THE BACTERIAL OXIDATION OF TRYPTOPHAN

I. A General Survey of the Pathways[1]

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Two different pathways for the bacterial oxidation of tryptophan have been proposed recently as a result of two independent investigations by the analysis of adaptive patterns. Suda, Hayaishi, and Oda (1949, 1950) used as the starting-point for their work certain earlier observations by Mirick (1943) on the adaptive responses of an unidentified soil bacterium. Mirick's organism, which was potentially capable of oxidizing all three *mono*-aminobenzoic acids, could not be adapted to anthranilic (*o*-aminobenzoic) acid by exposure to either *m*- or *p*-aminobenzoic acid; yet exposure to tryptophan, despite its very different chemical structure, caused an activation of the enzyme system oxidizing anthranilic acid equivalent to that produced by the specific substrate itself. The biochemical implication of these findings was comprehended by Suda, Hayaishi, and Oda, who formulated independently of others (Stanier, 1947; Karlsson and Barker, 1948) the concept that exposure to a primary substrate will activate adaptive enzymes which operate on the subsequent metabolic intermediates, and coined the name "successive adaptation" for the phenomenon. They proceeded to study the adaptive patterns caused by exposure to tryptophan of an unidentified *Pseudomonas* sp., deducing from their results the following metabolic sequence: L-tryptophan → L-kynurenine → anthranilic acid → catechol.

Independently, Stanier and Tsuchida (1949) made a study of the oxidation of tryptophan by another unidentified pseudomonad, using the analysis of adaptive patterns. The general technique had been developed earlier by Stanier (1947) under the name of "simultaneous adaptation" during studies on the oxidation of simple aromatic compounds, and it had seemed of interest to find out whether other bacterial oxidations could be analyzed usefully by the same method. The results obtained with tryptophan were clear-cut and suggested as the initial degradative steps: tryptophan → kynurenine → kynurenic acid, anthranilic acid being excluded as a later intermediate. Both isomers of tryptophan could be oxidized.

Thus two radically different pathways for the oxidation of tryptophan, diverging at the stage of kynurenine, have been reported as existing in two unidentified members of the genus *Pseudomonas*. Further work was clearly desirable, and we have consequently embarked on a comparative analysis of the

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pathways and enzymatic mechanisms for the oxidative degradation of tryptophan by bacteria. The first part of our program has consisted of a survey (primarily by the analysis of adaptive patterns) designed to reveal the broad course of tryptophan oxidation by many different strains of pseudomonads. The results of this initial survey, reported below, fully substantiate the existence of the two pathways proposed earlier. A few isolates could not be assigned to either biochemical category, but these were all organisms with enzymatic defects which resulted in blocked oxidations of tryptophan.

In the future, the pathway discovered by Suda, Hayaishi, and Oda will be designated as *the aromatic pathway*, and that discovered by Stanier and Tsuchida as *the quinoline pathway*.

TABLE 1

Patterns of adaptation shown by the two "reference strains" after growth on DL-tryptophan

ORGANISM	ADAPTED (+) OR UNADAPTED (−) TO OXIDIZE				
	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid	Kynurenic acid
Strain Suda, Hayaishi, and Oda (1949)	+	−	+	+	−
Strain Tr-7 of Stanier and Tsuchida (1949)	+	+*	+	−	+

* This and other strains employing the quinoline pathway often show a slight partial adaptive lag, expressed as an increasing rate of oxygen uptake, when provided with D-tryptophan after growth on DL-tryptophan. This lag, doubtless caused by preferential adaptation to the L-isomer, has been ignored in tabulating results, both here and in table 3.

MEDIA AND METHODS

Stock cultures of our collection of tryptophan-oxidizing pseudomonads were kept on yeast agar slants. Cell suspensions specifically adapted to a given compound were obtained in the usual manner, by growth on plates of a medium containing that compound as the sole or principal source of carbon (Stanier, 1947; Stanier and Tsuchida, 1949). In practice, we seldom used strictly synthetic media for this purpose; a supplement of yeast extract (0.2 per cent) was usually included, since experience showed that many strains grew more rapidly and profusely in a supplemented medium, while the specific adaptive patterns were not thereby distorted.

In preparing cell suspensions for manometric work, a standardized procedure was adopted. Cells were harvested after growth for 18 to 20 hr at 30 C on agar plates, washed once with phosphate buffer (M/60, pH 7.0), and then suspended in the same buffer mixture to a final turbidity corresponding to a reading of 425 with the Klett colorimeter, using a blue filter. This standardization permitted us to compare the enzymatic activities of different strains in a roughly quantitative, as well as in a qualitative manner.

A crude manometric screening technique was devised in order to reduce as

much as possible the labor of determining adaptive patterns in many different strains. The original strains studied by Stanier and Tsuchida (1949) and by Suda *et al.* (1949) display the adaptive patterns listed in table 1, after growth on a medium containing DL-tryptophan as the principal carbon source. Consequently, new strains were tested for their ability to oxidize the compounds listed in table 1, after growth on a standard medium of the following composition: DL-tryptophan, 0.4 per cent; yeast extract, 0.2 per cent; K_2HPO_4, 0.1 per cent; $MgSO_4$, 0.05 per cent; agar, 2.0 per cent; pH adjusted to 7.0. Limiting amounts of substrates (2.0 micromoles) were always used so as to permit detection of any anomalies in total oxygen uptake. It was felt that this screening test would allow new strains to be assigned with a fair degree of certainty to one or other of the previously established biochemical types and would also permit detection of any strains which metabolized tryptophan by other, unknown pathways.

BIOLOGICAL MATERIAL

We were fortunate in obtaining a large and varied collection of tryptophan-oxidizing pseudomonads from Dr. S. H. Hutner, who had originally isolated these organisms from enrichment cultures furnished either with tryptophan or with nicotinic acid as the sole source of carbon and nitrogen. Hutner's cultures carry the prefix "Tr-" or "Nic-" (indicating the enrichment medium from which they were isolated), followed by his strain number; these designations have been retained by us in order to facilitate later comparisons. It should be noted that the culture previously studied by Stanier and Tsuchida (1949) is a Hutner isolate (Tr-7). In addition, five strains were isolated locally from tryptophan enrichments: these carry the prefix "RS-", followed by a strain number. Lastly, we have worked with the strain originally studied by Suda, Hayaishi, and Oda (str. SHO).

Of these 31 strains, 12 are members of the *Pseudomonas fluorescens* species-group, including str. SHO. The rest have not been observed to produce the characteristic pigment of *P. fluorescens*, and will be referred to as *Pseudomonas spp.* Identifications would be desirable, but the taxonomy of the genus is at present in such an unsatisfactory state that the appending of a specific label has very little value. On the basis of superficial examination, the nonfluorescent strains appear to comprise at least three distinct groups.

RESULTS

Outcome of the screening tests. The adaptive patterns revealed by the screening tests were readily interpretable in all save a few cases. The great majority of strains (19) behaved in a manner which clearly indicated an oxidation of tryptophan by the aromatic pathway, while five strains revealed the expected biochemical patterns for an oxidation by the quinoline pathway. Six strains could not be definitely assigned to either pathway on the basis of the screening tests.

The results obtained with strains employing the aromatic pathway are summarized in table 2. It may be noted that this biochemical group includes all the fluorescent pseudomonads with the exception of a single aberrant strain (Tr-13)

to be described later; in addition it contains a considerable number of nonfluorescent pseudomonads. All of these organisms oxidize L-tryptophan, L-kynurenine, and anthranilic acid at a rapid and steady rate to the point of substrate exhaustion, which is reached after the same time interval by aliquots of a cell suspension given equimolar amounts of the three compounds. The majority of strains, like str. SHO, are completely incapable of metabolizing D-tryptophan;

TABLE 2

Results of screening tests on pseudomonads metabolizing tryptophan by the aromatic pathway

STRAIN NO.	ACTION OF DL-TRYPTOPHAN-GROWN CELLS ON:					O_2 UPTAKE, μL PER μ MOLE OF L-TRYPTOPHAN
	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid	Kynurenic acid	
A. Members of *Pseudomonas fluorescens* species-group						
Tr-10	+	±	+	+	−	160
Tr-19	+	−	+	+	−	150
Tr-20	+	−		+	−	157
Tr-23	+	−	+	+	−	166
Tr-26	+	−	+	+	−	162
Tr-27	+	−	+	+	−	150
Tr-33	+	−	+	+	−	Not determined
Tr-35	+	−	+	+	−	Not determined
RS-3	+	−	+	+	−	168
RS-5	+	−	+	+	−	162
B. Nonfluorescent pseudomonads						
Tr-9	+	−	+	+	−	Not determined
Tr-22	+	−	+	+	−	166
Tr-28	+	±	+	+	−	Not determined
Tr-34	+	−	+	+	−	Not determined
Nic-17	+	±		+	−	145
Nic-24	+	−	+	+	−	134
Nic-32	+	±	+	+	−	135
Nic-33	+	±		+	−	145
RS-2	+	−	+	+	−	150

+ = immediate oxidation at a high rate (comparable to the rate with L-tryptophan).

± = immediate oxidation at a low rate (less than 30 per cent of that with L-tryptophan).

− = no immediate oxidation.

a few of them attack it at a low rate, which is in no case greater than 30 per cent of the rate with the L-isomer, and generally much less. The total oxygen uptake per micromole of L-tryptophan oxidized (determined from the point of inflection of the curve for oxygen uptake, uncorrected for endogenous respiration) varied from 135 to 165 microliters. Part of this variation doubtless reflects differences in the endogenous rate of oxygen uptake; but it is also possible that there are slight differences between strains in the extent of oxidative assimilation.

The results obtained with strains employing the quinoline pathway are given

in table 3. All of them oxidize L-tryptophan, L-kynurenine, and kynurenic acid at a steady and rapid rate, the point of substrate exhaustion being reached simultaneously by aliquots of a cell suspension given equimolar amounts of the three compounds. Anthranilic acid is immediately oxidized by none. In marked contrast to the strains using the aromatic pathway, these organisms can all oxidize D-tryptophan at a relatively high rate, varying from 41 to 74 per cent of the rate with the L-isomer. After growth on the test medium containing DL-tryptophan, they often show an initially increasing rate of oxygen uptake with the D-isomer, which attains a steady value from 10 to 30 minutes after substrate addition. This is doubtless referable to a preferential adaptation to the L-isomer during growth on a medium containing both isomers, and has been ignored in tabulating the results. In connection with the relatively ready oxidizability of D-tryptophan by this biochemical group, it may be noted that the two locally-

TABLE 3

Results of screening tests on pseudomonads metabolizing tryptophan by the quinoline pathway

STRAIN NO.	ACTION OF DL-TRYPTOPHAN-GROWN CELLS ON:					O_2 UPTAKE, μL PER μ MOLE OF L-TRYPTOPHAN	RATE OF OXIDATION OF D-TRYPTOPHAN, RELATIVE TO L-TRYPTOPHAN
	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid	Kynurenic acid		
							per cent
Tr-6	+	+	+	−	+	162	50
Nic-SH_3	+	+	+	−	+	155	74
Nic-SH_2	+	+		−	+	173	41
RS-1	+	+	+	−	+	154	59
RS-4	+	+		−	+	163	47

+ = immediate oxidation at a high rate (see footnote to table 1 with respect to oxidation of D-tryptophan).

− = no immediate oxidation.

isolated strains included (RS-1 and RS-4) were both obtained from enrichment cultures furnished with DL-tryptophan, whereas the other three local isolates, belonging to the aromatic group, were obtained from enrichments with L-tryptophan. These facts suggest that a highly specific procedure for the isolation of pseudomonads employing the quinoline pathway could be developed by using the pure D-isomer of tryptophan as the enriching compound. Our supply of D-tryptophan was too small to permit an experimental verification of this point. The total oxygen uptake per micromole of tryptophan oxidized (again uncorrected for endogenous respiration) by strains employing the quinoline pathway ranged from 154 to 162 microliters. This variation is insignificant and suggests that the magnitude of oxidative assimilation is constant within the group.

Only six strains could not be clearly assigned to either of the foregoing classes; the results obtained with these strains are presented in table 4. Tr-24 and Tr-25 appear to comprise a subclass of the organisms that oxidize tryptophan by the aromatic pathway, characterized by a partial metabolic block at the stage of anthranilic acid. This is suggested both by the relatively low rate at which

anthranilic acid is oxidized, and by the very low total oxygen uptake with tryptophan at which a break in rate occurs. Following this initial break in rate of oxygen uptake with tryptophan, oxygen consumption proceeds at a rate approximately equal to the initial rate with anthranilic acid, and if the experiment is continued for a sufficient length of time, a second break can be observed, which corresponds to a total oxygen uptake of approximately 150 to 160 microliters per micromole of substrate. During growth on a tryptophan-containing medium, these two strains excrete large amounts of a substance which shows violet fluorescence upon ultraviolet illumination, a property also shown by anthranilic acid. A detailed study of the metabolism of Tr-25 is given in a later paper.

TABLE 4

Results of screening tests on atypical strains

STRAIN	ACTION OF DL-TRYPTOPHAN-GROWN CELLS ON					O_2 UPTAKE, μL PER μ MOLE OF L-TRYPTOPHAN
	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid	Kynurenic acid	
Tr-24	+	−	+	±	−	80*
Tr-25	+	−	+	±	−	92*
Tr-2	±	−		±	−	Not determinable
Tr-31	±	−		−	−	Not determinable
Tr-13	+	−	−	−	−	40
Tr-14	+	−	+	−	−	62

* First break in rate; thereafter, slow oxidation at approximately the rate for anthranilic acid.

\+ = immediate oxidation at a high rate.

± = immediate oxidation at a low rate.

− = no immediate oxidation.

Tr-2 and Tr-31 proved very difficult to study and the data recorded for them are of questionable significance. Growth of these two strains on tryptophan is accompanied by a brown discoloration of the medium, suggestive of the accumulation of polyphenols. Cells harvested from a tryptophan-containing medium appear to have suffered severe physiological damage, evidenced both by a negligible rate of endogenous respiration and by a very slow oxidation even of L-tryptophan. Even when the period of growth on the standard test medium was shortened to 12 hours, satisfactory manometric data were still unobtainable. Consequently, the pathway employed by these strains remains obscure.

Strain Tr-13, a fluorescent pseudomonad, oxidizes only L-tryptophan of the substrates included in the screening test. The total oxygen uptake per micromole of tryptophan is approximately 40 microliters, which is evidence for an early and complete metabolic block. The accumulating metabolite has been identified as indole.

Strain Tr-14 oxidizes L-tryptophan and L-kynurenine, but neither kynurenic nor anthranilic acid; as in the case of Tr-13, the total oxygen uptake with

tryptophan is exceptionally low (approx. 60 microliters per micromole), again indicating an early and complete metabolic block. This organism has been shown to accumulate a mixture of two cyclic end products, kynurenic and anthranilic acids. Thus of all the strains tested it is the only one which could be considered to employ a "mixed" pathway for the degradation of tryptophan, and its metabolism is so defective that the result can be more aptly described as a double cul-de-sac. Detailed studies on the metabolism of Tr-13 and Tr-14 will be published later.

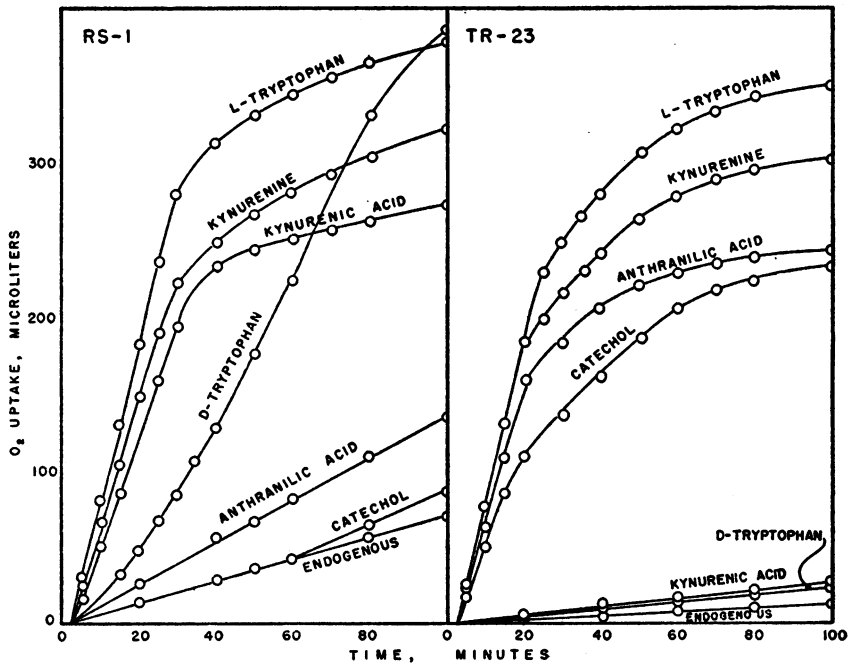


Figure 1. Oxidation of limiting amounts (2 micromoles) of various substrates by cells grown in the presence of DL-tryptophan. RS-1 decomposes tryptophan by the quinoline pathway, and Tr-23 by the aromatic pathway.

The adaptive nature of tryptophan oxidation by strains employing the aromatic and quinoline pathways. The adaptive nature of tryptophan oxidation was established for three strains employing the aromatic pathway (Tr-23, Tr-33, and RS-5), and two employing the quinoline pathway (Nic-SH_3 and RS-1). Each strain was grown on asparagine agar and then tested for its ability to oxidize L- and D-tryptophan, L-kynurenine, anthranilic acid, catechol, and kynurenic acid; in no case was there an oxygen uptake greater than the endogenous oxygen consumption with any of these compounds during the first 20 minutes after substrate addition. The typical adaptive patterns with respect to the previously listed compounds established by growth on DL-tryptophan are shown in figure 1 for a representative strain employing each pathway. From the results obtained with these five strains, it seems permissible to assume that the early steps in

tryptophan oxidation by all strains employing the aromatic and quinoline pathways are under strictly adaptive control.

The intermediate role of formylkynurenine. Mehler and Knox (1950) showed that formylkynurenine is an intermediate in the enzymatic oxidation of tryptophan to kynurenine by mammalian liver preparations. Independently, Amano, Torii, and Iritani (1950) proposed its intermediate role in the oxidation of tryptophan by bacteria employing the aromatic pathway, as a result of their observation that cells adapted to tryptophan are simultaneously adapted to formylkynurenine. Both Mehler and Knox and Amano, Torii, and Iritani used L-formylkynurenine, prepared by the ozonolysis of L-tryptophan. Dr. T. Sakan

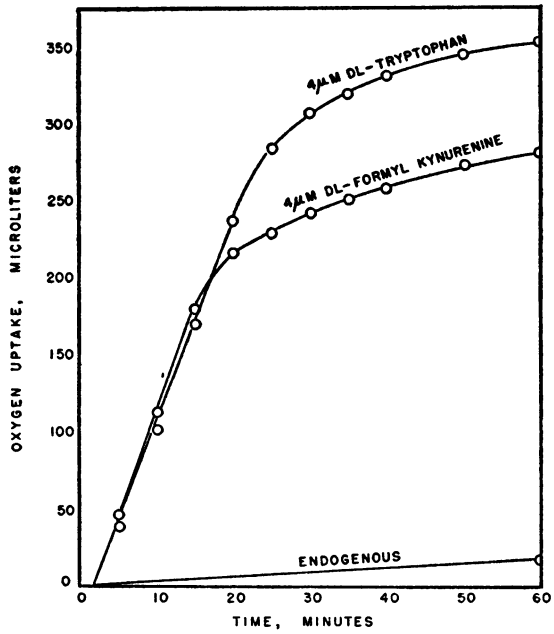


Figure 2. Oxidation of limiting amounts of DL-tryptophan and DL-formylkynurenine (4 micromoles of each) by strain Tr-23, grown in the presence of DL-tryptophan.

has recently developed a synthesis of formylkynurenine, and kindly provided us with a sample of synthetic DL-formylkynurenine which we used to test the intermediacy of this compound in the oxidation of tryptophan by our bacterial strains. Figure 2 shows the oxygen uptake by a tryptophan-grown strain employing the aromatic pathway (Tr-23) at the expense of equimolar amounts of DL-tryptophan and DL-formylkynurenine; there is complete simultaneous adaptation to formylkynurenine, as reported by Amano, Torii, and Iritani. A comparison of the total oxygen uptakes with DL-tryptophan and DL-kynurenine shows that only one isomer of formylkynurenine is oxidized. Tr-23 cannot attack D-tryptophan, and hence if both isomers of formylkynurenine were oxidizable, the total oxygen consumption at the expense of DL-formylkynurenine should be considerably greater than the total oxygen consumption at the expense of an equi-

molar amount of DL-tryptophan; in actual fact, it is approximately 65 microliters less, in moderate agreement with the difference of 45 microliters to be expected on the assumptions that only one half of each substrate (2 micromoles) has undergone oxidation, and that the transformation of tryptophan to formylkynurenine involves an uptake of two atoms of oxygen (Mehler and Knox, 1950). Presumably it is the L-isomer of formylkynurenine that is metabolized.

When analogous experiments were conducted using strains that employ the quinoline pathway (Tr-7 and RS-1), simultaneous adaptation to formylkynurenine was again observed. The behavior of these two strains with respect to utilization of the stereoisomers of formylkynurenine was wholly unexpected, however. Like other strains employing the quinoline pathway, Tr-7 and RS-1

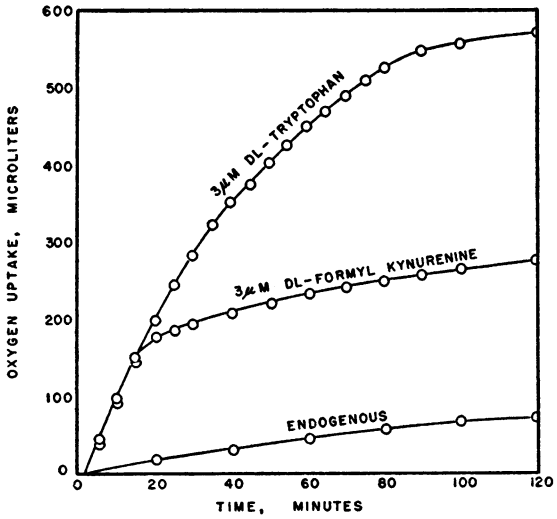


Figure 3. Oxidation of limiting amounts of DL-tryptophan and DL-formylkynurenine (3 micromoles of each) by strain Tr-7, grown in the presence of DL-tryptophan.

can oxidize both isomers of tryptophan, and we consequently expected that they would be able to oxidize both isomers of formylkynurenine; as shown by the data from a typical experiment (figure 3) this does not occur, the total oxygen uptake at the expense of DL-formylkynurenine being considerably less than half that with an equimolar amount of DL-tryptophan. The presence of unoxidized formylkynurenine at the termination of the experiment in the vessel originally furnished with formylkynurenine was confirmed by spectrophotometric analysis. The supernatant liquid from this vessel showed the characteristic ultraviolet absorption spectrum of formylkynurenine with peaks at 260 and 320 millimicra (Mehler and Knox, 1950), and the total absorption was of the magnitude expected on the assumption that only one half the substrate provided had been decomposed. The outcome of these experiments shows that the tentative conclusions of Stanier and Tsuchida (1949) concerning the mode of decomposition of D-tryptophan by strains employing the quinoline pathway are probably in-

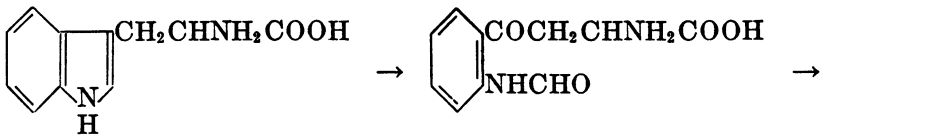
correct. The adaptive patterns with respect to the D-isomers of tryptophan and kynurenine observed by these authors suggested a degradation of D-tryptophan via D-kynurenine; but on such an hypothesis, D-formylkynurenine could be expected to act as an intermediate. Since only one isomer of formylkynurenine (presumably the L-isomer) can be attacked, it now seems more likely that the metabolism of D-tryptophan involves an initial racemization and further degradation by the pathway operative for the L-isomer of tryptophan. If this is true, the simultaneous adaptation to D-kynurenine as a result of adaptation to D-tryptophan observed by Stanier and Tsuchida must be considered as a non-specific adaptive response.

Tests of possible later intermediates on the quinoline pathway. The oxidation of kynurenic acid could lead eventually to the formation either of benzene or of pyridine derivatives, depending on which moiety of the quinoline nucleus is first subjected to attack. Since many benzene derivatives were available, their possible intermediacy was tested, using tryptophan-grown cells of strain Tr-7. Protocatechuic acid, *p*-hydroxybenzoic acid, and *m*-hydroxybenzoic acid were oxidized after marked adaptive lags by these cells; all other compounds tested proved completely unattackable. The list included: benzoic acid, salicylic acid, 2,3-dihydroxybenzoic acid, catechol, phloroglucinol, pyrogallol, resorcinol, and hydroquinone.

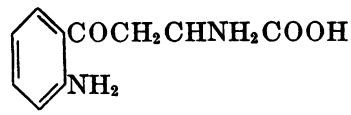
One quinoline derivative, xanthurenic acid, was also tested. It is oxidized by tryptophan-grown Tr-7 only after an adaptive lag. Thus at the present time, no later intermediates on the pathway through kynurenic acid can be postulated; the only definite established fact about this pathway is that it is biochemically completely different from the aromatic pathway.

DISCUSSION

The data reported above fully confirm the existence of the earlier-postulated aromatic and quinoline pathways for the bacterial oxidation of tryptophan, and provide a reasonably clear general picture of the early steps in the oxidative metabolism of tryptophan by members of the *Pseudomonas* group. With one exception (the indole-producing strain Tr-13), the initial reactions are common to all strains, and involve attack on the five-membered ring of the indole nucleus:



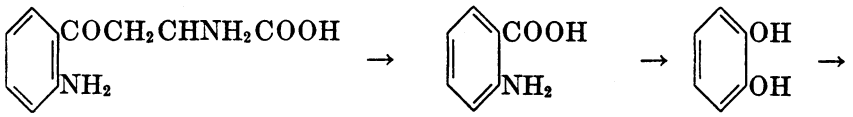
tryptophan formylkynurenine



kynurenine

Among strains which carry out a complete oxidation of tryptophan, there

is a strict and absolute dichotomy in the pathway of oxidation beyond kynurenine; some degrade it to anthranilic acid, and thence to catechol (aromatic pathway), while others degrade it to kynurenic acid (quinoline pathway). Later intermediates on the quinoline pathway have not yet been detected. Enzymatic studies on extracts of strains employing the aromatic pathway, which will be reported later, have shown that this degradation proceeds via *cis-cis*-muconic acid and β-ketoadipic acid:



kynurenine anthranilic acid catechol

$$HOOC{-}CH{=}CH{-}CH{=}CH{-}COOH \rightarrow HOOC{-}CH_2{-}CO{-}(CH_2)_2{-}COOH$$

cis-cis-muconic acid β-ketoadipic acid

The only strain whose metabolism of tryptophan involves both benzene and quinoline derivatives is the metabolically-aberrant Tr-14, which accumulates a mixture of anthranilic and kynurenic acids.

SUMMARY

The pathways of tryptophan oxidation in a large collection of pseudomonads have been investigated by the analysis of adaptive patterns.

Nineteen of the strains tested appear to oxidize tryptophan via kynurenine, anthranilic acid, and catechol (aromatic pathway).

Five strains appear to oxidize tryptophan via kynurenine and kynurenic acid (quinoline pathway).

Six strains do not conform in their adaptive patterns to the requirements for an oxidation via either aromatic or quinoline pathway. Two of these accumulate anthranilic acid, one indole, and one a mixture of anthranilic and kynurenic acids.

The evidence from adaptive patterns suggests that formylkynurenine is an intermediate between tryptophan and kynurenine for strains employing the aromatic and quinoline pathways.

Extensive tests of possible intermediates have failed to reveal any compounds on the quinoline pathway below kynurenic acid.

Strains employing the quinoline pathway can readily metabolize both isomers of tryptophan, but attack only one isomer of formylkynurenine, which suggests that the degradation of D-tryptophan involves a preliminary racemization to the L-isomer.

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