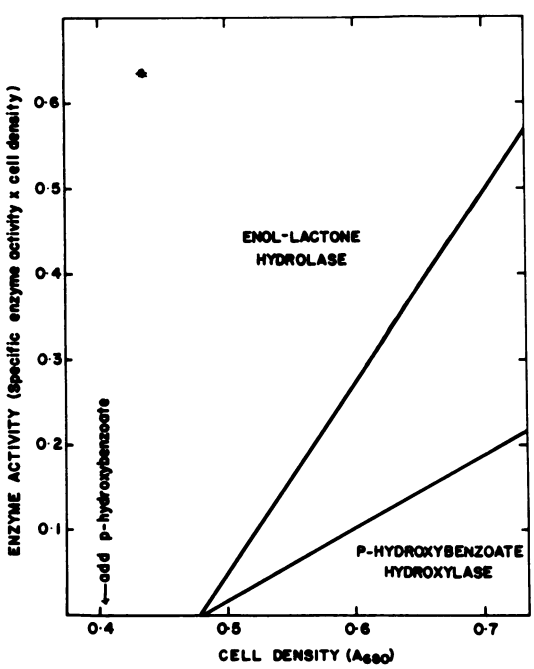

FIG. 4. *The kinetics of induction of catabolic enzymes by p-hydroxybenzoate in P. putida under semigratuitous conditions. The times of the initial appearance of p-hydroxybenzoate hydroxylase and of enol-lactone hydrolase are indistinguishable despite the fact that the biosynthetic controls of the two enzymes are separated by two sequential inductive steps (79).*

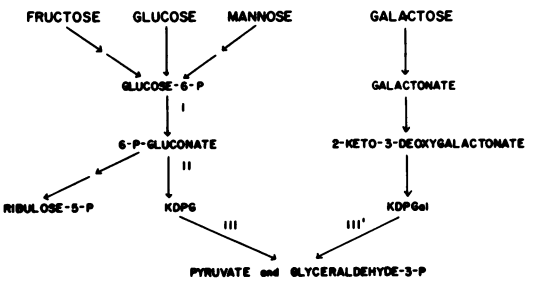

FIG. 5. *Pathways for the utilization of hexoses in Pseudomonas.*

phogluconate dehydratase and by 2-keto-3-deoxy-6-phosphogluconate aldolase (enzymes II and III, respectively, in Fig. 5). The synthesis of these enzymes has been shown to be inducible in *P. aeruginosa* (99); their formation is elicited by exposure of the bacteria to glucose, fructose, mannose, or gluconate, but not by growth with pyruvate, succinate, or citrate (61, 99). Indeed, citrate has been shown by Hamlin, Ng, and Dawes (62) to lower the rate of synthesis of the enzymes during growth in the presence of glucose, but this effect may not be due to repression. The uptake of glucose by *E. coli* (111) and by *Aspergillus nidulans* (125) is inhibited by a metabolite closely related to acetyl coenzyme A (CoA); the apparent repression observed in *P. aeruginosa* may be due to a similar inhibition of glucose permease of this organism by citrate or by catabolites derived therefrom (86).

The specific activity of glucose-6-phosphate dehydrogenase (enzyme I in Fig. 5) is substantially increased by growth of *P. aeruginosa* in the presence of glucose. Further metabolic control of this enzyme was demonstrated by Lessie and Neidhardt (99), who showed that either adenosine triphosphate (ATP) or guanosine triphosphate inhibited its activity by decreasing its ability to bind glucose-6-phosphate. They suggested that this mechanism prevented drainage of glucose-6-phosphate into the Entner-Doudoroff pathway during energy-rich conditions. Vander Wyk and Lessie (154) found that the glucose-6-phosphate dehydrogenase from *P. multivorans* was regulated in a similar manner. In addition, they demonstrated that ATP inhibited the activity of the enzyme with nicotinamide adenine dinucleotide (NAD) as substrate to a greater degree than the inhibition of activity with nicotinamide adenine dinucleotide phosphate (NADP) as substrate; this differential inhibition, they proposed, permits the cell to govern the relative rate of reduction of NAD and NADP by glucose-6-phosphate.

Despite its chemical similarity to D-glucose, D-galactose is catabolized via an independent route in *P. saccharophila* (138; Fig. 5). The galactose pathway, like the Entner-Doudoroff pathway, culminates in the aldol cleavage of a 2-keto-3-deoxy-6-phosphohexose to pyruvate and glyceraldehyde-3-phosphate (reaction III′ in Fig. 5). The two hexose derivatives that are cleaved differ only in the orientation of the hydroxyl group on the 4 carbon. Nevertheless, the two aldolases (enzymes III and III′) are physically separable. Neither enzyme shows activity with the analogous substrate from the convergent pathway, and antisera formed against one enzyme do not react with the other (138). The syntheses of the two enzymes are controlled independently; enzyme III is formed under all growth conditions but enzyme III′ is found only in cultures grown with D-galactose. The two enzymes do share many physical characteristics, indicating that they may have a homologous evolutionary origin.

Ruiz-Amil et al. (129) have shown that inductive control extends to glyceraldehyde-3-phosphate dehydrogenase in *P. putida*. Growth with glucose or glycerol increased the level of the enzyme in this organism to 100 times the specific activity found after growth with succinate, lactate, or acetate. The severity of this control prompted the authors to question the frequently assumed gluconeogenic role of glyceraldehyde-3-phosphate dehydrogenase in this species. Support for their view that gluconeogenesis is achieved by an as yet undescribed pathway in fluorescent pseudomonads derives from the inability of Lessie and Neidhardt to detect fructose-1,6-diphosphate aldolase in extracts of a strain of *P. aeruginosa* (99). Tiwari and Campbell (152), however, did detect the aldolase in another strain of the same species. A comparative study of carbohydrate metabolism in the pseudomonads may clarify this situation and, at the same time, shed new light on the problem of hexose synthesis.

Tartrate

The first studies on the metabolism of tartaric acid by *Pseudomonas* indicated that the enzymes involved were under specific inductive control. Shilo (136) established that an unidentified pseudomonad formed three different dehydrases, each of which converted one of the three optical isomers of tartrate to oxaloacetate; each dehydrase appeared to be induced by its substrate, but not by the other two tartrate isomers. Shilo and Stanier (137) extended these results and suggested that the rate of tartrate metabolism was also governed by specific permeases, each of which was induced by and functional upon a single isomer of tartrate. Conflicting results were obtained with another strain. Martin and Foster (110) showed that, after growth with L- or D-

tartrate, cultures of *P. fluorescens* oxidized all three isomers.

Since these early investigations, the biochemistry of tartrate utilization has been shown to be quite complex. Dagley and Trudgill (40) revealed that tartrate was metabolized by a pathway independent of oxaloacetate in a number of pseudomonads; extracts of appropriately induced *P. acidovorans* cultures converted either L- or *meso*-tartaric acid to glycerate and carbon dioxide. NAD was essential for this conversion. An extensive analysis by Kohn, Jacoby, et al. (92–96), who crystallized many of the enzymes involved, provided firm support for the conclusions of Dagley and Trudgill. It is now clear that *Pseudomonas* may catabolize tartaric acid via any of the routes depicted in Fig. 6. Indeed, one strain of *P. putida* forms a tartrate dehydrase when grown with the L-isomer (81), but catabolizes *meso*-tartrate via glycerate (40, 92); the enzymes involved are inducible, but their inducers have not been identified. In light of present biochemical knowledge, it should be possible (i) to determine the precise metabolic routes used for dissimilation of tartrate by individual strains of *Pseudomonas*, (ii) to identify the inductive mechanisms that govern the selection of specific pathways of tartrate catabolism, and (iii) to establish the degree to which these regulatory mechanisms are shared by taxonomically related strains.

Dicarboxylic Acids of the Tricarboxylic Acid Cycle

Succinate, malate, and oxaloacetate play a multiple role in aerobic metabolism; they serve as metabolic precursors for the synthesis of carbohydrates, acetyl CoA and the aspartate family of amino acids, they participate catalytically in the oxidation of acetyl CoA via the tricarboxylic acid cycle and, if their exogenous concentration is sufficient, they may serve as growth substrates. To be oxidized completely, the dicarboxylic acids first must be converted to acetyl CoA. The key enzyme initiating this sequence in *P. putida* appears to be the malic enzyme which, in the presence of malate and NAD, gives rise to pyruvate, reduced nicotinamide adenine dinucleotide, and carbon dioxide. Jacobson et al. (86) showed that the synthesis of malic enzyme is sharply repressed by acetate, a mechanism which conserves dicarboxylic acids in the presence of other sources of acetyl CoA. A converse control, the stimulation of the activity of enzymes that form dicarboxylic acids from C-3 compounds and carbon dioxide by acetyl CoA, has been observed in several bacteria (18, 19, 131). Jacobson et al. (86), however, found no inhibition

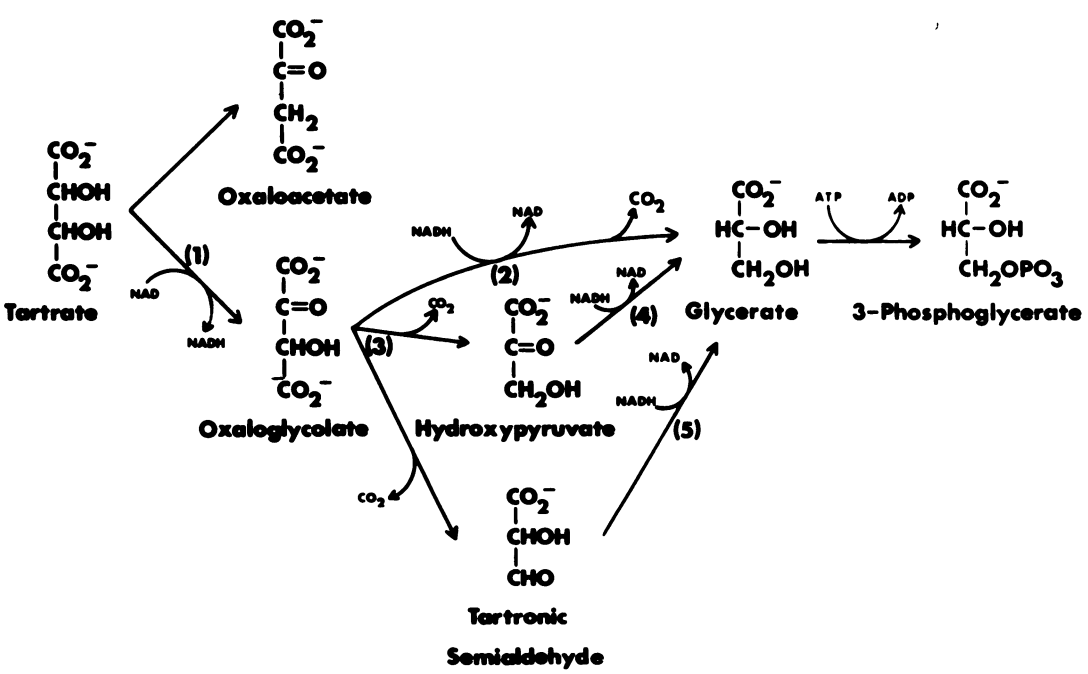

FIG. 6. *Catabolic fates of tartrate in different Pseudomonas species. Enzymes catalyzing reactions 1, 2, 4, and 5 have been crystallized.*

of the activity of malic enzyme by acetyl CoA or by related compounds.

Branched-Chain Dicarboxylic Acids

Cooper and Kornberg (35) showed that itaconate (Fig. 7) is converted to pyruvate and acetyl CoA by inducible enzymes in cultures of *P. acidovorans* (strain B_2aba). Growth with itaconate, but not with succinate, endowed the organism with the ability to oxidize itaconate and, in addition, mesaconate and citramalate (Fig. 7). Brightman and Martin (10), analyzing an unidentified pseudomonad, found a similar pattern of induction: after growth with itaconate, mesaconate, or citramalate, this organism oxidized all three compounds. The data of Brightman and Martin indicate that, unlike *P. acidovorans*, the pseudomonad they studied converted the branched-chain dicarboxylic acids to succinate via succinic semialdehyde. Thus, two different catabolic pathways appeared to be employed by the two strains examined; the specificity of inductive control is not rigorous in either instance.

Higher Dicarboxylic Acids

In an investigation of the metabolism of dicarboxylic acids, Hoet and Stanier (72) found isofunctional enzymes under independent inductive control. A single strain of *P. fluorescens* was shown to form two distinct enzymes that catalyze the transfer of CoA from succinyl-CoA to β-ketoadipate. One transferase was specifically induced by growth with β-ketoadipate or metabolic precursors thereof. The other transferase was not induced by β-ketoadipate but was found in extracts of cells that have been grown with unsubstituted dicarboxylic acids. Hence, the two transferases of overlapping specificity are subject to regulatory controls that dictate their metabolic activities: one enzyme is employed in the dissimilation of β-ketoadipate and the other initiates the catabolism of adipate. It should be noted that an even greater degree of inductive specialization was observed by Cánovas and Stanier (21), who found *three* inducible β-ketoadipate-succinyl-CoA transferases in *Acinetobacter* (*Moraxella*) *calcoacetica*. One enzyme was found in adipate-grown cells and appeared to be uniquely associated with the utilization of that compound. Two transferases effected the metabolic activation of β-ketoadipate; one of these was induced by *cis,cis*-muconate, a metabolic precursor of β-ketoadipate, and the other was induced by protocatechuate which is degraded to β-ketoadipate by a largely independent route.

Hoet and Stanier (71) found that higher dicarboxylic acids (pimelic, suberic, azelaic, and sebaric) were in large part catabolized in *P.*

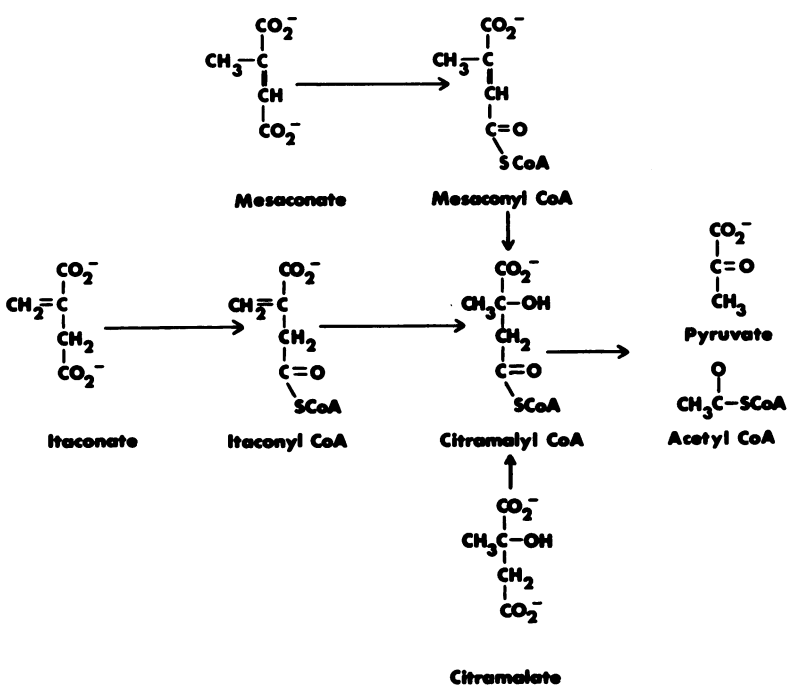

FIG. 7. *Possible pathways for the utilization of branched-chain dicarboxylic acids by P. acidovorans. The reaction sequence for the conversion of itaconate to pyruvate and acetyl CoA has been demonstrated by Cooper and Kornberg (35). The other reactions are hypothetical.*

fluorescens by a single set of enzymes under common inductive control. Growth with any of the four acids elicited the synthesis of the enzymes requisite for the complete oxidation of the other three. Two mutants, one deficient in the synthesis of a thiokinase and the other unable to form β-hydroxyacyl CoA dehydrogenase, were unable to grow at the expense of any of the dicarboxylic acids in the C_7–C_{10} series. In addition, the latter but not the former mutant had lost the ability to grow at the expense of adipate. An apparently inefficient biosynthetic control operated at the level of activation: growth with any of the acids containing from 6 to 10 carbons elicited the synthesis of both the transferase and the thiokinase, despite the fact that the former enzyme is associated solely with the activation of adipate (C_6) and that the latter enzyme is not required for growth with this compound.

Pathways Leading to α-Ketoglutarate

A wide variety of carbon compounds is catabolized in pseudomonads by pathways that converge upon α-ketoglutarate. Some of these growth substrates are listed in Table 1, which also indicates the routes by which they are converted to α-ketoglutarate. It should be noted that growth substrates as diverse as D-glucarate and L-hydroxyproline are utilized via a common intermediate, α-ketoglutarate semialdehyde. Indeed each of the 10 carbon sources listed is converted to one of three possible direct metabolic precursors of α-ketoglutarate. This metabolic convergence may not occur at the enzymic level: a single bacterium might contain the capacity to form five independent α-ketoglutarate semialdehyde dehydrogenases, each induced by growth with a different substrate. On the other hand, a single dehydrogenase might be elicited by growth with all precursors of α-ketoglutarate semialdehyde.

The first evidence relevant to this problem was gathered by Adams and Rosso (1), who purified and characterized two α-ketoglutarate dehydrogenases from extracts of *P. putida*. One form of the enzyme was formed constitutively; this enzyme possessed a greater affinity for glutaric semialdehyde than for the α-keto derivative and, consequently, was believed by the authors to play a role in the catabolism of lysine via glutaric acid. A second dehydrogenase, which was highly active with α-ketoglutaric semialdehyde as substrate, was isolated from cells that had been grown with hydroxyproline. A considerable amount of physical evidence indicated that this enzyme was identical to the dehydrogenase that was formed during growth at the expense of glucarate.

TABLE 1. *Catabolic pathways entering the tricarboxylic acid cycle via α-ketoglutarate*

Primary growth substrate	Direct metabolic precursor of α-ketoglutarate	References
L-Arabinose	α-Ketoglutarate semialdehyde (2,5-dioxovalerate)	41
D-Galactarate	α-Ketoglutarate semialdehyde (2,5-dioxovalerate)	41, 87
D-Glucarate	α-Ketoglutarate semialdehyde (2,5-dioxovalerate)	41
L-Hydroxyproline	α-Ketoglutarate semialdehyde (2,5-dioxovalerate)	56
D-Xylose	α-Ketoglutarate semialdehyde (2,5-dioxovalerate)	41
L-Histidine	L-Glutamate	63,80, 100
L-Tryptophan (quinoline pathway)	L-Glutamate	145
L-Arginine	L-Glutamate	97
L-Lysine	α-Hydroxyglutarate	120
Glutarate	α-Hydroxyglutarate	82

Subsequently, Gryder and Adams (56) showed that hydroxyproline is an inducer of all of the enzymes of the hydroxyproline pathway, including α-ketoglutaric semialdehyde dehydrogenase in *P. putida*. Therefore, it appears that either the same enzyme can be induced both by hydroxyproline and by glucarate (or by a metabolite derived therefrom) or that two different, but physically indistinguishable, enzymes are induced. This question will not be resolved until mutant strains deficient in the synthesis of the dehydrogenase are isolated.

Hydroxyproline

As mentioned above, the regulation of the four enzymes that convert hydroxy-L-proline to α-ketoglutarate in *P. putida* has been investigated in detail by Gryder and Adams (56). Either hydroxy-L-proline or allo-hydroxy-D-proline (the first intermediate in the pathway) elicits full induction of all four enzymes; the same compounds cause the synthesis of an active permease which concentrates either inducer.

In the same study, Gryder and Adams also investigated the in vivo stability of induced catabolic enzymes in *P. putida*. The intracellular levels of two induced enzymes of the hydroxy-

proline pathway decreased at a rate of about 5% per hour when the cells were shaken in the absence of inducer. After 7 hr the rate of enzyme disappearance decreased markedly. Induction of enzymes associated with other catabolic pathways did not increase the turnover of the hydroxyproline enzymes. On the contrary, the gratuitous preinduced proteins appeared to be stabilized in cells that were forming inducibly large quantities of other enzymes, even in the absence of an exogenous source of nitrogen. Since the endogenous amino acid pools are insufficient to account for the large amount of de novo synthesis that was observed, the source of nitrogen for synthesis of the induced enzymes remains an intriguing mystery.

Histidine

The regulation of L-histidine catabolism has been compared in a number of bacteria: the mechanism of induction has been explored in *Aerobacter aerogenes* (133), *Salmonella typhimurium* (11), *Bacillus subtilis* (29), *P. aeruginosa* (100, 112), *P. putida* (80), and *P. acidovorans* (63). In all but one of the examined species, urocanate, the first catabolite derived from L-histidine, plays a dual inductive role: it elicits the synthesis of the enzyme that gives rise to it in addition to inducing at least two of the enzymes that mediate its conversion to L-glutamate. Thus, in these organisms urocanate appears to serve a function analogous to that of the product inducer L-kynurenine in the L-tryptophan sequence. On the basis of this evidence it is tempting to conclude that the enzymes mediating early steps in the utilization of essential metabolites, such as L-histidine, are usually governed by product induction in bacteria. The strength of this generalization is vitiated, however, by the clear demonstration by Chasin and Magasanik (29) that L-histidine need not be converted to urocanate to elicit full induction of the catabolic pathway in *B. subtilis*. As Chasin and Magasanik pointed out, their results do not preclude the possibility that another product derived from histidine metabolism is the true inducer of the *B. subtilis* enzymes, but they do suggest that product induction may not be an essential control governing the catabolism of biosynthetic intermediates.

Lessie and Neidhardt (100) conducted a detailed examination of histidine catabolism in *P. aeruginosa*. In addition to establishing the central inductive role of urocanate, they found that the enzymes of the pathway were sensitive to catabolite repression in the presence of succinate. Ammonium salts enhanced the effect, suggesting that a nitrogenous compound derived from succinate effected the repression. A possible mechanism for the observed effect of succinate was suggested by Hug et al. (80), who examined another fluorescent pseudomonad, *P. putida*. They found that succinate was an effective inhibitor of urocanase. By itself, this interaction might be expected to enhance the level of inducer and hence to increase, rather than decrease, the rate of synthesis of histidine enzymes. Hug et al. further found, however, that urocanate inhibited the activity of histidine ammonia lyase, the enzyme that forms it from histidine. Consequently, it appears that the pool size of the inducer is governed, on a coarse level, by the relative intracellular concentrations of histidine ammonia lyase and of urocanase and, on a fine level, by small molecules which influence the activity of these enzymes.

P. acidovorans shares the regulatory pattern found for the histidine pathway in the fluorescent strains but shows more metabolic flexibility towards imidazole derivatives. A strain examined by Hassall (63) oxidized both imidazolepropionate (which had been used as a nonmetabolizable inducer by Lessie and Neidhardt) and imidazoleacetate. Both compounds elicited full synthesis of the enzymes that catabolize histidine.

Valine

As shown in Fig. 8, 10 different enzymes appear to be associated with the utilization of D- and L-valine via succinyl CoA in fluorescent pseudomonads (4). The biosynthetic regulation of six of these enzymes in *P. putida* has been studied by Marshall and Sokatch (109). Two of the four enzymes that convert D- and L-valine to methylacrylyl CoA are constitutive; the other two enzymes are induced by growth with L-valine but are controlled independently (Fig. 8). Kinetic experiments suggested that the two enzymes that catalyze the conversion of 3-hydroxyisobutyrate to propionyl CoA (reactions VI and VII in Fig. 8) may be coordinately controlled; of the enzymes examined, only these two were induced by growth with either L-valine or isobutyrate. Hence, it appears that the inducer of these two enzymes, unlike the inducer of the early enzymes in the pathway, is readily formed from both L-valine and isobutyrate. Studies with mutants suggest that L-valine is not an inducer of the dehydrogenase that converts 2-ketoisovalerate to isobutyryl CoA (reaction II in Fig. 8); rather the enzyme is induced by its substrate, 2-ketoisovalerate, suggesting that this compound plays a major role in the induction of the enzymes that initiate the pathway.

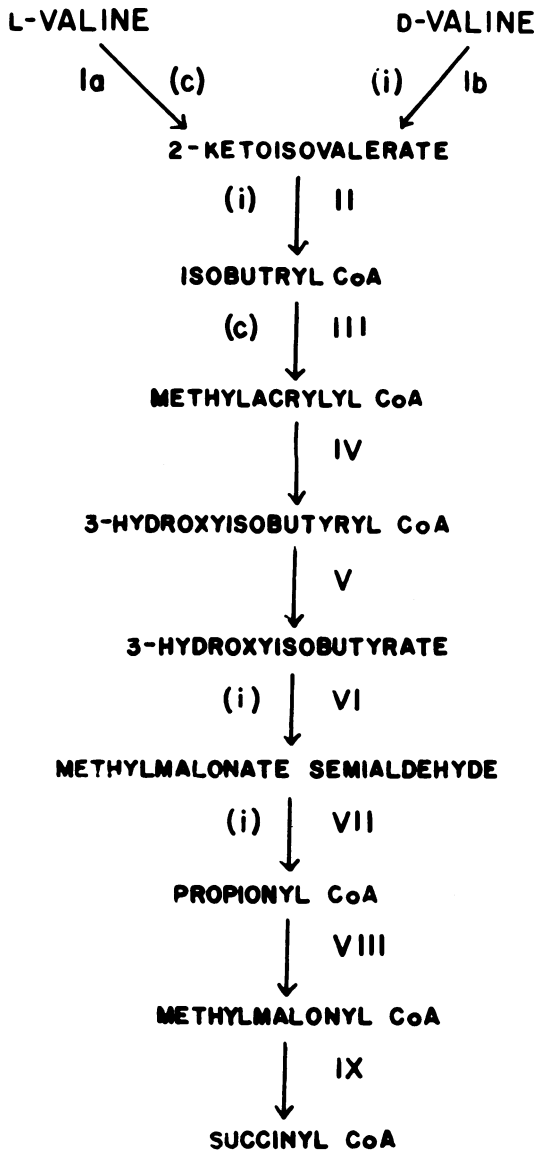

FIG. 8. *A suggested pathway (4) for the conversion of* D- *and* L-*valine to succinyl CoA in P. putida. The regulation of the six enzymes symbolized by underlined roman numerals was studied by Marshall and Sokatch (109). The symbol (c) indicates that the enzymes were found to be constitutive; the symbol (i) means that they were inducible. As symbolized by the bracket, the enzymes catalyzing reactions VI and VII appeared to be under coordinate control.*

Camphor

The four enzymic reactions that initiate the utilization of camphor in *P. putida* are depicted in Fig. 9. The first and the third reactions are catalyzed by monooxygenases, each of which is composed of three distinct proteins (59, 158). Hence, the conversion of (+)-camphor to 3,4,4, trimethyl - 5 - carboxymethyl - Δ^2 - cyclopentenone (compound V in Fig. 9) requires the activity of eight different proteins. All eight are fully induced by growth with compound V; this catabolite appears to induce directly since it is produced from camphor by a series of irreversible reactions. Nevertheless, (+)-camphor also elicits the synthesis of the enzymes very shortly (3 min) after its exposure to cells. On the basis of these results, Gunsalus et al. (59) suggested that both camphor and compound V, despite their radically different chemical structures, may be inducers of this group of enzymes.

Some of the enzymes of the camphor pathway possess a fairly broad specificity. As noted by Gunsalus et al., this biochemical flexibility, coupled with the coincident induction exerted by several intermediates in the pathway, may permit the organisms to utilize a wide variety of compounds with structures analogous to intermediates in the camphor pathway.

Several of the enzymes mediating early reactions in the camphor pathway appear to be subject to coordinate biosynthetic control. This suggested to Gunsalus et al. that they may be members of the same operon. Strong support for this conclusion comes from the demonstration by Gunsalus' group that some of the structural genes encoding the camphor enzymes are linked in a genetic element that can be transferred as a unit between different strains of *P. putida* (24).

Metabolism of compound V by *P. putida* produces isobutyrate which is converted to isobutyryl CoA and then metabolized via reactions common to valine catabolism (Fig. 8). Isobutyrate-grown cultures of *P. putida* oxidize neither camphor (58) nor L-valine (109). Thus, sequential inductive control permits the formation of enzymes that catalyze the reactions subsequent to metabolic convergence without necessitating the induction of the enzymes that catalyze the relatively specialized reactions associated with camphor and valine catabolism.

Acetamide

Clarke and her associates have exploited the relatively simple biochemistry of acetamide metabolism to analyze in detail the interplay between induction and repression that governs amide utilization in *P. aeruginosa* (30). A single, inducible enzyme hydrolyzes acetamide to acetate and ammonia in this organism. The acyl intermediate in this cleavage can be trapped as the hydroxamate derivative, permitting facile estimation of the activity of the hydrolase. Survey of a

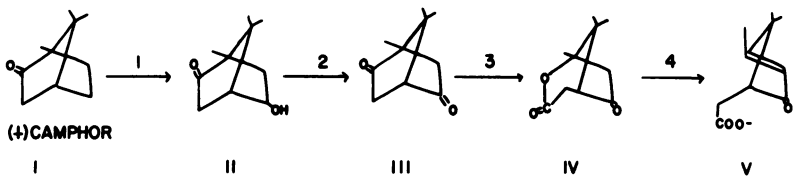

FIG. 9. *The initial reactions in the catabolism of (+)-camphor by P. putida (59).*

number of amides revealed that *N*-acetyl acetamide (Fig. 1) was an inducer but not a substrate of the enzyme. Formamide served as an enzyme substrate but was a weak inducer; furthermore, succinate strongly repressed formamide-induced enzyme synthesis. Consequently, Brammar, Clarke, and Skinner (9) were able to isolate regulatory mutant strains by selecting for organisms which, unlike the wild type, could grow in mineral medium containing succinate as carbon source and formamide as nitrogen source. Some of the mutants were shown to be magno-constitutive, forming amidase at a rate comparable to fully induced wild-type strains; others were partially constitutive and still subject to some inductive control. A third mutant category was characterized by an altered regulatory mechanism which enabled formamide to act as an effective inducer. Transductional analysis with the phage F116 demonstrated that the regulatory gene that was altered in the mutants is very close to the amidase structural gene (9).

Competition between induction and repression of amidase was explored by Clarke et al. (31, 32) in continuous cultures of wild-type *P. aeruginosa* growing at the expense of acetamide. At low dilution rates, relatively little amidase was produced, presumably because the intracellular levels of inducer were held low by enzymic hydrolysis. Higher dilution rates increased the rate of amidase synthesis, as would be expected if the intracellular concentration of inducer increased. Nevertheless, a dramatic decrease in the specific activity of amidase was observed as the dilution rate approached the maximal level. Clarke et al. attributed the decreased rate of enzyme synthesis to the rapid conversion of acetamide to metabolites that effect catabolite repression of the enzyme. To support their conclusion, they showed that mutant strains, resistant to catabolite repression by succinate, formed higher levels of amidase than did wild-type cells at high dilution rates. These results convincingly demonstrate the multiple variables introduced by studies with metabolizable inducers and further indicate that studies with batch cultures give a restricted view of the factors governing enzyme induction.

PATHWAYS CONVERGING UPON β-KETOADIPATE

Tryptophan

The first unambiguous identification of an inducer governing catabolic enzymes in *Pseudomonas* was achieved by Palleroni and Stanier (118). They examined the regulation of the enzymes that initiate the catabolism of L-tryptophan in strain Tr-23, a fluorescent pseudomonad. As shown in Fig. 10, this organism employs three enzymes to convert L-tryptophan to L-alanine and anthranilate; a hydroxylase converts anthranilate to catechol which is dissimilated via β-ketoadipate to acetyl CoA and succinate (Fig. 13). All of the enzymes of the pathway are inducible.

Examination of regulatory patterns in blocked mutants established that kynurenine is the only one of the first three intermediates of the pathway that is an inducer. In addition to eliciting the coordinate synthesis of the two enzymes that give rise to it, L-kynurenine coincidently induces kynureninase, the third enzyme of the pathway, which is controlled independently. The fourth enzyme, anthranilate hydroxylase, is induced sequentially by anthranilate. The conclusions of Palleroni and Stanier were further supported by results obtained with nonmetabolizable chemical analogues of L-kynurenine which induced solely the first three enzymes of the pathway (Fig. 1).

The results of Palleroni and Stanier permit an accurate description of the events that follow exposure of an uninduced culture of strain Tr-23 to an exogenous supply of L-tryptophan. Upon entering the cells, the potential growth substrate is converted to L-kynurenine by the combined action of two enzymes, tryptophan oxygenase and *N*-formylkynurenine formamidase, which are present in uninduced cells at 2% of their highest levels. The two enzymes are induced by L-kynurenine, the product of their action, leading to an increased rate of formation of the inducer. In addition to exerting product induction, L-kynurenine stimulates the synthesis of the enzyme that hydrolyzes it to L-alanine and anthranilate. Thus, by regulating the rate of both its formation and its removal, L-kynurenine in-

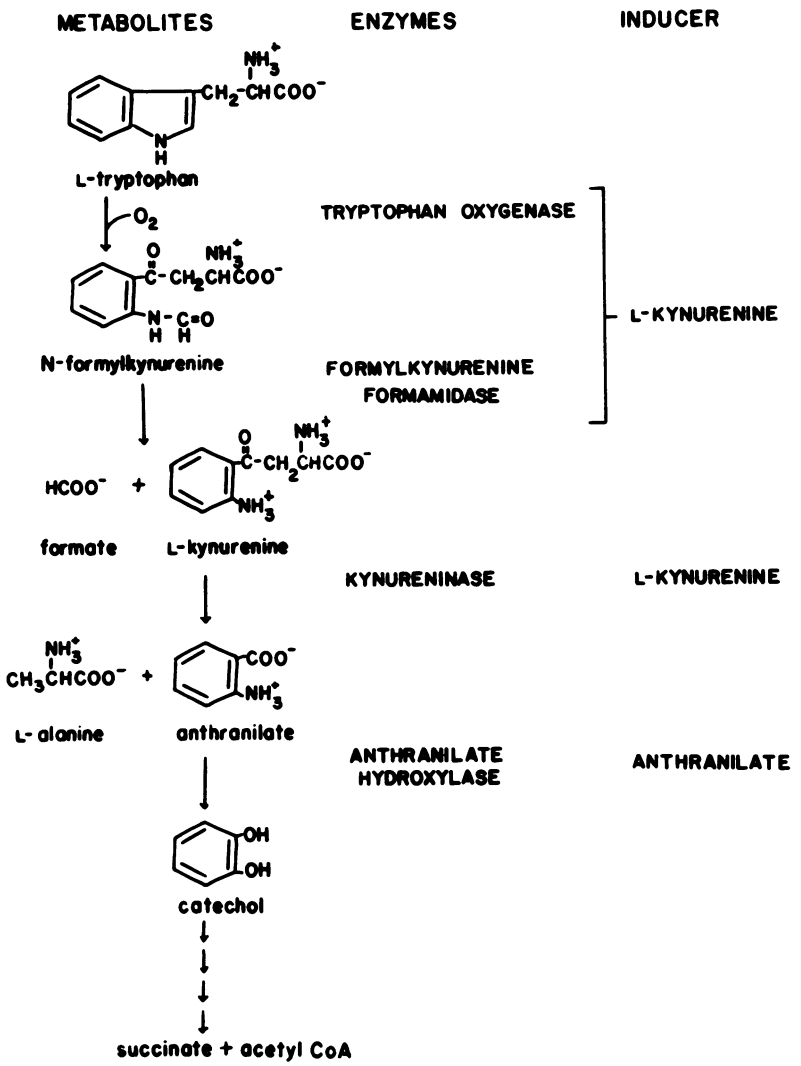

FIG. 10. *Regulation of the L-tryptophan pathway in the fluorescent Pseudomonas strain Tr-23. The bracket indicates that tryptophan oxygenase and formylkynurenine formamidase are under coordinate biosynthetic control.*

fluences its own endogenous concentration. As anthranilate is formed, it effects the first sequential inductive step in the pathway by eliciting the synthesis of the enzyme that converts it to catechol.

The most striking feature of the regulatory system described by Palleroni and Stanier (118) is the induction exerted by the metabolic product L-kynurenine upon the enzymes that form it. The authors suggested that a possible factor leading to the selection of this indirect control was the fact that the primary substrate of the pathway, L-tryptophan, is an essential metabolite and therefore present in the cells under all growth conditions. If biosynthetically formed L-tryptophan triggered the synthesis of enzymes for its catabolism under all growth conditions, a debilitating cycle could result: in the absence of exogenous L-tryptophan, energy consumed in the synthesis of the amino acid would be lost if it were catabolized. On the other hand, if the pool size of L-tryptophan were maintained at a level substantially below the Michaelis constants of the catabolic enzymes that initiate its dissimilation, the L-kynurenine would not be formed at an appreciable rate and the catabolic enzymes would not be induced in the absence of an exogenous supply of the amino acid. Support for the inter-

pretation of Stanier and Palleroni was presented by Magasanik who showed that product induction governed the synthesis of the catabolic enzymes of L-histidine in *A. aerogenes* (133). As noted earlier, comparative studies have revealed a similar mechanism governing L-histidine catabolism in all species examined, with the sole exception of *B. subtilis*.

The regulatory mechanism governing tryptophan catabolism in strain Tr-23 is not unique to this organism. Palleroni and Stanier showed that two other fluorescent pseduomonads [later characterized as representatives of *P. putida* biotype B and *P. fluorescens* biotype G (147)] shared a similar control. Rosenfeld and Feigelson (127, 128) extended the study to *P. acidivorans* which differs both nutritionally and metabolically from the fluorescent pseudomonads (147). Like the fluorescent pseudomonads, *P. acidovorans* cleaves tryptophan to kynurenine, but the catabolic pathways diverge at this point (144). In the quinoline pathway, employed by *P. acidovorans*, L-kynurenine is converted to kynurenic acid and utilized via glutamate (6, 145). Despite this marked metabolic divergence, *P. acidovorans* also employs L-kynurenine as a product inducer of tryptophan oxygenase and *N*-formylkynurenine formamidase (128). The presence of a similar control in these disparate groups suggests that it may have substantial selective value.

Mandelate

The dissimilation of D-mandelate by *P. putida* is initiated by a group of five enzymes that converts the compound to benzoate (Fig. 11); the same enzymes catalyze the conversion of *p*-hydroxymandelate to *p*-hydroxybenzoate (57, 60, 143, 150). Both benzoate and *p*-hydroxybenzoate are metabolized to succinate and acetyl CoA by the convergent β-ketoadipate pathway (Fig. 13). Any one of the first three compounds in the mandelate sequence may serve as a growth substrate for *P. putida*. By examining the inductive effect of these metabolites in mutant strains with lesions in structural genes, Hegeman (67) established that the five mandelate enzymes are subject to coordinate control and that either D-mandelate or benzoylformate is capable of eliciting their full induction; the inductive effect of L-mandelate has not been established. A sequential inductive step intervenes at the level of benzoate. Inducers of the mandelate enzymes do not induce benzoate dihydroxylase, the enzyme catalyzing the next step in the sequence, and benzoate-grown cells do not oxidize mandelate without a lag for induction of the mandelate enzymes (66). Hegeman showed that the enzymes

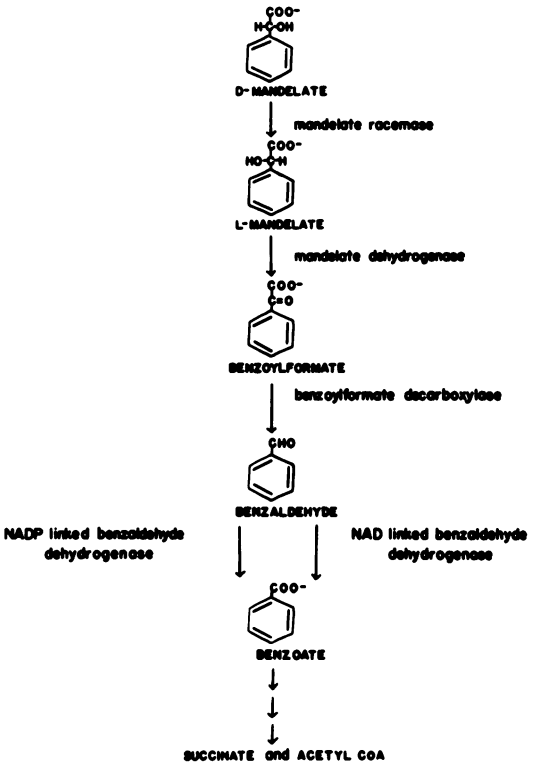

FIG. 11. *The mandelate pathway of P. putida. All five of the enzymes that participate in the conversion of* D-*mandelate to benzoate are governed as a regulatory unit that can be induced by* D-*mandelate or benzoylformate.*

of the mandelate pathway are under coordinate synthetic control: their relative rates of synthesis remain constant when cells are grown in the presence of various concentrations of either the metabolite inducers or the nonmetabolizable inducer analogue phenoxyacetate. Hegeman isolated mutant strains of *P. putida* that formed substantial levels of the five mandelate enzymes under all growth conditions. The mutations in these constitutive strains decreased the specificity of the inductive response; benzoate, for example, increased the level of the mandelate enzymes 20-fold in the mutant, although it does not induce the enzymes in the wild-type strains. Other metabolites that stimulated the synthesis of the mandelate enzymes in the constitutive strains were phenylalanine and tyrosine; indeed, endogenous induction by these biosynthetic intermediates may be responsible for the constitutivity of the mutant strains when they are grown in the absence of any aromatic supplement. The genetic basis for the constitutive mutation is unknown.

The physiological studies of Hegeman (66, 68) clearly demonstrated that the mandelate enzymes

of *P. putida* share a common regulatory gene and suggested that the structural genes coding for the enzymes are genetically contiguous in an operon. Strong support for this view came from the genetic analysis of Chakabarty and Gunsalus (26) who used the phage pf16h_2 to mediate the transfer of the entire mandelate unit from one strain of *P. putida* to another. Using the same transducing phage, Wheelis and Stanier (156) demonstrated the close genetic linkage of the structural genes mandelate dehydrogenase and benzoylformate decarboxylase in *P. putida*. A different genetic arrangement was observed in *P. aeruginosa* by Rosenberg and Hegeman (126), who found that the structural genes for these two enzymes were not cotransducible and that the genetic region encoding the benzoate enzymes appeared to lie between them.

A curious aspect of the mandelate pathway in *P. putida* is the induction of two benzaldehyde dehydrogenases with the mandelate regulatory unit. Each enzyme demonstrates absolute specificity for its pyridine nucleotide substrate, so the two enzymes may be necessary for the reduction of both NAD and NADP in the early steps of mandelate catabolism. A third benzaldehyde dehydrogenase was identified by Stevenson and Mandelstam (150), who found that either benzaldehyde or *p*-hydroxybenzaldehyde repressed the synthesis of the entire mandelate regulatory unit, including both benzaldehyde dehydrogenases, in *P. putida*. Upon exposure to either aldehyde, cells formed inducibly an aldehyde dehydrogenase that was governed independently of the mandelate regulatory unit.

Mandelstam and Jacoby (108) found that benzoate, catechol, and succinate, catabolites derived directly from mandelate, all inhibited the induction of the mandelate enzymes in *P. putida*. They suggested that this effect was due to a specific repression exerted by each of these metabolites upon the synthesis of the mandelate regulatory unit and hence named it multisensitive end-product repression. Since the repressive effect was exerted by succinate, the apparent repression by catechol and by benzoate might have been attributed to catabolite repression exerted by the end products of the aromatic pathway. Mandelstam and Jacoby excluded this interpretation by showing that both catechol and benzoate inhibited the induction of the mandelate enzymes in mutant strains that were unable to catabolize these aromatic metabolites. As the authors pointed out, the inhibition of the induction of the mandelate unit was not necessarily due to a repressive effect: the aromatic catabolites could have blocked the permeation of mandelate into the cell and thus might have lowered the endogenous concentration of inducer. The inhibition of the synthesis of the mandelate enzymes by benzoate, catechol, or succinate can be overcome by increasing the concentration of mandelate in the growth medium. Thus, the effect appears to be competitive. Mandelstam and Jacoby generally added mandelate to a final concentration of 250 μg/ml. By increasing this concentration fourfold, they largely prevented the apparent repression exerted by aromatic metabolites and by succinate. The competitive effect accounts for the apparent discrepancy between the results of Mandelstam and Jacoby and those of Hegeman (66), who found that succinate did not repress the mandelate enzymes. Hegeman employed a relatively high mandelate concentration in his growth media.

Shikimate

Shikimate and quinate are dissimilated via protocatechuate in *P. putida*; the step reactions in this convergent metabolic pathway are shown in Fig. 12. Tresguerres et al. (153) showed that

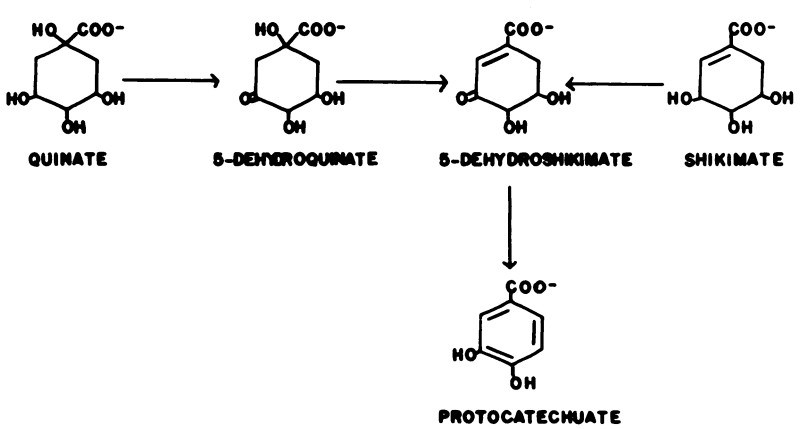

FIG. 12. *Metabolic pathways for the conversion of quinate and shikimate to protocatechuate in P. putida.*

protocatechuate is the inducer of these enzymes in *Acinetobacter*. This does not appear to be the case in *P. putida*; after growth at the expense of protocatechuate, cultures of this species do not oxidize quinate or shikimate (156). Quinate is not an inducer in a mutant of *P. putida* that lacks the ability to convert dehydroquinate to dehydroshikimate; these results suggest that the most likely inducer of the enzymes is 5-dehydroshikimate, although shikimate may also play an inductive role.

The β-Ketoadipate Pathway

The β-ketoadipate pathway (Fig. 13) is a metabolic mechanism for the formation of succinate and acetyl CoA from protocatechuate or catechol (116, 117). These diphenols occur frequently in the metabolism of pseudomonads because they are the last aromatic intermediates in the dissimilation of a wide variety of benzenoid compounds. Catechol, for example, is produced directly from salicylate, phenol, benzoate, anthranilate, and benzene (38). All of these compounds occur in the natural environment and, in addition, most of them are intermediates formed in the catabolism of more complex aromatic growth substrates. Hence, the β-ketoadipate pathway plays a major role in the nutrition of the metabolically versatile pseudomonads.

Catechol and protocatechuate are converted to a common intermediate, β-ketoadipate enol-lactone, by a series of three chemically analogous reactions (117). Oxygenative cleavage between the hydroxyl groups yields *cis,cis*-muconate from catechol and β-carboxy-*cis,cis*-muconate from protocatechuate; strictly analogous enzyme reactions convert the two muconates to muconolactone and γ-carboxymuconolactone, respectively. Decarboxylation of γ-carboxymuconolactone forces the migration of the double bond within the lactone ring to yield β-ketoadipate enol-lactone; deprotonation of the γ-carbon of muconolactone gives rise to the same product via an analogous mechanism. Despite the marked similarity of the reactions of the catechol and protocatechuate branches of the β-ketoadipate pathway, they are all catalyzed by physically separable enzymes of high specificity (113, 114). Protocatechuate

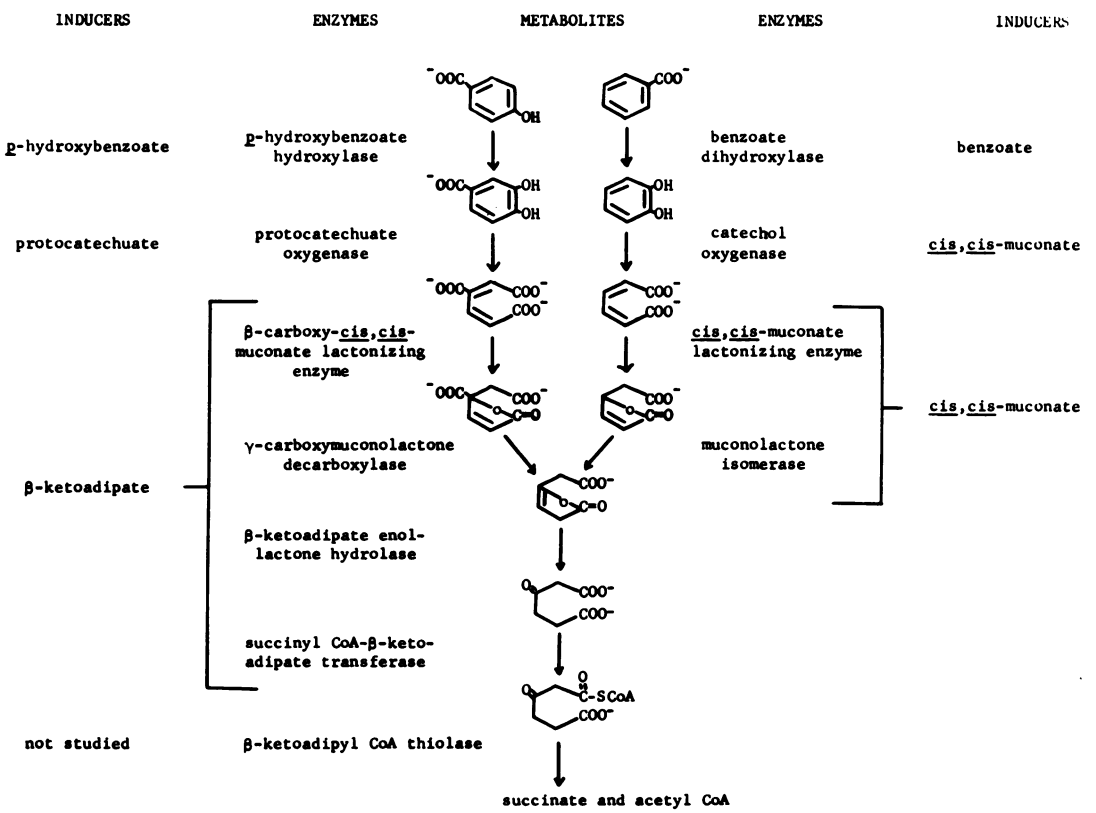

FIG. 13. *The β-ketoadipate pathway. The enzymes that are subject to coordinate biosynthetic control in P. putida are enclosed in brackets.*

oxygenase from *P. putida* cleaves catechol at 2% of the rate it acts upon its natural substrate; catechol oxygenase does not act upon protocatechuate. Muconate lactonizing enzyme exhibits no detectable activity towards β-carboxy-*cis*,*cis*-muconate, and the carboxymuconate lactonizing enzyme does not catalyze the lactonization of *cis*,*cis*-muconate. Muconolactone isomerase and γ-carboxymuconolactone decarboxylase also show no activity with the substrate analogue from the parallel pathway. Nevertheless, the enzymes catalyzing analogous reactions do share some physical properties, which suggests that they may share a homologous evolutionary origin (117).

A single series of enzymes catalyzes the conversion of β-ketoadipate enol-lactone to succinate and acetyl CoA in the pseudomonads (117, 146). Hydrolysis of the enol-lactone yields β-ketoadipate which is activated to β-ketoadipyl CoA and cleaved by type reactions common to fatty-acid catabolism.

As shown in Fig. 13, the enzymes of the two branches of the β-ketoadipate pathway are governed by quite different inductive mechanisms in the pseudomonads (115). Whereas *cis*,*cis*-muconate is the inducer of the enzymes in the catechol branch, β-carboxy-*cis*,*cis*-muconate does not play an inductive role. Furthermore, coordinate regulation in the protocatechuate branch extends much further than in the catechol branch: the carboxymuconate regulatory unit contains enol-lactone hydrolase and succinyl CoA:β-ketoadipate transferase, enzymes that are essential for the utilization of both protocatechuate and catechol. The operation of these different inductive controls can be shown by reviewing the physiological events that follow the exposure of an uninduced *Pseudomonas* culture to *p*-hydroxybenzoate, a metabolic precursor of protocatechuate, or to benzoate, a precursor of catechol.

When *p*-hydroxybenzoate enters a *Pseudomonas* cell that can use it as a growth substrate, it triggers the synthesis of the hydroxylase that converts it to protocatechuate. As noted above, protocatechuate is a major site of catabolic convergence; a sequential inductive step caused by endogenously accumulated protocatechuate initiates the synthesis of the oxygenase that gives rise to carboxymuconate. The next three intermediates in the pathway are not inducers; the conversion of carboxymuconate to β-ketoadipate, the inducer of the carboxymuconate regulatory unit, is mediated by three enzymes, carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase, which are present at 2% of their fully induced levels in uninduced cells. Once formed, β-ketoadipate induces the carboxymuconate regulatory unit of enzymes, and the utilization of *p*-hydroxybenzoate via β-ketoadipyl CoA commences.

Like *p*-hydroxybenzoate, benzoate induces only the enzyme for which it serves as substrate in *Pseudomonas*. A sequential inductive step takes place at the level of catechol, but catechol itself is not an inducer. The activity of catechol oxygenase, which is present at less than 0.1% of its fully induced level in uninduced cells, is essential to form *cis*,*cis*-muconate, which triggers the synthesis of the three enzymes of the catechol branch. Two separate regulatory units are induced by *cis*,*cis*-muconate. Muconate-lactonizing enzyme and muconolactone isomerase are regulated as a unit; their synthesis is far less sensitive to catabolite repression than that of catechol oxygenase. Once they are induced, the three enzymes of the catechol branch catalyze the net conversion of catechol to β-ketoadipate enol-lactone. A sequential inductive step takes place at this site, but once more the first metabolite in the sequence is not the inducer: the enol-lactone must be hydrolyzed to β-ketoadipate by the basal levels of enol-lactone hydrolase before the hydrolase and the transferase can be induced. The induced synthesis of these enzymes and of the thiolase permits benzoate to be used as a growth substrate.

Despite their independently governed synthesis, catechol oxygenase and the muconate regulatory unit share a common regulatory gene; a mutant of *P. putida* that is constitutive for the muconate regulatory unit also forms catechol oxygenase in the absence of inducer (157). The synthesis of the oxygenase remains relatively sensitive to catabolite repression in the mutant strain, indicating that this enzyme is governed by two regulatory genes: one, shared with the muconate regulatory unit, controls induction and the other dictates catabolite repression.

An apparent physiological burden is placed upon *Pseudomonas* strains because of the regulatory linkage of the enzymes governing the hydrolase and the transferase with carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase, enzymes that are unique to the protocatechuate sequence. Since all four enzymes are under coordinate inductive control, the latter two enzymes, which serve no metabolic function in the catabolism of catechol, are fully induced during the utilization of this compound. When this regulatory pattern was first observed in *P. putida*, it was suggested that chemical properties could have restricted the selection of a metabolite as an inducer for the carboxymuconate regulatory unit (115). β-Carboxy-*cis*,*cis*-muconate, γ-carboxymucono-

lactone, and β-ketoadipate enol-lactone are chemically unstable. If this, or another chemical property, precluded their use as metabolic effectors, then the enzymes of the carboxymuconate unit would have to be induced either "from the top" by protocatechuate, or "from the bottom" by β-ketoadipate. The use of β-ketoadipate as an inducer forces the gratuitous synthesis of carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase during the utilization of catechol by *Pseudomonas*. Another genus of nutritionally versatile bacteria, *Acinetobacter*, has been shown to employ protocatechuate as an inducer of all of the enzymes that convert it to β-ketoadipyl CoA (16, 17, 21, 22). As a consequence of this induction from the top, *Acinetobacter* does not induce any of the protocatechuate enzymes during growth with precursors of catechol. These bacteria synthesize a second form of enol-lactone hydrolase and succinyl CoA:β-ketoadipate transferase that is controlled in coordination with muconate lactonizing enzyme and muconolactone isomerase; this regulatory unit is induced by *cis,cis*-muconate in *Acinetobacter* (21). Thus, both *Pseudomonas* and *Acinetobacter* appear to govern the enzymes of the carboxymuconate unit with less than optimal efficiency. The former bacteria synthesize gratuitous enzymes under some growth conditions and the latter bear redundant genetic information. The development of these seemingly awkward control systems is most easily explained as a consequence of the inability of β-carboxy-*cis,cis*-muconate, γ-carboxymuconolactone, or β-ketoadipate enol-lactone to serve as inducers.

The α-Ketoacid Pathways

Some *Pseudomonas* species initiate the catabolism of protocatechuate and catechol by cleaving the aromatic rings on one side of, rather than between, the neighboring hydroxyl groups (5, 8, 37, 49, 54, 123). The catabolic pathways that follow this mechanism of "*meta*"-ring cleavage yield α-ketoacids by the sequences depicted in Fig. 14. Although the reactions of the two pathways are analogous, they appear to be mediated by physically separable enzymes that are subject to independent inductive control.

The specificity of the enzymes of the catechol α-ketoacid pathway is not absolute. Indeed, Dagley and his associates (36, 37) proposed that this sequence serves as a general reaction for the dissimilation of methyl-substituted aromatic compounds. In support of this view, Ribbons (121, 122) showed that growth of *P. aeruginosa* with phenol, 2-methyl phenol, 3-methyl phenol, or 4-methyl phenol elicits the synthesis of the

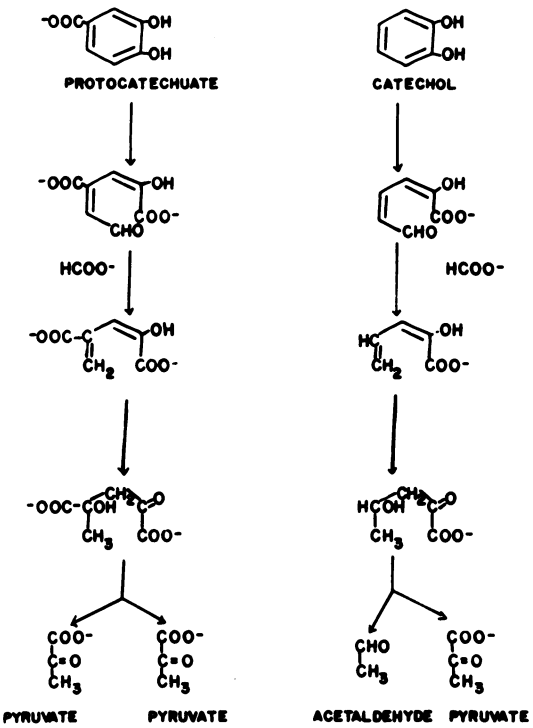

FIG. 14. *The α-ketoacid pathways for the utilization of protocatechuate and catechol.*

enzymes of the catechol α-ketoacid pathway. The methyl phenols are converted to methyl catechols and dissimilated by the same enzymes that act upon catechol.

The regulatory mechanisms governing the synthesis of the enzymes of the α-ketoacid pathways assumed new importance when Davies and Evans (42) showed that a single *Pseudomonas* strain possessed the ability to form the catechol enzymes of both the α-ketoacid pathway and the β-ketoadipate pathway. When grown with napthalene or salicylate, the organism converted the growth substrate to catechol which was dissimilated via the α-ketoacid pathway. On the other hand, growth of the same organism with benzoate elicited specifically the enzymes of the β-ketoadipate pathway. Similar results were reported from studies with a different strain by Azoulay and Senez (2, 3). Cain and Farr (15) found that benzenesulfonates elicited the synthesis of the α-ketoacid catechol pathway in *P. aeruginosa* but that growth with benzene always induced the enzymes of the β-ketoadipate catechol pathway.

An indication of the chemical basis for the selective utilization of cleavage mechanisms emerged from a survey conducted by Seidman et al. (134) with a strain of *P. aeruginosa*. They

found that aromatic growth substrates bearing electron-withdrawing groups, such as protocatechuate and cinnamic acid, elicited the synthesis of the enzymes of the β-ketoadipate pathway but that substitution of the aromatic ring of growth substrates with electron-donating alkyl groups led to the induction of the enzymes of the α-ketoacid pathway. The intralactonic rearrangements of the β-ketoadipate pathway probably would be hindered by electron-donating groups since they are dependent upon either nucleophilic attack (in lactonization) or deprotonation (in isomerization). This is not true of the open-chain hydrates involved in the α-ketoacid pathways. Consequently, the inductive mechanisms appear to select the enzymes of the metabolic pathway that require the least activation energy.

The physiological basis for the selective utilization of the aromatic pathways was elucidated by Feist and Hegeman (52, 53), who examined a strain of *P. putida* that can use either the β-ketoadipate or the α-ketoacid pathways for the utilization of catechol. The results of their studies, shown in Fig. 15, clearly demonstrate how the choice of inducers can determine the pathway employed. Phenol and its methyl derivatives directly induce all of the enzymes of the α-ketoacid pathway. This coincident inductive control assures that the formation of catechol 2,3-oxygenase is simultaneous with that of the hydroxylases that mediate the formation of catechol or its methyl derivatives. Consequently, catechol (or methyl catechol) does not accumulate within the cells after exposure to phenol (or methyl phenol); rather the induced catechol 2,3-oxygenase maintains a low concentration of the diphenolic compound. On the other hand, the inductive effect of benzoate is restricted to the enzyme complex that mediates its conversion to catechol. Upon exposure to benzoate, uninduced cells synthesize benzoate dihydroxylase which forms catechol. The accumulated catechol is converted to *cis,cis*-muconate by the uninduced levels of catechol 1,2-oxygenase; as it is formed, muconate elicits the synthesis of the enzymes of the catechol branch of the β-ketoadipate pathway.

In summary, the α-ketoacid pathway appears

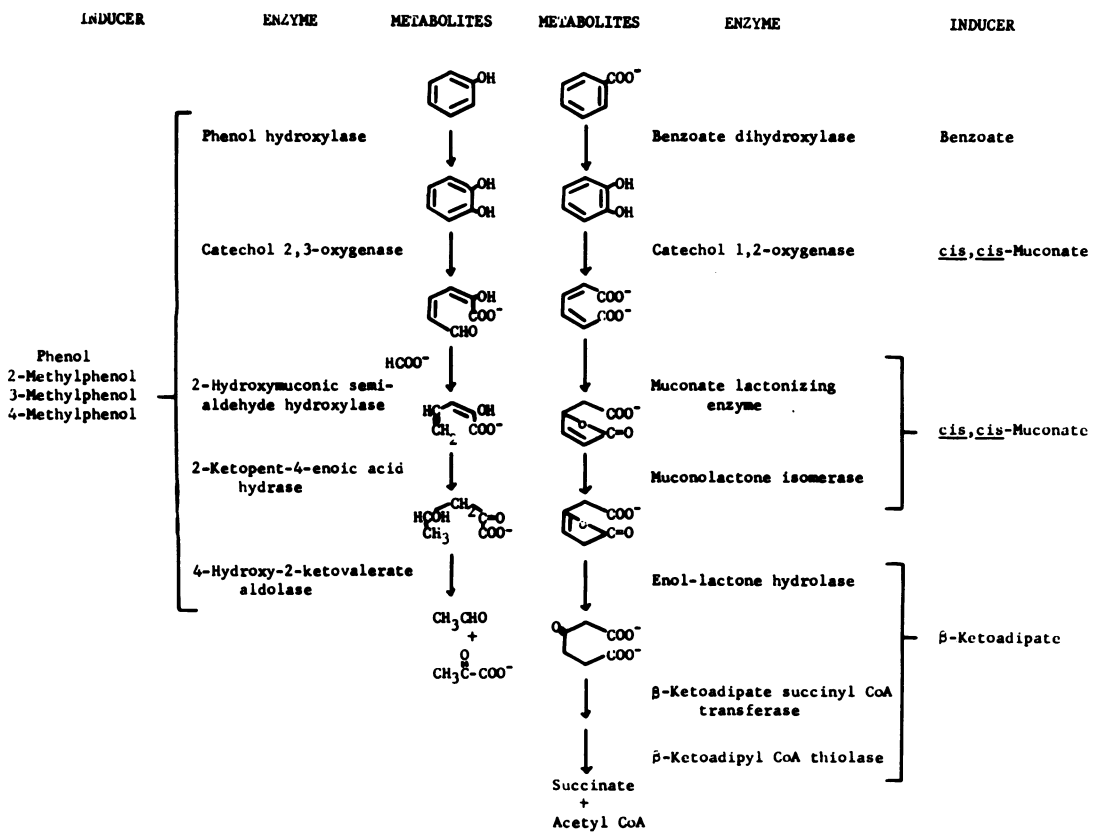

FIG. 15. *Regulation of the α-ketoacid and the β-ketoadipate pathways in a single strain of P. putida. Enzymes that are subject to coordinate biosynthetic control are enclosed in brackets (53).*

to be a general mechanism for the dissimilation of alkyl-substituted phenolic compounds; these growth substrates directly induce the synthesis of all of the enzymes of the pathway. In contrast, the β-ketoadipate pathway appears to be a more specific metabolic sequence; apparently alkyl derivatives of catechol cannot be catabolized via this route. Consequently, the product induction of catechol 1,2-oxygenase may confer a considerable selective benefit upon cells by enhancing the specificity of the inductive response. For catechol or a derivative thereof to elicit the synthesis of catechol 1,2-oxygenase, the carbon chain of the compound must interact specifically with biological macromolecules in two very different chemical environments. First, the diphenol must be cleaved by the enzymes; second, the dicarboxylic muconic acid must interact with the regulatory apparatus. An alkyl substitution might not completely prevent the oxygenative cleavage of catechol, but the electron-donating effect of the substitution might be expected to exert a significant influence on the dissociation constants of muconic acid and thus to strongly influence its interaction with the macromolecule that governs the synthesis of the oxygenase. By exercising strict inductive control, cells prevent the accumulation of large quantities of alkyl-substituted muconic acids that in all probability cannot undergo the intramolecular rearrangements of the β-ketoadipate pathway.

EVOLUTIONARY FORCES THAT DETERMINE THE REGULATION OF CATABOLIC PATHWAYS

Enzymes that catalyze neighboring catabolic reactions in *Pseudomonas* are frequently governed as units of metabolic function subject to coincident inductive control exerted by a single metabolite; therefore, a sequential inductive step often initiates the synthesis of a group of related enzymes. The use of semisequential inductive control is perhaps best illustrated by the regulatory mechanisms which govern the enzymes of the convergent metabolic sequence for the utilization of aromatic acids by fluorescent pseudomonads. As shown in Fig. 16, 10 inductive events are sufficient to elicit the synthesis of the 25 enzymes of these pathways. Thus, on the average, between two and three enzymes are subject to coincident control. In some instances, however, a single metabolite induces as many as five enzymes, whereas at other sites only a single enzyme is induced by its substrate. Figure 16 also reveals a varied pattern with respect to the selection of inducers: some enzymes are induced by their substrates, some by their products and, in the mandelate sequence of *P. putida*, at least two different metabolites induce a single regulatory unit. These observations prompt two questions. First, what evolutionary forces determine the sites of sequential inductive steps in catabolic

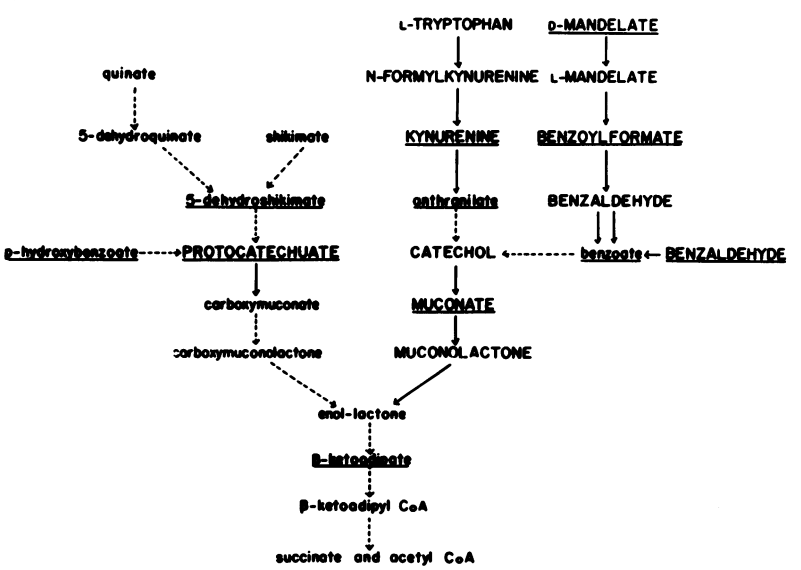

FIG. 16. *Regulation of the pathways for catabolism of aromatic acids by fluorescent pseudomonads. Metabolites are designated by name and the enzymes that catalyze their interconversion are symbolized by arrows. A sequential shift in inductive control is indicated by a shift between solid and dashed arrows. For emphasis, the substrates of enzymes symbolized by solid arrows are in upper case type and the substrates of enzymes symbolized by dashed arrows are in lower-case type; thus, a sequential shift is also indicated by a shift in type. Metabolites known to be inducers are underlined.*

pathways? Second, what factors favor the selection of certain metabolites as inducers? These questions are the subjects of the following two sections.

The Sites of Sequential Inductive Steps

Two counterbalancing selective factors appear to have directed the evolution of semisequential inductive control. One of these is the economy of protein synthesis that derives from the formation of inducible enzymes in the presence, but not in the absence, of their substrates. The other selective force is the economy of information permitted by the use of a common regulatory mechanism to govern the synthesis of several enzymes; the consequences of this force are exemplified by the mandelate pathway of *P. putida* (Fig. 16) in which only 3 sequential inductive steps are required to trigger the synthesis of the 12 enzymes that convert the primary substrate to succinate and acetyl CoA. By uniting the inductive control over enzymes catalyzing neighboring reactions, cells restrict the amount of regulatory information required to achieve the full regulation of enzyme synthesis. Since the replication and expression of regulatory information, like the synthesis of enzymes, drains the biosynthetic capacity of cells, regulatory efficiency would be expected to be selectively advantageous.

In response to selective pressure for the maximal economy of protein synthesis governed by a minimal amount of regulatory information, cells appear to have developed coincident inductive control for sets of enzymes which act as *units of metabolic function*. In most natural environments, enzymes that comprise a unit of metabolic function do not act singly; rather, they normally act in concert to achieve a net metabolic transformation. For example, it appears that as the pathway for catabolism of L-tryptophan evolved in fluorescent pseudomonads, this amino acid was a more commonly occurring growth substrate than *N*-formylkynurenine or L-kynurenine. Hence, as the regulation of these catabolic enzymes evolved, the demand for the induced synthesis of kynureninase was almost always accompanied by a demand for the synthesis of L-tryptophan oxygenase and *N*-formylkynureninase; therefore, the selective pressures favoring unified biosynthetic control overcame those favoring strict sequential induction, and the three enzymes were subjected to coincident control. It should be noted that the inductive role of L-kynurenine permits this compound to be utilized for growth, but coincident inductive control forces the gratuitous synthesis of tryptophan oxygenase and *N*-formylkynureninase under these conditions. Evidently the biosynthetic burden imposed by the occasional demand for the gratuitous synthesis of these two enzymes is not as great as the drain of energy and carbon required for the continuous synthesis of regulatory molecules to govern sequentially the three enzymes of the sequence. Anthranilate and L-alanine can serve as growth substrates in the natural environment; consequently, the synthesis of the enzymes that initiate the utilization of these compounds is under independent inductive control.

In general, the enzymes of catabolic pathways are governed as units of metabolic function that mediate the interconversion of metabolites that either exist in the natural environment (as do L-tryptophan and anthranilate) or that are formed by convergent metabolic sequences (as are catechol and β-ketoadipate enol-lactone). The only obvious exception to this pattern in the pathways shown in Fig. 16 is the sequential inductive step that occurs in the protocatechuate pathway at the level of β-carboxymuconate. As mentioned in the discussion of the β-ketoadipate pathway, this curious control appears to result from the inability of β-ketoadipate enol-lactone to act as an inducer. In the catechol pathway, a sequential inductive step does take place at β-ketoadipate enol-lactone, the site of metabolic convergence.

Although existing regulatory mechanisms obviously are the products of evolution, little attention has been paid to the genetic mechanisms that underlie this process. As theories develop to account for the evolution of enzymes, they should be extended to encompass the evolution of regulatory mechanisms, because it is most likely that both structural and regulatory genes were formed by analogous genetic events and by similar environmental pressures.

What is the evolutionary origin of genes encoding functional units of enzymes? Despite a paucity of supporting data, proposals have been formulated to account for two kinds of biochemical evolution (70): the formation of enzymes with new *catalytic* capabilities and the modification of *binding* sites so that enzymes with given catalytic functions can accommodate new substrates (Table 2, line 1). The fundamental difference in the two theories lies in the information that was believed to have been selected initially in the evolving structural genes (Table 2, line 2). Horowitz (77, 78), in his hypothesis of retroevolution, proposed that slight modification of an enzyme with an appropriate binding site could permit it to subject a substrate to either a strain or a charge interaction that caused a new kind of catalysis. Once a functional enzyme was formed, subsequent mutations favoring an enzyme with a more efficient catalytic site could have been selected. Since the metabolites of some pathways share

TABLE 2. *Proposals accounting for the evolutionary origin of metabolic pathways*

Property	Vertical evolution	Horizontal evolution
1. Information acquired by mutation of the evolving genes	Catalytic sites	Binding sites
2. Information initially selected from the evolving genes	Binding sites for enzymes within a pathway	Catalytic sites for enzymes from other pathways
3. Original loci of genes encoding enzymes that catalyze sequential metabolic reactions	Linked	Scattered

similar chemical structures, it has been suggested that the duplication of genes coding for some enzymes within a pathway could have given rise to the evolutionary precursors of structural genes for other enzymes *within the same pathway* (77, 78). If common intermediary metabolites (e.g., intermediates in the tricarboxylic acid cycle) are regarded as at the bottom of the evolutionary scale and complex chemicals (e.g., phenylalanine or camphor) as at the top, then metabolic pathways can be considered vertical; given these assumptions, homologous enzymes within the same pathway can be defined as the products of *vertical evolution* (Table 2). Several lines of evidence (70, 98) indicate that new metabolic pathways could have developed by *horizontal evolution*, the modification of a structural gene coding for an enzyme that participates in one pathway so that it can act upon a new substrate and permit the functioning of a new pathway. In this *interpathway* evolution, an enzyme was initially selected for its catalytic activity. The enzyme was then modified by mutation so that it could bind a new substrate more efficiently.

The mechanism of formation of new structural genes could be expected to have a profound effect on their genetic and regulatory linkage (Table 2, line 3). Horowitz (77, 78) observed that vertical evolution is a particularly attractive hypothesis because it could account for the observed linkage of many genes encoding sequential metabolic reactions in operons within bacteria; this genetic linkage can be interpreted as the consequence of the initial evolutionary development of the pathway through tandem duplication (101), which could have produced neighboring regions of redundant genetic information. The ease with which unified regulatory control can be exerted over operons could offer a selective benefit to organisms that maintain them. On the other hand, horizontal evolution offers no direct mechanism to permit the genetic or regulatory linkage of enzymes within a pathway. Translocation mutations could have produced genetic linkage, but such mutations would have been retained only in the presence of environmental forces that favored their selection.

A major difficulty in accounting for the evolution of complex pathways is the identification of selective pressures that could have enriched organisms possessing only part of a metabolic sequence. This problem was addressed directly by Horowitz (77), who suggested that biosynthetic path ways developed in a stepwise fashion "from the top." According to his hypothesis of retroevolution, the prebiotic environment contained biosynthetic building blocks and, in addition, the chemical precursors of these compounds. As the supply of a building block was depleted, selective pressure was exerted in favor of organisms that could form it from its chemical precursor. Repetition of this process could have selected for organisms that had acquired an entire biosynthetic pathway one evolutionary step at a time. This simple proposal cannot be applied to many pathways because their intermediates are chemically unstable, would not be expected to accumulate in the natural environment, and do not readily permeate bacterial membranes. It appears that at least some catabolic sequences were acquired as *units of metabolic function*. For example, all of the metabolites formed in the conversion of protocatechuate to succinate and acetyl CoA are too unstable to accumulate in the natural environment; it seems most likely that the ability to utilize protocatechuate was acquired by an organism with the necessary genetic background as the result of a single mutation (117). Once the pathway was established, further mutations that increased its efficiency were selected.

The same environmental pressures that force the evolution of catabolic pathways as units of metabolic function favor the concomitant exchange of genetic material encoding metabolic units. For a genetic recombinant to be enriched in a population, it must acquire genetic information sufficient to permit its growth in a selective

environment. Wheelis and Stanier (156) pointed out that since only a small segment of the chromosome is transferred during genetic exchange among *Pseudomonas*, genetic translocations that link structural genes encoding related metabolic processes would be enriched in the population because they permit entire units of metabolic function to be selected simultaneously in recombinants. Regardless of their evolutionary origin, genes coding for metabolically related enzymes would be linked if they were subject to frequent coincident selection. In *Pseudomonas* species, genes for the enzymes of peripheral catabolic pathways often are linked in supraoperonic clusters (126, 156), suggesting that they are subject to frequent coincident genetic transfer in the natural environment. In contrast, genes coding for biosynthetic processes are widely scattered (50, 74); since anabolic processes are essential for the free-living pseudomonads under almost all growth conditions, mutants with defects in these pathways would be counter-selected rapidly. Consequently, the selection of recombinants with structural genes for the biosynthetic enzymes of *Pseudomonas* is probably a relatively infrequent event outside of the laboratory. If partial genetic exchange favors the linkage of structural genes for units of catabolic function, it should be expected also to favor the linkage of these genes to the genes that regulate their expression. The extreme form of such linkage is in a cluster like that of the amidase structural and regulatory genes in *P. aeruginosa* (9) or the catechol genes in *P. putida* (157).

Interstrain gene transfer obviates the necessity for gene duplication prior to horizontal evolution. Representatives derived from a single strain could develop in separate ecological niches, each of which forced the selection of organisms with differing modifications of a preexisting set of structural genes. Thus, different but homologous pathways could develop within a species. Information encoding for two homologous pathways could then be assimilated within a single strain as a result of interstrain gene transfer. Such genetic exchange may be widespread. Chakrabarty and Gunsalus (27) recently demonstrated the interspecies transfer of genes between *P. putida* and *P. aeruginosa*.

A newly evolved functional unit of enzymes permits organisms that possess it to utilize a potential growth substrate. In addition, it opens new avenues of metabolic potential: additional units of metabolic function that lead into the newly formed pathway can then be selected. By the sequential addition of units of metabolic function away from the central pathways of the cell (from the bottom), complex catabolic pathways like the one shown in Fig. 16 could have evolved. Since the selective pressures that guided the evolution of catabolic pathways are similar to those that determine the sites of coincident induction, it could be argued that on an enzymic level ontogeny recapitulates phylogeny.

The Selection of Inducers

To elicit the synthesis of a functional unit of enzymes, an inducer must bind to a specific recognition site within the cell. The best characterized inducer recognition site, the *lac* repressor of *E. coli*, is a protein (55); it is likely that the inducer recognition sites of *Pseudomonas* are proteins as well. The only known property of *Pseudomonas* inducer recognition sites is their ability to bind metabolites; this limited knowledge focuses attention on the hypothesis of vertical evolution to account for the evolutionary origin of inducer recognition molecules: if the initial selection of binding sites could have directed the evolution of metabolic pathways, could not the same process have played a role in the determination of which metabolites possess inductive function? Speculation of this nature suggests that the evolutionary selection of an inducer for a metabolic unit of function was largely a matter of chance. The inducer recognition site would have been formed from whatever duplicated structural gene was initially modified so that it could assume a regulatory role. A comparison of inductive patterns, however, indicates that the selection of certain metabolites as inducers was not merely a matter of chance. On the contrary, chemical and physiological factors appear to have played a determining role in the selection of inducers for metabolic units of function.

An indication of the restrictions chemical factors can place on the selection of inducers is gained by comparison of the regulation of the β-ketoadipate pathway in different bacterial groups. In *Pseudomonas*, most of the enzymes of the protocatechuate pathway are under coordinate biosynthetic control and are induced by β-ketoadipate (Fig. 16). In *Acinetobacter*, all of the enzymes of the pathway are members of a regulatory unit that is induced by protocatechuate (21). In both genera, regulatory linkage extends past a point of metabolic convergence at β-ketoadipate enol-lactone. As noted above, this control places an apparently gratuitious biosynthetic burden on the cells. The extensive regulatory linkage in the protocatechuate pathway could be the consequence of its evolution as a unit of metabolic function. Nevertheless, it is remarkable that subsequent evolutionary events did not permit the development of independent control to permit a sequential inductive step at the level of

β-ketoadipate enol-lactone. The absence of this relatively efficient mechanism in either genus suggests that some chemical factor, perhaps the instability of β-ketoadipate enol-lactone, γ-carboxymuconolactone, and β-carboxymuconate, precluded their use as inducers and forced the selection of protocatechuate or β-ketoadipate. Thus, just as the shape of metabolic pathways appears to be guided by the feasibility of chemical reactions, the selection of metabolites as inducers may be restricted by chemical factors.

Physiological factors also appear to play a determining role in the choice of inducers for catabolic enzymes. Some functional units of enzymes possess broad specificity and can act upon a number of chemically related substrates. Examples of these are the enzymes that act upon camphor, mandelate, alkyl-substituted diphenols (via the α-ketoacid pathways), and higher straight-chain dicarboxylic acids. The inductive control of these groups of enzymes appears also to be of broad specificity: a number of different metabolites associated with a unit of function can elicit its synthesis. As pointed out by Gunsalus et al. (59), the broad specificity of both activity and inductive control permits these enzymes to confer maximal metabolic flexibility upon cells that possess them; a single set of genetic information can be used for the dissimilation of a wide range of substrates.

On the other hand, some enzymes that participate in specialized catabolic sequences are under highly specific inductive control. Examples of these are the functional units of enzymes employed to initiate the utilization of the amino acids L-tryptophan and L-histidine and for the dissimilation of catechol via the β-ketoadipate pathway. Within *Pseudomonas*, all of these functional units are governed by product induction; the primary substrate must be metabolized by at least one enzyme in the functional unit before induction commences. As described in the earlier section on the regulation of histidine and tryptophan catabolism, product induction can serve to prevent the triggering of a vicious cycle for the catabolism of these metabolites as they are formed biosynthetically. Catechol is not a known biosynthetic intermediate, but the specificity of induction conferred by the regulation of catechol 1,2-oxygenase by *cis,cis*-muconate offers a clear selective benefit. Product induction of the oxygenase forces dual scrutiny of the catechol molecule for possible substitutions before induction can begin; thus, chemical substitutions that might prevent the complete catabolism of the diphenol may be recognized either on the benzene ring or as a component of the dicarboxylic muconic acid. The specific control of induction decreases the possibility of the bacteria initiating a catabolic pathway that they cannot complete. The by-product induction of β-galactosidase by β-galactosyl alcohols in *E. coli* can be regarded as a control analogous to that of catechol oxygenase in *Pseudomonas*. Lactose must be cleaved by β-galactosidase before induction of the *lac* operon can take place. Some naturally occurring disaccharides may be concentrated by the permease but not hydrolyzed by the enzyme. Under normal ecological conditions, by-product induction of the operon may restrict synthesis of the energy-consuming permease to those circumstances in which it concentrates a disaccharide that can be cleaved by β-galactosidase.

Product induction can permit the synthesis of a metabolic unit of function on those presumably rare occasions in which only the enzymes catalyzing the later reactions in the sequence are required for growth. Muconate, for example, cannot be used as a growth substrate by wild-type fluorescent pseudomonads because it cannot permeate their cell membranes. Populations of these organisms, however, contain frequent mutant strains with altered permeability that permits growth at the expense of muconate. If catechol rather than muconate were the inducer of the enzymes of the catechol pathway, two mutations would be required for growth with muconate; the alteration in permeability would have to be accompanied by a change in regulation to permit synthesis of the muconate enzymes. Thus, product induction maintains among the fluorescent pseudomonads a moderately high frequency of organisms that can grow at the expense of muconate.

Thus far, inductive patterns have prompted speculations about the interaction of bacteria with their environment and, more specifically, the evolutionary origins of metabolic pathways. Fragile as these proposals are, they do permit predictions about the sites of sequential inductive steps and the identity of inducers in yet-to-be-studied sequences. As further studies sharpen the concept of a unit of metabolic function and give greater insight into the specificity of inductive control, they may also reveal the evolutionary events that combined to form the complex catabolic pathways of bacteria.

ACKNOWLEDGMENTS

In 1964, I. C. Gunsalus and R. Y. Stanier convened a *Pseudomonas* conference in Cold Spring Harbor to discuss, among other topics, the regulation of catabolic pathways. This productive gathering was just one of their many contributions to the development of the ideas discussed in this paper.

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