THE BACTERIAL OXIDATION OF TRYPTOPHAN

III. ENZYMATIC ACTIVITIES OF CELL-FREE EXTRACTS FROM BACTERIA EMPLOYING THE AROMATIC PATHWAY[1]

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Analysis of the adaptive patterns of many tryptophan-oxidizing pseudomonads indicates that a majority of these organisms metabolize tryptophan *via* kynurenine, anthranilic acid, and catechol—the so-called "aromatic pathway" (Stanier, Hayaishi, and Tsuchida, 1951). In order to confirm the existence of this pathway, we have undertaken a study of the enzymatic activities of cell-free extracts. Only one previous investigation of the enzymes in pseudomonads that oxidize tryptophan by the aromatic pathway has been reported. Hayaishi and Hashimoto (1950) obtained a cell-free preparation that brought about the oxidation of catechol; the enzyme, named "pyrocatechase," was purified about 30-fold, and the product of the reaction with this purified system was identified as *cis-cis*-muconic acid.

MATERIALS AND METHODS

Sources of chemicals. Our work would not have been possible without the generosity of many individuals who supplied us with compounds which are commercially unavailable. We are indebted to Dr. R. E. Kallio and to Dr. D. Bonner for L-kynurenine; to Dr. T. Sakan for DL-α-hydroxytryptophan and DL-formylkynurenine; to Dr. W. C. Evans for *cis-cis-* and *cis-trans*-muconic acids; to Dr. B. Witkop for DL-acetylformylkynurenine; to Dr. P. K. Stumpf for flavinadeninedinucleotide and triphosphopyridinenucleotide; to Dr. W. W. Umbreit for pyridoxal phosphate; to Dr. T. C. Tung for N-methyltryptophan; to Dr. H. N. Rydon for the 4, 5, 6 and 7-monomethyltryptophans; and to Dr. J. Monod for notatin.

Methods for growing cells and preparing extracts. In most of our work a single strain (Tr-23) showing the characteristic adaptive patterns of the aromatic pathway was employed. On a few occasions, extracts were prepared from other strains using this pathway, and showed identical enzymatic activities. Cells were grown for 16 to 20 hr at 30 C on a mechanical shaker in a liquid medium of the following composition: DL-tryptophan, 0.2 per cent; K_2HPO_4, 0.1 per cent; KH_2PO_4, 0.1 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; pH adjusted to 7.0. As a rule, the medium also contained 0.1 per cent Difco yeast extract, which greatly increased the yield of cells without decreasing specific activity. After being

harvested by centrifugation, the cells were washed once with dilute phosphate buffer (pH 7.0) and then subjected to extraction, drying, or other treatments.

A considerable number of methods for obtaining enzymatically-active preparations were tested during the course of our work; they included acetone drying, supersonic disintegration, slow vacuum drying over phosphorus pentoxide, and autolysis of heavy cell suspensions. Most of these methods yielded preparations that could oxidize catechol, but not tryptophan, kynurenine, or anthranilic acid. On a few occasions, vacuum-dried cells or extracts therefrom showed a slow oxygen uptake when provided with tryptophan. The one technique which consistently produced extracts able to oxidize all the previously mentioned compounds was McIlwain's (1948) method of grinding wet cells with alumina. This very rapid and simple procedure appears to be the method of choice for obtaining enzymatically-active extracts from bacterial cells, and its use has now extended successfully to the isolation of other bacterial enzyme systems in our laboratories. Since McIlwain's method does not seem to have received the adoption that it merits, a brief account of our experience with it will be presented.

McIlwain used a British brand of alumina, but we have obtained excellent results with two American brands, sold as "levigated alumina" by Adolph Buehler and by Norton Abrasives. Both are supplied as dry powders, and in our experience can be used without any treatment; a preliminary washing with distilled water to remove possible impurities may in some cases be a wise precaution. A paste of wet bacterial cells is mixed with 2 to 3 times its weight of dry alumina and ground by hand in a well-chilled mortar until cellular disintegration occurs. As a rule, vigorous grinding for 3 to 4 minutes breaks the vast majority of the cells. With a little experience, one can tell from the physical appearance of the mixture when grinding has been sufficient; the originally friable mass becomes softer, moister, and slightly tacky. At this point, chilled buffer is added to the mortar and well mixed with the paste. We commonly extracted with 5 ml of M/50 phosphate buffer (pH 7.0) per gram of wet cells. The resultant slurry is centrifuged in the cold at high speed in order to separate the extract from the alumina and insoluble cellular debris. With the high-speed attachment of an International refrigerated centrifuge, operation at top speed for 15 minutes causes a satisfactory separation. The supernatant thus obtained is a clear, yellow solution rich in proteins and nucleic acids; if fresh and very concentrated, it often shows a reddish fluorescence. In the case of pseudomonads, the appearance of the insoluble cellular debris, which sediments on top of the alumina during centrifugation, provides a good indication of the success of the extraction. Whereas intact cells are buff-colored, the packed debris resulting from their destruction is brick-red in color and on resuspension in water yields a milky liquid showing an intense Tyndall effect, entirely different in appearance from a suspension of intact cells. We have not attempted to study the enzymatic activity of the particulate layer, all the experiments reported hereafter having been conducted with the clear supernatant extracts.

Procedures for determining enzymatic activity. The Warburg apparatus was

employed for the measurement of oxygen uptake, carbon dioxide evolution, and acid formation, all experiments being conducted at a temperature of 30 C. Ultraviolet spectrophotometry was extensively used, both as an adjunct to manometric experiments to determine the fate of added substrates, and as an independent technique for following certain enzymatic reactions. In the former case, ultraviolet absorption spectra over the range from 220 to 360 millimicra or portions thereof were determined on the contents of Warburg vessels, suitably
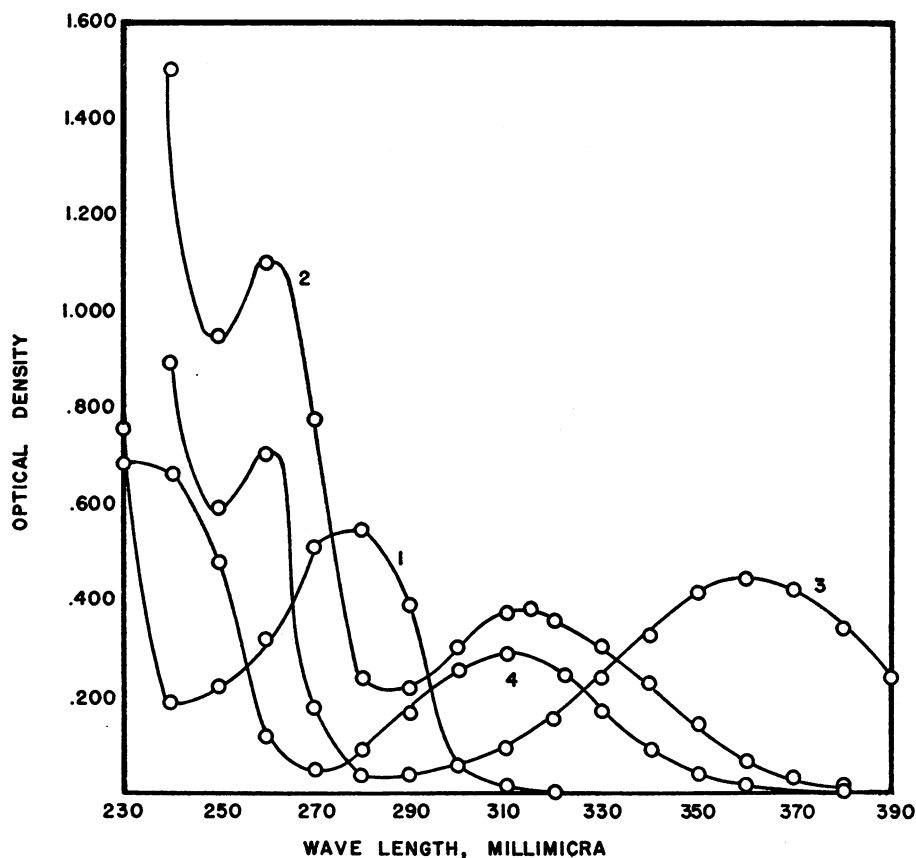

Figure 1. Absorption spectra of tryptophan (1), formylkynurenine (2), kynurenine (3), and anthranilic acid (4). Concentrations: 0.0001 M. pH: 7.0. Depth of cell: 1 cm.

diluted, after termination of the reaction. As a rule, such solutions were deproteinized by treatment with dilute sulfuric acid and, subsequently, reneutralized before optical measurements were made; if deproteinization was omitted, a control cuvette, containing the bacterial extract alone at an equivalent concentration, was included to provide a correction for light absorption by the extract. When spectrophotometry was employed directly as a means of following an enzymatic reaction, extract and substrate were mixed in the cuvettes at appropriate dilutions, and the optical density was read periodically at a suitable

fixed wavelength; if absolute measurements of optical density were required, a control cuvette, containing extract alone, was included in the series to provide a correction. The enzymatic reactions with which we were concerned are well suited for study by optical means, since most of the compounds involved show strong absorption maxima at different points in the ultraviolet region (figure 1). β-Ketoadipic acid was determined by catalytic decarboxylation as described by Stanier, Sleeper, Tsuchida, and MacDonald (1950).
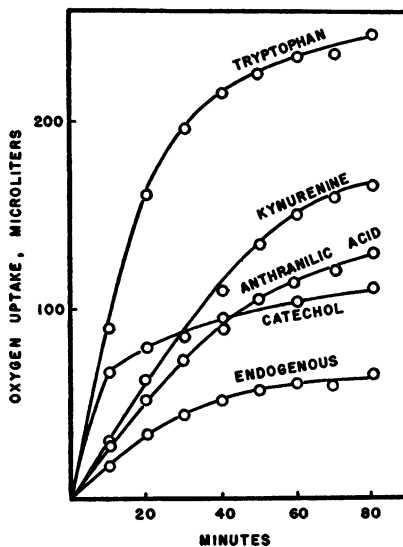

Figure 2. Oxygen consumption by a fresh cell-free extract at the expense of L-tryptophan, L-kynurenine, anthranilic acid, and catechol (2 micromoles of each). Each vessel contained 1.0 ml of extract (= 1.3 mg of protein), 0.8 ml of M/25 tris-(hydroxymethyl)aminomethane buffer (pH 7.5), substrates as indicated, and KOH in the center well.

RESULTS

General activity of extracts. A freshly-prepared extract, if tested at a high concentration, oxidizes tryptophan, kynurenine, anthranilic acid, and catechol with the respective consumption of 8, 5, 3, and 2 atoms of oxygen per mole of substrate supplied (figure 2). These oxidations all result in the formation of β-ketoadipic acid, which has never been observed to undergo decomposition in the presence of extracts. The stoichiometry of β-ketoadipate formation from tryptophan was established by catalytic decarboxylation of the reaction product with aniline citrate; the total carbon dioxide evolution was 98 per cent of theory (assuming formation of one mole of β-keto acid per mole of tryptophan decomposed), and the rate of decarboxylation was identical with that of synthetic β-ketoadipic acid. Fresh extracts also show a large endogenous oxygen consumption, which falls rapidly as they are allowed to age.

When extracts are diluted, aged for several days in the cold, or shaken in Warburg vessels for several hours at 30 C, certain of the step-reactions between

tryptophan and β-ketoadipic acid no longer occur. As a result of these treatments, the oxygen consumed per mole of tryptophan supplied falls to a value of 3 atoms, and the ultraviolet absorption of the reaction mixture following the oxidation shows that anthranilic acid has accumulated (figure 3). Some aged extracts have been observed to consume only 2 atoms of oxygen per mole of tryptophan decomposed, still with the formation of anthranilic acid as an end product. Kynurenine and anthranilic acid no longer give rise to an oxygen up-
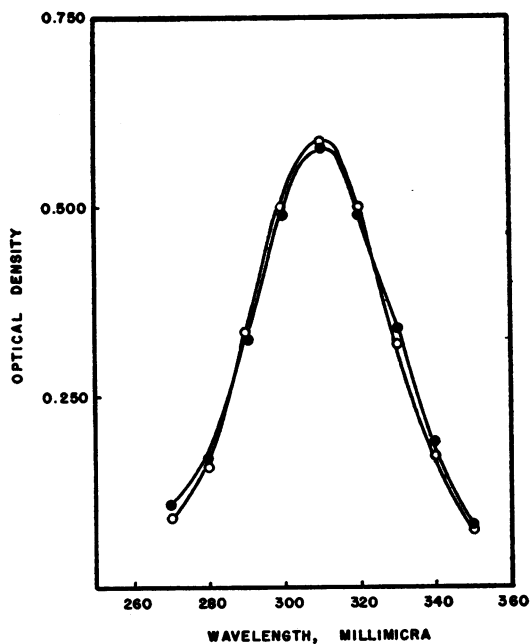
Figure 3. Ultraviolet absorption spectra of 0.0002 M synthetic anthranilic acid (dots) and of the product formed by the oxidation of tryptophan with a cell-free extract aged for 3 days at 2 to 5 C (circles). The amount of tryptophan supplied was 2 micromoles. Upon completion of the oxidation (followed manometrically) the reaction mixture was deproteinized and diluted to a final concentration equivalent to 0.0002 M on the basis of the tryptophan supplied. Spectrophotometric measurements made at pH 7.0 with a cell depth of 1 cm.

take when added to aged or diluted extracts, but the total oxygen uptake at the expense of catechol remains unchanged, and its oxidation still results in the accumulation of β-ketoadipic acid. These findings are summarized in table 1.

Under anaerobic conditions, neither methylene blue nor 2,6-dichlorophenolindophenol can function as an alternate hydrogen acceptor for any of these oxidations, which suggests that the enzymes involved are all oxidases or peroxidases.

Properties of the tryptophan-oxidizing component of the system. When kept in a frozen state, extracts retain a large part of their tryptophan-oxidizing activity for several weeks, but when kept slightly above 0 C their tryptophan-

oxidizing activity declines very markedly in a few days. This is evidenced both by a fall in the maximal rate of oxygen consumption and by the development of a brief lag in oxygen uptake when tryptophan is added to the enzyme. Dialysis for a short period of time completely inactivates the system, which cannot be reactivated subsequently by the addition of flavinadeninedinucleotide, flavinmononucleotide, diphosphopyridinenucleotide, triphosphopyridinenucleotide, adenosine triphosphate, pyridoxal phosphate, adenylic acid, Mg^{++}, methylene blue, or bacterial kochsaft, either individually or in combination. Dilution causes a very marked inactivation. The optimal pH for tryptophan oxidation, as measured by the initial rate of oxygen uptake, lies close to 7.8. Cyanide at a concentration of 0.001 M causes about 50 per cent inhibition; 8-hydroxyquinoline, and 7-methyltryptophan at the same concentration have no inhibitory effect.

Only the L-isomer of tryptophan can be attacked, as shown by the fact that the total oxygen consumption with a given amount of the racemic mixture is

TABLE 1

A comparison of the oxidative abilities of fresh and aged cell-free extracts

SUBSTRATE	FRESH EXTRACT		AGED EXTRACT	
	Total O$_2$ uptake, atoms/mole	β-Ketoadipic acid formation*	Total O$_2$ uptake, atoms/mole	β-Ketoadipic acid formation*
L-Tryptophan	8	+	2–3	−
L-Kynurenine	5	+	0	−
Anthranilic acid	3	+	0	−
Catechol	2	+	2	+
β-Ketoadipic acid	0		0	

* Qualitatively determined by Rothera reaction.

exactly half that with the same amount of the L-isomer. D-Tryptophan does not appear to inhibit the oxidation of the L-isomer. N-Methyltryptophan (abrin), which was reported by Sung and Tung (1950) to be readily converted to tryptophan by mammalian tissues and by *Escherichia coli* and *Serratia marcescens*, is unoxidizable. Growth tests show that it cannot serve as a source of carbon and nitrogen for Tr-23, suggesting that this bacterium is devoid of demethylase. Monomethyltryptophans substituted in the 4, 5, 6, and 7 positions likewise proved unoxidizable by extracts.

α-Hydroxytryptophan, which was postulated as an intermediate in the oxidation of tryptophan by Kotake and Masayama (1936), is not oxidized by extracts. This finding confirms the observation that it is not metabolized by tryptophan-adapted cells (Sakan and Hayaishi, 1950) and excludes impermeability as an explanation of such nonutilization. Mason and Berg (1951) have likewise found that α-hydroxytryptophan cannot be metabolized by mammalian liver slices which oxidize tryptophan.

Effect of a peroxide donor on the metabolism of tryptophan by aged extracts. The general properties of the tryptophan-oxidizing system as described in the
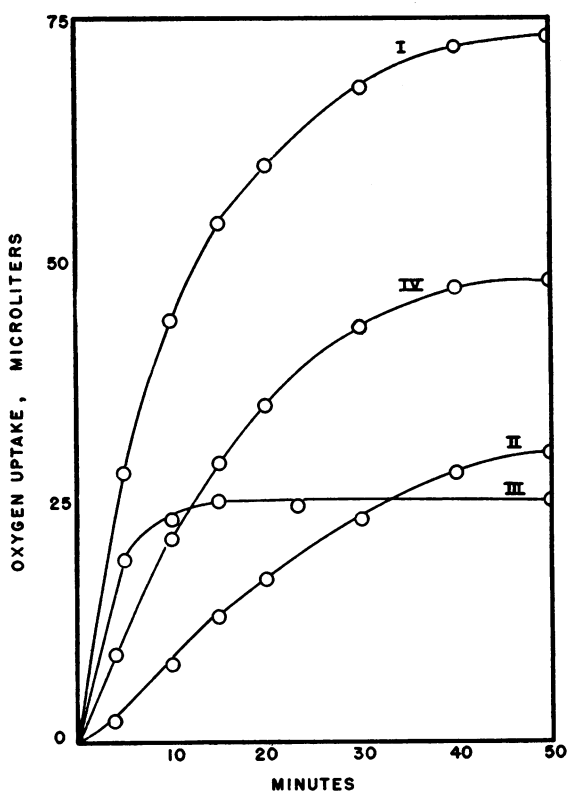
preceding paragraphs indicated its similarity to the system responsible for the oxidation of tryptophan to formylkynurenine by mammalian liver extracts. This system, which was recently discovered by Knox and Mehler (1950), consists of a tryptophan peroxidase coupled to an oxidase, which converts the product of the peroxidase reaction (whose structure is not known) to formylkynurenine with accompanying formation of hydrogen peroxide. The hydrogen peroxide thus generated is used in the first step of the reaction:

$$\text{tryptophan} + H_2O_2 \xrightarrow[\text{(tryptophan peroxidase)}]{} \text{intermediate}$$

$$+ O_2 \xrightarrow[\text{(oxidase)}]{} \text{formylkynurenine} + H_2O_2$$

The rapid decline in activity caused by dilution of fresh extracts or by aging, and the marked lag in oxygen uptake at the expense of tryptophan shown by aged extracts, both appeared most readily explicable in terms of the removal of extraneous peroxide-donating systems, originally present in the fresh extracts and required both for the priming of the oxidation and for its maintenance at maximal rate in the presence of catalase, with which our extracts were plentifully endowed. In order to test this hypothesis, we examined the effect of an extraneous peroxide-donating system (glucose plus notatin) on the oxidation of tryptophan by an aged extract. The results of a typical experiment are shown in figure 4. Oxygen uptake was determined in parallel upon three vessels, each containing both notatin and bacterial extract; one was furnished in addition with a limiting amount of tryptophan, one with a limiting amount of glucose, and one with a mixture of the two. By subtracting the oxygen uptake with glucose alone (curve III) from that with glucose and tryptophan (curve I), it is possible to obtain a curve (IV) for oxygen consumption resulting from the oxidation of tryptophan in the presence of glucose. Comparison with the curve (II) for oxygen consumption with the same amount of tryptophan in the absence of glucose, shows that the simultaneous oxidation of glucose caused a substantial increase in the oxygen uptake with tryptophan and abolished completely the usual characteristic initial lag. Aged extracts convert tryptophan to anthranilic acid, and since both tryptophan and anthranilic acid can be estimated spectrophotometrically in mixtures (Stanier and Hayaishi, 1951), it was possible to confirm the increased activity of the tryptophan-oxidizing system during accompanying glucose oxidation by subsequent spectrophotometric analysis. In the number of such experiments, increased formation of anthranilic acid from tryptophan as a result of accompanying glucose oxidation could be shown, and good checks between the manometric and spectrophotometric data were obtained; a typical result is shown in table 2.

Demonstration of the formation of kynurenine from tryptophan. The oxidation of tryptophan by aged extracts results in an accumulation of anthranilic acid. Such extracts also convert kynurenine to anthranilic acid in a nonoxidative reaction whose other product is alanine; this reaction is catalyzed by kynureninase, a very active enzyme and one of the most stable members of the sequence, whose properties will be described in detail elsewhere (Hayaishi and Stanier,

Figure 4. Effect of an extraneous source of peroxide on the oxygen uptake of an aged extract with tryptophan. Each vessel contained 0.3 ml of an aged extract, 0.3 ml of notatin (1 mg per ml), and 1.2 ml of M/10 phosphate buffer (pH 7.0), together with KOH in the center well. Curve I: 10 micromoles of tryptophan plus 2 micromoles of glucose. Curve II: 10 micromoles of tryptophan. Curve III: 2 micromoles of glucose. Curve IV: oxygen uptake with tryptophan and glucose, corrected for that with glucose alone.

TABLE 2

The effect of a peroxide-donating system (notatin + glucose) on the oxidation of tryptophan by an aged extract

Conditions

Each vessel contained 0.4 ml of aged extract, 0.1 ml of notatin (1 mg per ml), 2.0 ml of M/60 phosphate buffer (pH 7.0), and substrates as indicated (KOH in center well). Oxygen uptakes were determined, after which the contents of the vessels were diluted and assayed spectrophotometrically for anthranilic acid and tryptophan, with appropriate correction for light absorption by the extract and notatin.

Results

SUBSTRATES	O₂ UPTAKE	ANTHRANILIC ACID FORMED	RESIDUAL TRYPTOPHAN	MOLAR RECOVERY TRYPTOPHAN + ANTHRANILIC ACID
	μM	μM	μM	%
5 μM tryptophan + 10 μM glucose....	2.1*	1.9	2.7	92
5 μM tryptophan....................	0.8	0.7	4.0	94

* Corrected for oxygen uptake in a control vessel furnished with 10 μM of glucose alone.

1951). As a preliminary to demonstrating the formation of kynurenine from tryptophan, several attempts were made to fractionate crude extracts so as to separate kynureninase from the tryptophan-oxidizing system, but without success. An accumulation of kynurenine from tryptophan was eventually demonstrated by conducting the oxidation with a crude extract pretreated with semicarbazide. This substance is a powerful inhibitor of kynureninase, provided that it is added to the enzyme prior to the addition of substrate (Hayaishi and Stanier,
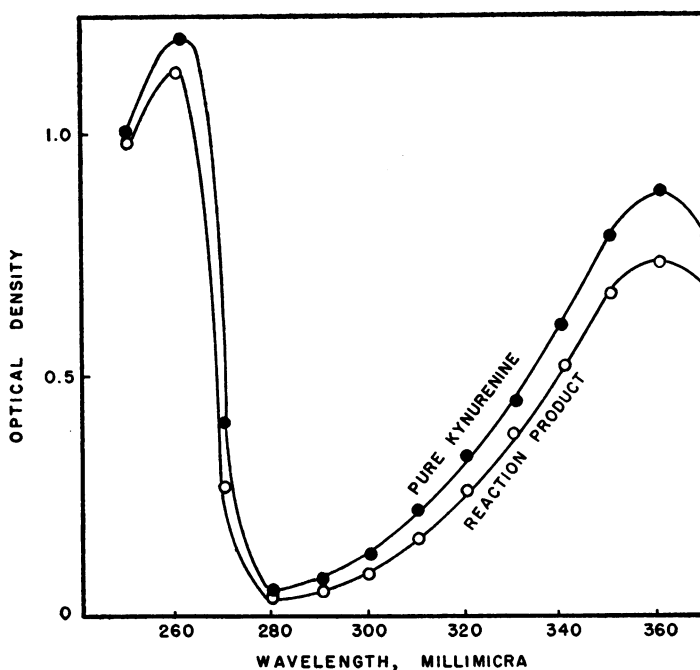
Figure 5. Ultraviolet absorption spectra of 0.0002 M kynurenine and of the product formed from tryptophan by an extract pretreated with semicarbazide. The enzymatic reaction was conducted in a vessel containing 0.2 ml of 0.01 M L-tryptophan, 0.5 ml of cell-free extract, 0.2 ml of 0.1 M semicarbazide (mixed with extract prior to substrate addition), and 1.1 ml of M/5 phosphate buffer (pH 8.5). After completion of the oxidation, the mixture was deproteinized and diluted to a final concentration equivalent to 0.0002 M on the basis of the tryptophan supplied. Spectrophotometric measurements made at pH 7.0 with a cell depth of 1 cm.

1951). As shown in figure 5, the oxidation of tryptophan under these circumstances results in a stoichiometric accumulation of kynurenine.

The decomposition of formylkynurenine. Mehler and Knox (1950) established the intermediate role of formylkynurenine in the oxidation of tryptophan by mammalian liver extracts, and showed that it is hydrolyzed to yield kynurenine and formic acid under the influence of an enzyme which they named formylase. Since it had already been established (Amano, Torii, and Iritani, 1950; Stanier, Hayaishi, and Tsuchida, 1951) that *Pseudomonas* strains employing the aromatic pathway are simultaneously adapted to oxidize formylkynurenine as a

result of adaptation to tryptophan, extracts were examined for the presence of formylase. In view of the fact that these extracts contained an active kynureninase, it appeared probable that formylkynurenine, if attacked, would be converted to anthranilic acid, rather than to kynurenine, which had been the end product in the mammalian system studied by Mehler and Knox (1950). Under anaerobic conditions, such a reaction should yield two equivalents of acid, as shown by the following equation:

formylkynurenine + 2H$_2$O → anthranilic acid + formic acid + alanine

Accordingly, the presence of a formylase was tested by adding formylkynurenine to an extract in bicarbonate buffer with an atmosphere of 95 per cent N$_2$—5 per cent CO$_2$, and determining acid formation manometrically by the release of carbon dioxide. The formylkynurenine available to us was a racemic mixture, only half of which (presumably the L-isomer) was oxidized by living cells. This material gave rise to an immediate rapid formation of acid when tested anaerobically with the extract, but the total acid produced was always considerably less than that expected on the assumption of a complete decomposition of one isomer according to the preceding equation. The ultraviolet absorption spectra of the resulting reaction mixtures suggested the presence of formylkynurenine, kynurenine, and anthranilic acid; however, the overlapping between the spectra of these three compounds is so great that a quantitative analysis of the data did not appear feasible. The combined manometric and optical data indicated that part of the formylkynurenine supplied had been decomposed to kynurenine, which was incompletely converted to anthranilic acid, despite the presence of an active kynureninase in the system.

The explanation of these somewhat unexpected findings was provided by an experiment in which the reaction was followed by determining changes in optical density at 360 millimicra. At this wavelength kynurenine has an absorption maximum, whereas the absorption of anthranilic acid is negligible, and that of formylkynurenine relatively small (figure 1). Changes of optical density were measured following the separate addition of formylkynurenine and kynurenine to aliquots of a diluted extract, and to similar aliquots treated beforehand with sufficient semicarbazide to inactivate kynureninase. At the concentration and wavelength employed the extract alone had a negligible optical density (0.030), and the semicarbazide was completely transparent; hence the measured optical densities reflect almost exclusively absorption by the substrates supplied and the products of their decomposition. The results are shown in figure 6. Curves I and II represent the changes in optical density occurring in the vessels supplied with kynurenine in the presence (II) and absence (I) of semicarbazide. It can be seen that the considerable kynureninase activity of the untreated extract is almost completely abolished by semicarbazide. Curves III and IV show the changes in optical density occurring in vessels supplied with DL-formylkynurenine in the presence (IV) and absence (III) of semicarbazide. With semicarbazide, there was rapid increase in optical density from the initial value of 0.200 (caused largely by the absorption of formylkynurenine) to a constant final

value of 0.696. Since only one isomer of formylkynurenine can be attacked, the optical density of residual formylkynurenine was approximately 0.100, and by subtraction the net increase in optical density therefore amounted to approximately 0.590. The initial concentration of formylkynurenine was 0.0003 M, and hence a 50 per cent conversion to kynurenine should result in a final concentration of 0.00015 M kynurenine. The net increase in optical density is very close to
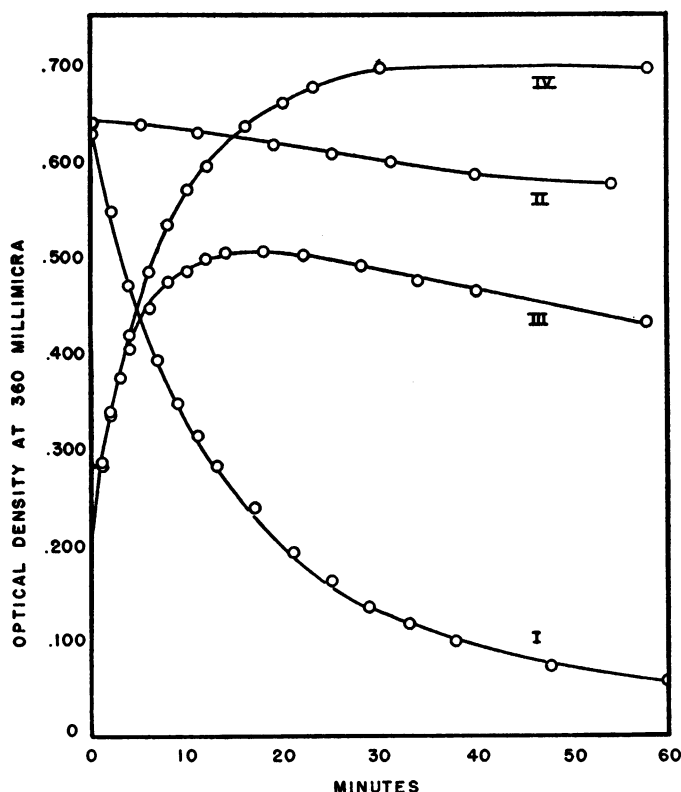

Figure 6. Spectrophotometric assays of formylase and kynureninase in an extract under comparable conditions. Each cuvette contained 0.03 ml of cell-free extract in a total volume of 3.2 ml (diluent, M/60 phosphate buffer, pH 7.0). Other components were: I, 0.00015 M L-kynurenine; II, 0.00015 M L-kynurenine plus 0.005 M semicarbazide; III, 0.0003 M DL-formylkynurenine; IV, 0.0003 M DL-formylkynurenine plus 0.005 M semicarbazide. The semicarbazide was mixed with the extract prior to addition of substrate. Depth of cell, 1 cm.

the expected value for this concentration of kynurenine, as shown by the initial optical densities on curves I and II, which represent the absorption by 0.00015 M kynurenine solutions.
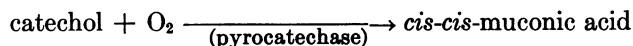
In the absence of semicarbazide (curve III) the decomposition of formylkynurenine also resulted in a rapid increase of optical density, the rates with and without semicarbazide being identical for the first 2 minutes. Thereafter the rate without semicarbazide declined, and after 15 minutes the optical density reached

a steady value of 0.503, which remained unchanged for 10 minutes and then slowly diminished. Assuming that half the formylkynurenine had undergone decomposition at the peak value for optical density, the kynurenine present would be responsible for an absorption of approximately 0.400 density units. This figure corresponds to a molar concentration of 0.0001; but as curve I shows, the rate of decomposition of kynurenine at this concentration is still high and very much in excess of the rate of decomposition shown in curve III during the final period of declining optical density. It therefore follows that kynureninase activity is inhibited by DL-formylkynurenine. Unless highly inhibitory impurities were present in the formylkynurenine that we used, this inhibition must be ascribed to the presence of the D-isomer. A conclusive proof would require tests with the pure D- and L-isomers of formylkynurenine, which are not at present available; but since it has been reported that D-kynurenine inhibits mammalian kynureninase (Wiss, 1949), it would not be surprising if D-formylkynurenine exerted a similar effect.

We have not studied the fate of alanine and formic acid, which are derived enzymatically from kynurenine and formylkynurenine, respectively; the fresh extracts contain enzymes which consume one atom of oxygen per mole of formate and one mole of oxygen per mole of alanine.

The enzymatic oxidation of anthranilic acid. The system responsible for the oxidation of anthranilic acid is the most unstable component of the chain of enzymes that convert tryptophan to β-ketoadipic acid. Only in fresh extracts with a high endogenous respiration could an oxidation of anthranilic acid be observed; dilution, aging in the cold, or shaking for a short time at 30 C in Warburg vessels caused inactivation. Dialysis likewise destroyed activity. The pH optimum for the reaction, measured by the initial rate of oxygen uptake with anthranilic acid, lies near 7.5.

Conversion of catechol and cis-cis-muconic acid to β-ketoadipic acid. As mentioned in the introduction, Hayaishi and Hashimoto (1950) purified a catechol-oxidizing enzyme (pyrocatechase) from extracts of a strain employing the aromatic pathway, and demonstrated that their purified enzyme oxidized catechol to *cis-cis*-muconic acid, identified by melting point and elementary analysis. In the present work it has always been found that *crude* extracts convert catechol to β-ketoadipic acid, a reaction first described by Stanier, Sleeper, Tsuchida, and MacDonald (1950), who worked with extracts of *Pseudomonas fluorescens* grown at the expense of mandelic or benzoic acid. The apparent discrepancy suggested by these facts has been removed by the discovery of Evans and Smith (1951) that *cis-cis*-muconic acid is an intermediate in the conversion of catechol to β-ketoadipic acid by extracts from cells of *P. fluorescens* and of a *Vibrio* sp. grown at the expense of benzoic acid. Thus the enzymatic conversion of catechol to β-ketoadipic acid consists of the following step-reactions:

$$\text{catechol} + O_2 \xrightarrow[\text{(pyrocatechase)}]{} \textit{cis-cis}\text{-muconic acid}$$

$$\textit{cis-cis}\text{-muconic acid} + H_2O \xrightarrow[\text{(unnamed enzyme)}]{} \beta\text{-ketoadipic acid}$$
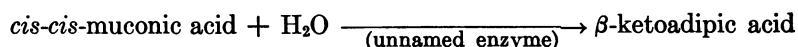
which, added together, give the equation for the reaction catalyzed by crude extracts:

$$\text{catechol} + O_2 + H_2O \rightarrow \beta\text{-ketoadipic acid.}$$
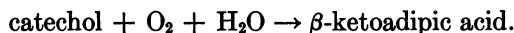
We have confirmed the observations of Evans and Smith (1950), using extracts of Tr-23 grown on tryptophan. Table 3 shows the uptake of oxygen and formation of β-keto acid at the expense of catechol, cis-cis-muconic acid, and cis-trans-muconic acid. The results agree in every respect with those of Evans and Smith; cis-cis-muconic acid is nonoxidatively converted to an equimolar amount of β-keto acid, whereas the cis-trans-isomer does not give rise to appreciable keto acid formation. The products formed enzymatically from catechol and cis-cis-muconic acid are decarboxylated by aniline citrate at a rate identical with that

TABLE 3

Action of an extract upon catechol, cis-cis-muconic acid, and cis-trans-muconic acid, as determined by oxygen uptake and β-keto acid formation

Conditions

A. For enzymatic reactions. Each vessel contained 0.2 ml of extract, 1.8 ml of M/5 phosphate buffer (pH 7.0), and 10 micromoles of substrate. Reactions were allowed to proceed until the oxygen uptake in the vessel furnished with catechol had ceased.

B. For subsequent catalytic decarboxylations. See "Materials and methods."

Results

SUBSTRATE	OXYGEN UPTAKE			β-KETO ACID FORMATION		
	Micro-liters	Micro-moles	Moles/mole of substrate	Micro-liters	Micro-moles	Moles/mole of substrate
Catechol	228	10.2	1.02	226	10.1	1.01
Cis-cis-muconic acid	0	0	0	236	10.5	1.05
Cis-trans-muconic acid	0	0	0	5	0.2	0.02

for pure β-ketoadipic acid. Cis-cis-muconic acid itself does not give rise to carbon dioxide formation under the conditions of keto acid determination employed.

The enzymatic decomposition of cis-cis-muconic acid and the inertness of the cis-trans-isomer were also demonstrated spectrophotometrically. As shown in figure 7, both muconic acids have a strong absorption in the short ultraviolet, while β-ketoadipic acid, which does not contain a resonating system of double bonds, is nearly transparent. Thus, the enzymatic conversion of cis-cis-muconic acid to β-ketoadipic acid can be followed by determining the change in optical density at 260 millimicra. Data from such an experiment are given in figure 8. It can be seen that the optical density of a mixture of the cis-cis-isomer with diluted extract diminished to a negligible value, while the optical density of a mixture containing the cis-trans-isomer remained constant. Very dilute solutions of the acids had to be employed, owing to their high extinction coefficients, which probably explains the marked departure from linearity of the rate of the reaction with cis-cis-muconic acid.

In connection with the absorption spectra for the pure muconic acids shown

in figure 7, it may be noted that the maxima do not occur at the same wavelength, as claimed by Elvidge, Linstead, Sims, and Orkin (1950); that for the cis-cis-isomer is located at 257.5 millimicra, and that for the cis-trans-isomer shows the expected shift to a slightly longer wavelength (262.5 millimicra). Our determinations of the maximal molar extinction coefficients (16,450 and 25,650 for cis-cis- and cis-trans-isomers, respectively) are in good agreement with those of Elvidge et al. (1950) (17,000 and 25,600, respectively).

Levels of specific enzymatic activities in extracts from unadapted cells. After growth on a medium containing asparagine as the principal carbon source,
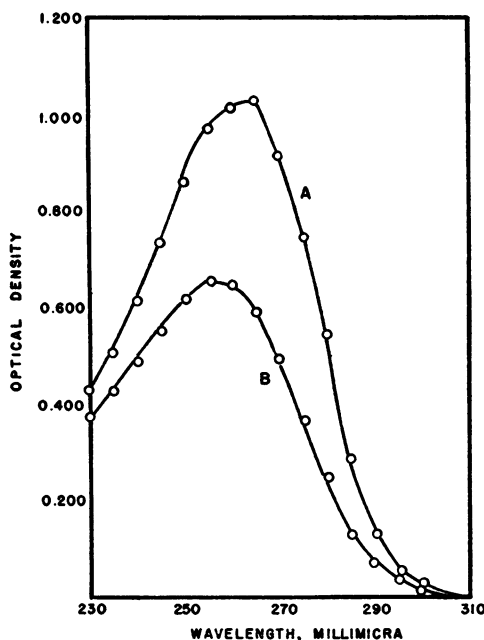
Figure 7. Absorption spectra of pure cis-cis- and cis-trans-muconic acids. Concentrations: 0.00004 M. pH: 7.0. Depth of cell: 1 cm. Curve A: cis-trans-isomer. Curve B: cis-cis-isomer.

Tr-23 shows a very marked adaptive lag in oxygen uptake when exposed to any compounds of the aromatic pathway; generally an hour or more elapses before the rate of oxygen consumption becomes measurably greater than the endogenous rate. We have always interpreted such lags as implying that the cells in question are initially devoid of the relevant enzymes, but it seemed desirable in connection with the present studies to obtain a formal proof for this contention, particularly since certain authors (e.g., Sevag, 1946) have attempted to interpret adaptive lags in other ways. Accordingly, levels of three specific enzymes—tryptophan peroxidase, kynureninase, and pyrocatechase—were determined in parallel on extracts from adapted and unadapted cells, prepared simultaneously and as far as possible in an identical manner. Unadapted cells were grown in a medium of the composition given earlier, except that the tryptophan was replaced by 0.4

per cent asparagine; adapted cells were grown in the usual tryptophan-containing medium. Both sets of cultures were started from identical inocula, and cultivated and harvested under identical conditions. Cell-free extracts were then prepared by alumina grinding as described earlier. In order to ascertain the degree of extraction, aliquots of the two extracts were diluted 1:100 with phosphate buffer and subjected to determinations of optical density at 260 and 280 millimi-
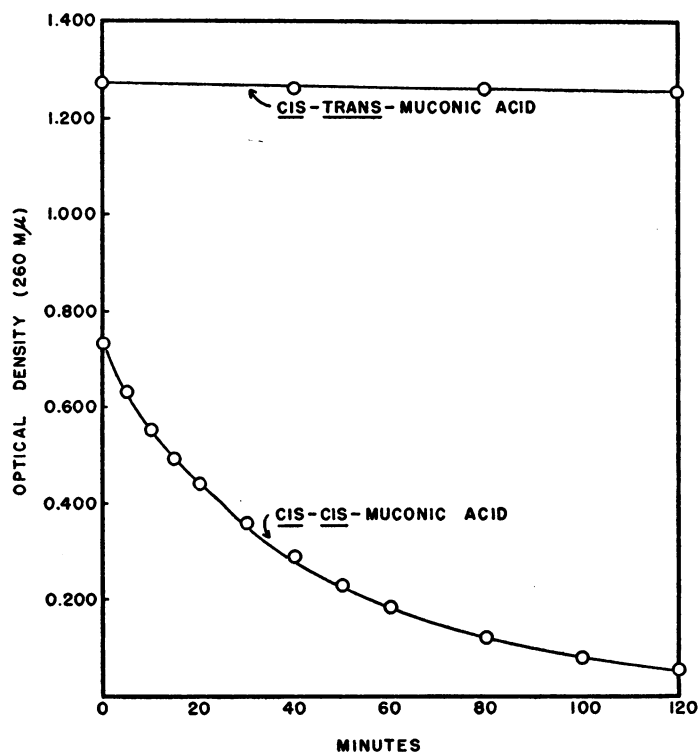

Figure 8. Spectrophotometric analysis of the action of a crude extract on the muconic acids. Each cuvette contained 0.02 ml of extract in a total volume of 3.4 ml (diluent, M/60 phosphate buffer, pH 7.0). The initial concentrations of the muconic acids were 0.00005 M; depth of cell, 1 cm. The plotted optical densities have been corrected for light absorption by the extract alone.

cra. The diluted extract from tryptophan-grown cells had an $O.D._{280}$ of 0.885, and the ratio $O.D._{280}/O.D._{260}$ was 0.56. The comparable figures for the diluted extract from asparagine-grown cells were 1.057 and 0.55, respectively. The close correspondence between the two $O.D._{280}/O.D._{260}$ ratios shows that both extracts contained the same relative amount of nucleic acid and protein, while the somewhat greater $O.D._{280}$ of the diluted extract from asparagine-grown cells shows that its protein concentration was slightly higher than that of the extract from tryptophan-grown cells. Parallel assays for enzymatic activity were then immediately conducted with the two extracts. The results (table 4) show that the extract from

asparagine-grown cells contained negligible amounts of the three enzymes, whereas the extract from tryptophan-grown cells was rich in them all. The high endogenous respiration of the extract from asparagine-grown cells observed in the assay for tryptophan peroxidase provides additional evidence that the extrac-

TABLE 4

Assays for tryptophan peroxidase, kynureninase, and pyrocatechase in extracts prepared from tryptophan-adapted and unadapted cells

A. *Manometric assays for tryptophan peroxidase*

Test system: 1.0 ml of bacterial extract; 1.0 ml of M/50 phosphate buffer (pH 7.0); KOH in center well. Vessels with substrate contained 5 micromoles of L-tryptophan.

SOURCE OF EXTRACT	TYPE OF RESPIRATION	MICROLITERS OF OXYGEN CONSUMED AFTER:		
		30 min	60 min	90 min
Unadapted cells.....	With substrate	52	77	90
	Endogenous	44	70	83
	Difference	8	7	7
Adapted cells........	With substrate	229	308	377
	Endogenous	46	79	106
	Difference	183	229	271

B. *Spectrophotometric assays for kynureninase*

Test system: 0.02 ml of bacterial extract; 1.0 ml of 0.001 M L-kynurenine; M/60 phosphate buffer (pH 7.0) to make 3.0 ml.

SOURCE OF EXTRACT	DECREMENT IN LOG $(I_0/I)_{360}$ mμ AFTER		
	5 min	9 min	13 min
Unadapted cells.........................	—	—	0.006
Adapted cells..........................	0.257	0.450	0.610

C. *Manometric assays for pyrocatechase*

Test system: 0.2 ml of bacterial extract; 2.0 ml of M/10 phosphate buffer (pH 7.0); 5 micromoles of catechol; KOH in center well.

SOURCE OF EXTRACT	MICROLITERS OF OXYGEN CONSUMED AFTER 60 MIN
Unadapted cells..	0
Adapted cells..	107

tion was performed in a satisfactory manner, and that the absence of the three specific enzymes was not a consequence of manipulative errors.

DISCUSSION

The aromatic pathway for the oxidation of tryptophan was postulated as a result of studies on the adaptive patterns of tryptophan-grown cells (Suda,

Hayaishi, and Oda, 1950; Stanier, Hayaishi, and Tsuchida, 1951), which suggested intermediate roles for formylkynurenine, kynurenine, anthranilic acid, and catechol. The observed enzymatic activities of cell-free extracts reported in the present paper confirm completely the inferences drawn from the analysis of adaptive patterns. With such extracts, we have shown the conversion of tryptophan to kynurenine and to anthranilic acid: the conversion of formylkynurenine to kynurenine: the conversion of kynurenine to anthranilic acid: and the oxidation of both anthranilic acid and catechol to β-ketoadipic acid. Furthermore, parallel studies on extracts from adapted and unadapted cells have furnished a direct proof that unadapted cells do not contain certain of the enzymes active in tryptophan metabolism—specifically, tryptophan peroxidase, kynureninase, and pyrocatechase, which act at rather widely separated points in the metabolic sequence. These data constitute the most detailed evidence so far adduced for the validity of biochemical inferences drawn from the analysis of adaptive patterns.

Investigation of the behavior of cell-free extracts has also provided new information about the biochemistry of the aromatic pathway. It is now established that the six-membered ring of the tryptophan molecule is converted via catechol to β-ketoadipic acid, a discovery which demonstrates the metabolic connection between this oxidation and the various bacterial oxidations of aromatic compounds previously known to give rise to the formation of β-ketoadipic acid (Stanier, 1950). The apparently anomalous report of Hayaishi and Hashimoto (1950), that a purified enzyme from an organism using the aromatic pathway converts catechol to cis-cis-muconic acid, has been explained by the discovery of Evans and Smith (1951), which we have confirmed, that crude bacterial extracts capable of forming β-ketoadipic acid by the oxidation of catechol can also convert cis-cis-muconic acid nonoxidatively to β-ketoadipic acid. Thus, there is good reason to believe that the immediate product of catechol oxidation is cis-cis-muconic acid, which undergoes a nonoxidative rearrangement, accompanied by water addition, to yield β-ketoadipic acid.

Detailed study of the enzymatic step-reactions which result in the conversion of tryptophan to anthranilic acid indicates a close similarity to the analogous reactions in the mammal. The bacterial oxidation of tryptophan to formylkynurenine involves a coupled peroxidation like that shown by Knox and Mehler (1950) to occur in liver extracts. The conversion of formylkynurenine to kynurenine is catalyzed by a hydrolytic enzyme similar to the mammalian formylase which was discovered by Mehler and Knox (1950). Lastly, the cleavage of kynurenine to anthranilic acid and alanine is brought about by another enzyme, kynureninase, which is virtually identical in its properties with the mammalian enzyme recently described by Wiss (1949) and Braunshstein, Goryachenkova, and Paskhina (1949).

The conversion of anthranilic acid to catechol is a step about which we have been able to learn little, primarily on account of the extreme ease with which the enzyme system becomes inactivated. The analogous oxidations of phenol and benzoic acid to catechol, and of p-hydroxybenzoic acid to protocatechuic acid, have likewise not as yet proved amenable to study in vitro (Stanier, 1950). These

oxidations all result in a hydroxylation of the benzene ring and probably represent variations on a single biochemical theme. Certain features of the anthranilic acid-oxidizing system, notably its dependence on a high rate of endogenous respiration and its ready inactivation by simple dilution, suggest that a coupled reaction of some sort (perhaps a peroxidation) is involved. The oxidations of phenol and p-hydroxybenzoic acid are probably the best systems with which to study the mechanism of these aromatic hydroxylations, since they are not complicated by concomitant decarboxylation and deamination.

Crude and preliminary though our studies on the enzymatic mechanisms of tryptophan metabolism have been, they provide an indication of the extreme complexity of the enzymatic changes that a cell may undergo when it adapts to a newly-provided substrate. Every step in the aromatic pathway from tryptophan through β-ketoadipic acid is under adaptive control, and even on the basis of our incomplete enzymatic analysis it is clear that at least eight distinct enzymes are involved: tryptophan peroxidase and oxidase, formylase, kynureninase, an anthranilic acid oxidase, pyrocatechase, a cis-cis-muconic acid hydrase, and an enzyme acting upon β-ketoadipic acid. The number of steps beyond β-ketoadipic acid that are under adaptive control are not known, and several of the earlier steps (notably the conversion of anthranilic acid to catechol) probably involve participation of more than one enzyme; hence, the grand total of new enzymes synthesized by the cell in response to the presence of tryptophan may easily exceed twelve. Since tryptophan can serve as the sole source of carbon, nitrogen, and energy for growth, the cell must synthesize each of the enzymes concerned with its degradation in substantial quantities. It is consequently no exaggeration to say that the enzymatic reorganization imposed on the cell by adaptation to metabolize tryptophan is roughly equivalent to that which would be involved in the construction of the entire enzymatic apparatus of glycolysis.

SUMMARY

Cell-free extracts capable of oxidizing L-tryptophan to β-ketoadipic acid have been obtained by grinding tryptophan-adapted cells of pseudomonads with alumina.

With such extracts, tryptophan undergoes an initial peroxidation to yield formylkynurenine. Formylkynurenine is in turn hydrolyzed to yield kynurenine, which then undergoes a nonoxidative decomposition to anthranilic acid and alanine. Anthranilic acid is further oxidized via catechol to cis-cis-muconic acid, and this compound is converted in a nonoxidative reaction to β-ketoadipic acid.

Certain properties of the enzymes responsible for this sequence of reactions are described.

Extracts prepared in identical fashion from cells not adapted to tryptophan (grown with asparagine as a carbon source) do not contain detectable amounts of the enzymes necessary for the dissimilation of tryptophan, kynurenine, and catechol, a fact which demonstrates that adaptation to metabolize tryptophan involves an actual synthesis by the cell of the relevant enzyme systems.

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