THE MECHANISM OF ALLANTOIN DEGRADATION BY A PSEUDOMONAS[1]

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Animals and plants are known to convert allantoin to glyoxylic acid and urea via allantoic acid (Laskowski, 1951). Bacteria and yeasts are known to decompose allantoin, but evidence as to the pathway is very incomplete. Barker (1943) has reported that *Streptococcus allantoicus* degrades allantoin anaerobically, giving rise to ammonia, urea, carbon dioxide, formic, acetic, lactic, and oxamic acids and possibly glycolic acid. It has been postulated from growth studies with bacteria (Young and Hawkins, 1944) and yeast (DiCarlo *et al.*, 1953) that the aerobic decomposition of allantoin occurs via allantoic acid yielding glyoxylic acid and urea.

The present paper reports on the mechanism of allantoin degradation by an unidentified species of *Pseudomonas* isolated by enrichment culture techniques. Cell-free extracts of this organism decompose allantoin yielding urea, carbon dioxide, and water. The intermediates along the degradative pathway have been isolated and identified as allantoic, glyoxylic, and formic acids

EXPERIMENTAL METHODS AND RESULTS

Isolation of the organism. An enrichment medium containing allantoin, 0.3 per cent; yeast extract (Difco), 0.1 per cent; and K_2HPO_4, 0.1 per cent in distilled water, pH 7.2, was inoculated with San Francisco bay mud. Abundant growth was obtained in 24 hours at 30 C in an aerobic culture incubated on a shaker. Successive transfers were made in the same medium at daily intervals for one week and finally plated out on the same medium supplemented with 1.5 per cent agar. The predominant strain was isolated and designated as 2RCC-1. This organism is an aerobic, gram negative, motile rod. Cultural and biochemical characteristics have placed it in the nonpigmented Pseudomonas group.

Preparation of cell suspensions and cell-free extracts. Cells grown in the allantoin medium at 30 C for 18 hours were harvested by centrifugation, washed 3 times with potassium phosphate buffer, 0.01 M, pH 7.0, and resuspended in the same buffer. Cell suspensions thus prepared were used in manometric experiments.

Cell-free extracts were prepared by grinding with alumina (McIlwain, 1948). Washed cells were ground with twice their weight of levigated alumina (B.K.H. Co., San Francisco) at 0 C for 5 to 10 minutes. The paste was extracted with 2 ml of 0.01 M phosphate buffer, pH 7.0, per g of bacteria and centrifuged at 0 C in a high speed International refrigerated centrifuge at 6,000 G for 15 minutes. The resulting clear, straw-colored supernatant was used as the enzyme extract. Cell-free extracts were also prepared by sonic vibration. Washed cells (4 g wet weight per 40 ml phosphate buffer, 0.01 M, pH 7.0) were treated for 10 to 15 minutes in a Raytheon 9 kc sonic oscillator. After treatment the extract was centrifuged at 0 C as above to remove cellular debris. Freshly harvested cells and cell-free extracts were frozen and stored at −10 C until needed. Cells which were stored at −10 C for one week prior to alumina or sonic treatment showed no appreciable loss in activity.

Methods. Manometric measurements were carried out by conventional methods (Umbreit *et al.*, 1949). Unless otherwise stated, manometric experiments were conducted at 30 C using air as the gas phase.

Allantoin, allantoic acid, and glyoxylic acid were estimated by the method of Young and Conway (1942), oxalic acid by the method of Calkins (1943), and glycine by the method of Christensen *et al.* (1951). The protein content of extracts was estimated colorimetrically by the method of Lowry *et al.* (1951).

C^{14} activity was measured with a thin-walled,

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end window Geiger-Muller tube. Carboxyl labeled glyoxylic acid was prepared from carboxyl labeled glycine using pig kidney glycine oxidase. The oxidase was isolated and purified according to the method of Ratner *et al.* (1944). The oxidase was incubated with carboxyl labeled glycine and flavine adenine dinucleotide for 6 hours at 30 C. The glyoxylic acid formed was isolated as the bisulfite addition complex, recrystallized in the cold, and acidified to give the free acid. The identity of glyoxylic acid was established by the mp of the 2,4-dinitrophenylhydrazone. The free acid was degraded with 0.43 N sodium periodate and the carboxyl carbon collected and counted as barium carbonate (Krasna *et al.*, 1952). The labeled glyoxylic acid had a specific activity of 4,000 cpm per μM.

Manometric studies. Oxygen uptake and carbon dioxide evolution by a suspension of resting cells grown on allantoin were determined with allantoin, allantoic, glyoxylic, formic, and oxalic acids, formaldehyde or urea as the substrate. With allantoin, allantoic acid, and glyoxylic acid, approximately 1 μM of oxygen was consumed and 2 μM of carbon dioxide were evolved per μM of substrate used. With formic acid, 0.5 μM of oxygen was taken up and 1 μM carbon dioxide evolved per μM substrate. No decomposition of oxalic acid, formaldehyde, or urea was detected manometrically. Cell-free extracts also failed to decompose these substrates. Typical manometric data are given in table 1.

When the organism was grown on glucose or nutrient broth, there was a 3 to 4 hour lag period before O_2 uptake and CO_2 evolution were observed with allantoin. That this lag was due to the adaptive formation of allantoin degrading enzymes was verified by a determination of allantoin degrading activity of cell-free extracts of cells before and some hours after exposure to allantoin.

Manometric experiments with cells cultured on allantoic acid gave essentially the same results as those obtained with allantoin grown cells. Cells grown on glyoxylic acid oxidized glyoxylic and formic acids immediately but showed a long lag before allantoin decomposition started. Formate grown cells exhibited the same behavior, rapidly oxidizing formate and requiring a period of adaptation before degradation of allantoin, allantoic acid, or glyoxylic acid occurred. The adaptive nature of all these systems was further verified by studies with cell-free extracts.

TABLE 1

Manometric data of Pseudomonas, strain 2RCC-1, grown on allantoin

Substrate	O_2 Uptake	CO_2 Evolution
	μM	μM
Allantoin	9.75	19.78
Allantoic acid	10.20	19.96
Glyoxylic acid	10.00	20.03
Formic acid	4.88	9.95

Each Warburg vessel contained 10 μM substrate, 25 mg (dry weight) of cell material in a total volume of 2.0 ml of 0.01 M phosphate buffer, pH 7.3. In O_2 uptake experiments, 0.2 ml of 10 per cent KOH was in the center well. In CO_2 evolution experiments, 0.2 ml of 0.5 N HCl was tipped from a side arm at the end of the incubation period to release bound carbon dioxide. The values have been corrected for endogenous activity.

Neither cell suspensions nor cell-free extracts produce gas from the above substrates in the absence of air. Analysis of the reaction media, however, revealed that allantoin and allantoic acid are converted to glyoxylic acid and urea under these conditions. The observed gas exchange with these substrates in the presence of air, therefore, probably results from the oxidation of glyoxylic acid formed by a hydrolytic decomposition of the ureide compounds. The formation of glyoxylic acid from allantoin under aerobic conditions was demonstrated by adding semicarbazide or 2,4-dinitrophenylhydrazine as trapping agents to the reaction mixture. Under these conditions, no gas exchange occurred and the glyoxylic acid derivative accumulated and was identified by paper chromatography and melting point determinations.

Nakada and Weinhouse (1953) have demonstrated that in rat liver homogenates the oxidation of formate to CO_2 and water is due to a peroxidative effect of catalase. That this is not the case for Pseudomonas, strain 2RCC-1, was shown by using cell-free extracts which were devoid of catalase activity. Oxygen uptake and CO_2 evolution were the same in the absence or presence of catalase.

Isolation and identification of intermediates in allantoin degradation. An analysis of reaction mixtures revealed that urea, carbon dioxide, and

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water are the end products of allantoin metabolism by Pseudomonas, strain 2RCC-1. In order to establish the mechanism of allantoin degradation, it was necessary to attempt isolation and identification of the intermediates along the degradative pathway. The intermediates isolated and identified are given below:

(1) *Allantoic acid.* The conversion of allantoin to glyoxylic acid and urea by cell-free extracts is inhibited by the addition of 0.01 M iodoacetate. As a result of this inhibition, allantoic acid accumulates stoichiometrically. It was isolated as the silver salt, converted to the acid with hydrogen sulfide, and hydrolyzed with 0.5 N HCl to glyoxylic acid and urea. Allantoin is not hydrolyzed under these conditions (Young and Conway, 1942).

(2) *Urea.* Urea was determined by the manometric method of Krebs and Henseleit (1932) using urease. It was found that 1.98 μM of urea per μM of allantoin accumulated as end products of allantoin metabolism by cell-free extracts. Urease has no action on allantoin or allantoic acid.

(3) *Glyoxylic acid.* This compound was isolated as the semicarbazone by the addition of 0.1 M semicarbazide to the reaction mixture. The semicarbazone derivative was isolated in both aerobic and anaerobic experiments using cell extracts. In aerobic experiments, the isolation of the glyoxylic acid derivative was possible only when the enzymatic oxidation was not allowed to proceed to completion. Following the addition of semicarbazide, the protein was removed by adjusting the pH to 5.0 with acetate buffer and heating for a few minutes in a boiling water bath. Upon standing overnight at 0 C, a semicarbazone was precipitated. It was recrystallized 2 times from hot water and dried *in vacuo* over P_2O_5. The melting point was 215 to 216 C with decomposition as was the mixed melting point with known glyoxylic acid semicarbazone.

Paper chromatography of the semicarbazone derivative in a wide variety of solvents was unsuccessful. Therefore, the free acid was regenerated from the semicarbazone by the addition of concentrated HCl. The regenerated free acid, authentic glyoxylic acid, and a mixture of the two were subjected to ascending paper chromatography in a solvent of *n*-butanol saturated with 2 N formic acid. The papers were dried and developed by the method of Magasanik and Umbarger (1950). The samples all gave Rf values of 0.63 $\pm$ 0.01.

TABLE 2

The effect of hypophosphite on the degradation of allantoin, allantoic acid, and glyoxylic acid by Pseudomonas, strain 2RCC-1

Substrate	O_2 Uptake	CO_2 Evolution	Formate Accumulation
	μM	μM	μM
Allantoin	4.98	9.94	9.97
Allantoic	5.0	9.98	9.95
Glyoxylic	5.1	10.02	10.00
Formic	0	0	10.00

Conditions were the same as in table 1 except the cells had been preincubated for 1 hour at 30 C with 0.002 M hypophosphite prior to use. Similar results were obtained with cell-free extracts preincubated with hypophosphite.

The 2,4-dinitrophenylhydrazone of glyoxylic acid was prepared for paper chromatography by the method of Block *et al.* (1952). Aliquots of the derivative in ethanol were chromatographed in a tertiary amyl alcohol-ethanol-water (5:1:4) solvent. Both ascending and descending methods were employed. The Rf values of the reaction mixture derivative, known glyoxylic acid derivative, and a mixture of the two were identical, 0.24 $\pm$ 0.01.

The crystalline 2,4-dinitrophenylhydrazone, recrystallized twice from methanol and water and dried *in vacuo* over P_2O_5, gave a mp and a mixed mp with authentic glyoxylic 2,4-dinitrophenylhydrazone of 189 to 191 C.

(4) *Formic acid.* Potassium hypophosphite (KH_2PO_2) has been shown to be a specific inhibitor of formate decomposition by *Escherichia coli* (Pine, 1953, *personal communication*; Takamiya, 1953). The oxidation of formate by extracts or cell suspensions of Pseudomonas, strain 2RCC-1, is likewise inhibited by 0.002 M hypophosphite (HP). The inhibition occurs, however, only after the enzyme system and the inhibitor have been preincubated for one hour at 30 C prior to the addition of substrate. Upon addition of allantoin, 0.5 μM of oxygen is taken up and 1 μM of CO_2 evolved per μM of substrate, utilized, and formate accumulates stoichiometrically. Similar results were obtained when allantoic acid or glyoxylic acid was the substrate (see table 2). The formate was isolated by

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steam distillation, titrated with 0.01 N carbonate-free NaOH, made alkaline to pH 10, and evaporated to dryness. The residue was taken up in water, transferred to a Y tube (Calvin *et al.*, 1949), and oxidized to CO_2 with 10 per cent mercuric sulfate in 2 N H_2SO_4. All of the titrable acidity could be accounted for as formate. The *p*-bromophenacyl ester of the acid, isolated from large scale runs, was prepared by the method of Hurd and Christ (1935). The derivative melted at 140 to 142 C, and the mixed mp with known *p*-bromophenacyl formate was the same.

Cofactor requirements. A study to determine the cofactor requirements of allantoin degradation was carried out using extracts which had been repeatedly dialyzed against distilled water or treated with Dowex-1 chloride according to the method of Stadtman *et al.* (1951) in order to remove nucleotides. Such extracts retained their ability to hydrolyze allantoin and allantoic acid to glyoxylic acid and to oxidize formate to CO_2 and water, but they were unable to oxidize glyoxylic acid. Of a large number of cofactors tested, including coenzyme A and lipoic acid, only thiamin pyrophosphate (TPP) in combination with either Mg^{++} or Mn^{++} could reactivate the extracts. The addition of 2×10^{-4} micromoles of thiamin pyrophosphate plus 1×10^{-4} M $MgCl_2$ or $MnCl_2$ restored complete activity. The role of thiamin pyrophosphate in glyoxylic acid metabolism is now under further investigation.

Tracer experiments. In order to determine the fate of the carbon atoms of glyoxylic acid, C_{14} tracer experiments were carried out. Carboxyl labeled glyoxylic acid was used in tracer experiments with cell-free extracts of Pseudomonas, strain 2RCC-1, which had been preincubated with 0.002 M hypophosphite. The CO_2 was collected as barium carbonate, washed, and counted. Formate was isolated and oxidized to CO_2 as previously described and counted as $BaCO_3$. The results presented in table 3 show that the carboxyl carbon of glyoxylate is converted to formate.

Campbell (1953, *unpublished data*) has established that Pseudomonas, strain 2RCC-1, will oxidize glycine to NH_3, CO_2, and H_2O through glyoxylic and formic acids as intermediates. C_{14} alpha labeled glycine was, therefore, employed in experiments to determine the fate of the alpha carbon of glyoxylic acid. The data in table 3 show that the alpha carbon of glyoxylate is converted to carbon dioxide.

DISCUSSION

The data presented in the present study indicate that the aerobic degradation of allantoin by Pseudomonas, strain 2RCC-1, occurs as follows:

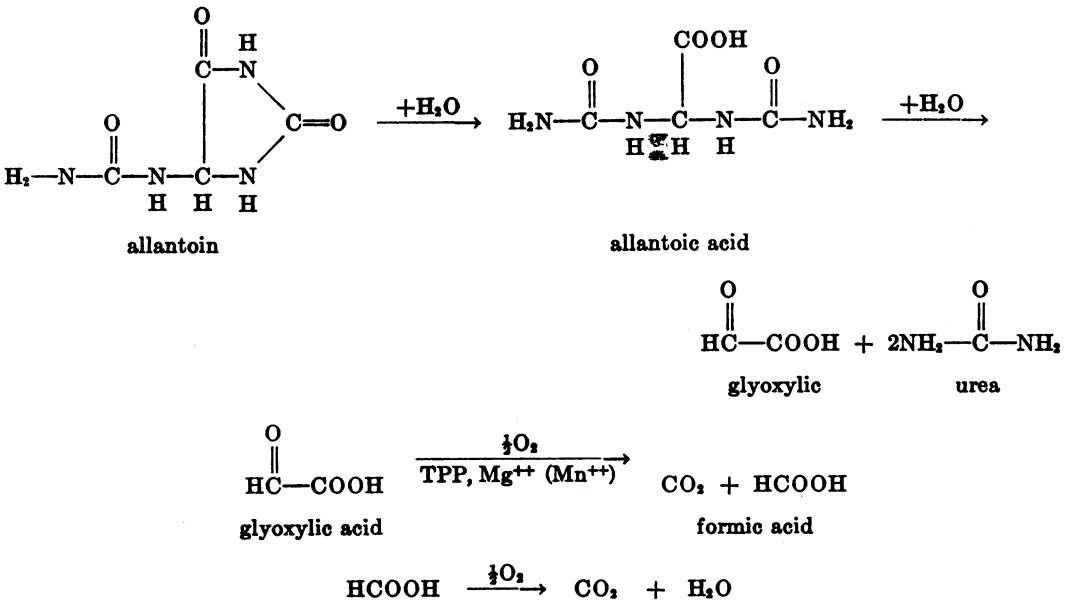

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TABLE 3

Distribution of C^{14} in products of glyoxylic acid oxidation by Pseudomonas, strain 2RCC-1

Substrate	Specific Activity		
	Substrate	CO_2	HCOOH
	cpm per μM		
Glyoxylate-1-C^{14}	4,000	350*	2,897
Glycine-2-C^{14}	1,000	782	0

Reaction mixture contained 0.01 M phosphate buffer (pH 7.2), 50 mg of cell-free extract (alumina), 200 μM of substrate in a total volume of 3.5 ml. Flasks were shaken at 30 C for 6 hours with air as the gas phase.

* This count can be accounted for as CO_2 arising from the degradation of formate since hypophosphite did not cause a complete inhibition of the reaction in this experiment.

It is obvious that the aerobic degradation of allantoin carried out by this organism is markedly different from the anaerobic decomposition by *Streptococcus allantoicus* reported by Barker (1943). Although the end products of allantoin catabolism are quite different in the two systems, the initial hydrolytic steps in the cleavage of the ureide compounds may be identical.

Nakada and Weinhouse (1953) have reported that rat liver homogenates carry out an oxidative decarboxylation of glyoxylic acid yielding formate and carbon dioxide. In tracer experiments, they demonstrated that the alpha carbon was converted to formate and the carboxyl to carbon dioxide. In contrast to this report, the tracer data in the present study reveal that Pseudomonas, strain 2RCC-1, converts glyoxylate to these products in a different manner. The data presented in table 3 show conclusively that the alpha carbon of glyoxylate is converted to carbon dioxide and the carboxyl carbon is converted to formate. These results are reminiscent of the phosphoroclastic split of pyruvate by *E. coli* in which formate originates from the carboxyl carbon (Chantrenne and Lipmann, 1950). These workers found that coenzyme A was required for the fixation of formate in pyruvate. Strecker (1951) reported, however, that thiamin pyrophosphate and Mn^{++} stimulate the incorporation of formate in the absence of coenzyme A. Thiamin pyrophosphate and Mg^{++} (Mn^{++}) may play similar roles in the phosphoroclastic decomposition of pyruvate and in the enzymatic transformations of glyoxylate by Pseudomonas extracts.

The fixation of formate and carbon dioxide into glyoxylate and the role of thiamin pyrophosphate and Mg^{++} (Mn^{++}) in the oxidation and fixation reactions are now under investigation. Fractionation and purification of the enzymes in the crude extracts responsible for the various degradation steps are also in progress.

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SUMMARY

Cell suspensions and cell-free extracts of Pseudomonas, strain 2RCC-1, isolated by enrichment culture techniques, carry out the aerobic degradation of allantoin to urea, carbon dioxide, and water in the following manner: allantoin → allantoic acid → glyoxylic acid + urea; glyoxylic acid → formate + carbon dioxide; formate → carbon dioxide + water. Thiamin pyrophosphate and Mg^{++} or Mn^{++} are required for the oxidation of glyoxylate to formate and carbon dioxide. Tracer experiments have demonstrated that the alpha carbon of glyoxylate is converted to carbon dioxide, and the carboxy carbon is converted to formate.

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