Isolation of New Limonoate Dehydrogenase from *Pseudomonas*

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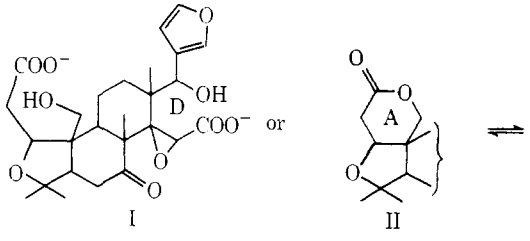
A new limonoid-metabolizing bacterium was isolated from soil and designated "*Pseudomonas*-sp. 321-18." This organism metabolized limonoate, mainly through deoxylimonin, but cell-free extracts contained considerable amounts of limonoate dehydrogenase activity which had properties quite different from the dehydrogenase (limonoate–NAD oxidoreductase) of *Arthrobacter globiformis* previously studied. The new enzyme was characterized as a limonoate–NAD(P) oxidoreductase. It required Zn ions and sulfhydryl groups for its catalytic action, used both NAD and NADP as cofactors, had a wide pH activity range with the optimal at pH 8.0, and had low affinity for DEAE cellulose. The enzyme has potential industrial use for improving the flavor of citrus fruit juices and products whose quality is impaired by limonin bitterness.

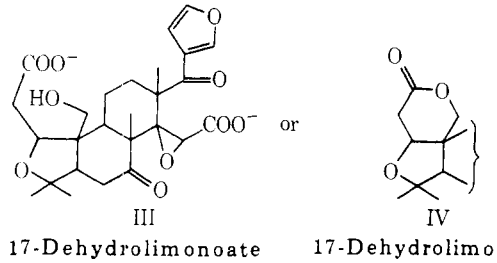
Bitterness due to limonin in citrus juices has become a serious economic problem. The naturally occurring form and precursor of limonin is limonoate A-ring lactone (II) (Maier and Margileth, 1969). We have searched for microbial enzymes which convert either the precursor or limonin to nonbitter compounds.

A limonoate dehydrogenase (limonoate–NAD oxidoreductase) isolated from *Arthrobacter globiformis* catalyzed the conversion of limonoate (I) or limonoate A-ring lactone (II) to nonbitter 17-dehydrolimonoate (III) or 17-dehydrolimonoate A-ring lactone (IV) (Hasegawa *et al.*, 1972b). With this enzyme the limonin-debittering reaction was demonstrated in orange juice (Hasegawa *et al.*, 1971).

Limonoate dehydrogenase



I Limonoate — II Limonoate A-ring lactone



III 17-Dehydrolimonoate — IV 17-Dehydrolimonoate A-ring lactone

In a continuing survey of microorganisms, a new bacterium was isolated from soil and appeared to metabolize limonoate through two pathways, one *via* 17-dehydrolimonoate and the other *via* deoxylimonin (V). The organism intracellularly produced a limonoate dehydrogenase (limonoate–NAD(P) oxidoreductase) which differed markedly from the dehydrogenase of *A. globiformis*.

This paper reports details of characterization of the bacterium, isolation and characterization of the new dehydrogenase, and also points out the difference between the dehydrogenase of *Pseudomonas*-sp. 321-18 and that of *A. globiformis*.

EXPERIMENTAL SECTION

Assay Methods. Protein concentration was measured by the procedure of Lowry *et al.* (1951). Limonoate dehydrogenase activity was determined by measuring the initial rate of NADP reduction (Hasegawa *et al.*, 1972b). Activity was assayed in 1 ml of a reaction mixture consisting of 0.01 *M* limonoate, 0.07 *M* Tris buffer at pH 8.0, 0.5 m*M* NADP, and 1–4 munits of enzyme. One unit of limonoate dehydrogenase activity is defined as the amount which catalyzes the formation of 1 μmol of NADPH/min under the conditions used.

Growth of Cells. Unless otherwise stated, the organism was grown by inoculating 1.0% of a 48-hr culture into a 2-l. Erlenmeyer flask containing 400 ml of 0.5% limonoate medium with the same mineral salts used for the growth of *Arthrobacter globiformis* (Hasegawa *et al.*, 1972b). The mixture was incubated at room temperature on a shaker or with vigorous aeration by sparging for 98 hr. Cells then were collected by centrifugation at 20,000 × *g* for 10 min, washed twice with 0.1 *M* phosphate buffer at pH 7.5, and kept in a freezer until used.

Characterization of Metabolites. Silica gel G plates were used for thin-layer chromatography (tlc). Plates were developed with benzene–ethanol–water–acetic acid (200:47:15:1), ethanol, cyclohexane–ethyl acetate (30:70), and dichloromethane–methanol (96:4), and were revealed by the Ehrlich's reagent method (Dreyer, 1965) or by spraying with 50% H_2SO_4 followed by heating. Nmr spectra were run at 100 MHz in $CDCl_3$ using a JNM-PS-100 spectrometer.

Isolation of Limonoate Dehydrogenase. Cells were thawed in 10 vol of 0.1 *M* phosphate buffer (pH 7.5) containing 0.01 *M* dithiothreitol and were ruptured in an ice bath with a Branson sonifier, J-22. The supernatant from centrifugation at 20,000 × *g* for 10 min was used as the starting material for purification. The crude extract was first subjected to $(NH_4)_2SO_4$ precipitation, followed by separation on two columns of DEAE cellulose by a previously described procedure (Hasegawa *et al.*, 1972b).

RESULTS

Identification of Bacterium. The organism isolated from soil was a yellow, short (0.5 × 1.5 μ), gram-negative rod, and was motile with polar flagella. It was highly aerobic and did not produce fluorescence. The yellow pigment extracted with methanol had absorption peaks in that solvent at 424–428, 448–450, and 476–478 mμ, and in petroleum ether (bp 30–60°) at 424, 450, and 480 mμ.

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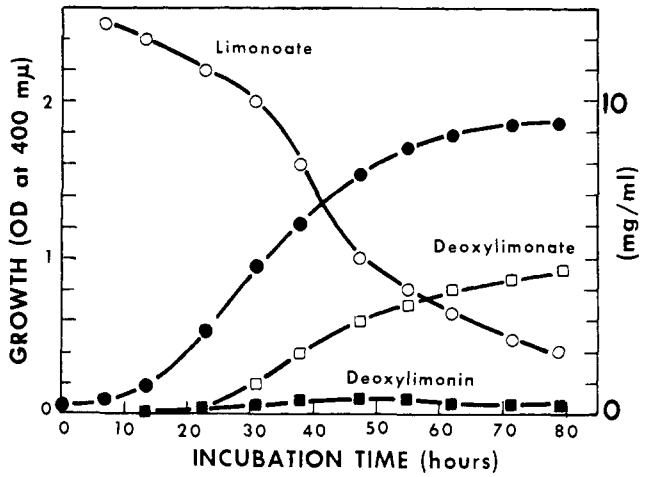


Figure 1. Growth of *Pseudomonas*-sp. 321-18 on a limonoate medium (— —) and correlation with decrease in substrate concentration and formation of metabolites. Growth conditions are described in the text.

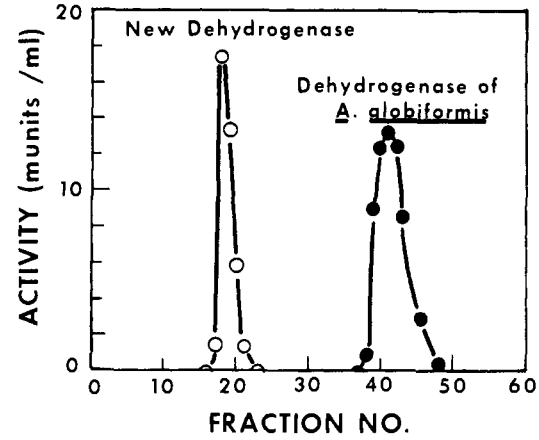


Figure 3. Separation of limonoate dehydrogenase of *Pseudomonas*-sp. 321-18 from that of *Arthrobacter globiformis* on DEAE cellulose column. The column (2.6 × 20 cm) was eluted by a linear gradient consisting of 150 ml of 0.01 *M* phosphate buffer at pH 7.0 and 150 ml of the same buffer containing 0.8 *M* NaCl. The effluent was collected in 5 ml fractions.

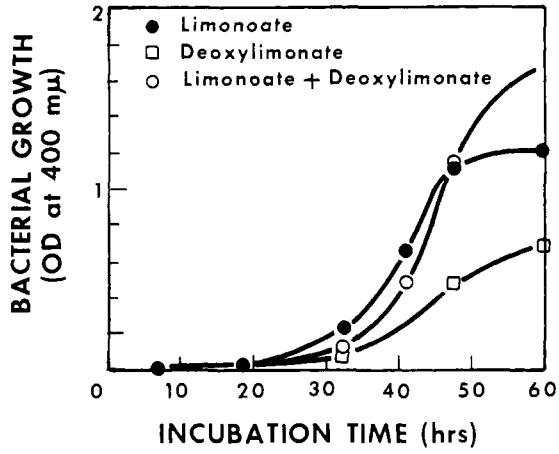


Figure 2. Growth of *Pseudomonas*-sp. 321-18 on three limonoid media. The cultural conditions are described in the text.

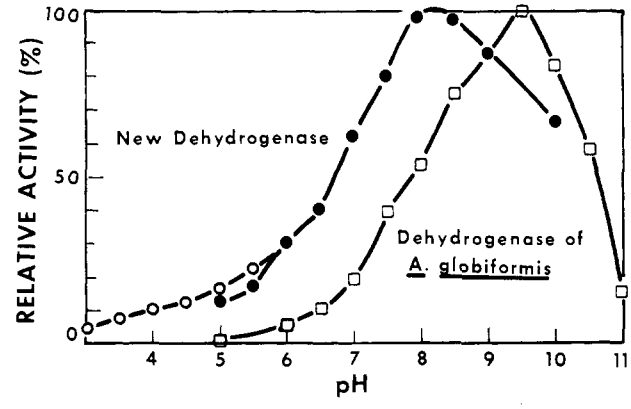


Figure 4. Comparison of effects of pH on limonoate dehydrogenase activity of *Pseudomonas*-sp. 321-18 and *Arthrobacter globiformis*. Incubation mixtures consisted of 0.01 *M* limonoate, 0.1 m*M* NADP, 6.25 munits of enzyme, and 0.1 *M* phosphate-Tris buffer for pH above 5 (—●—, —□—) and 0.1 *M* acetate-phosphate buffer for pH below 5 (—○—) in 1 ml.

Acid was weakly produced from glucose under oxidative conditions using the sensitive medium of Dye (1962). Tests for utilization of sugars, both mono- and disaccharides including sucrose, glucose, and galactose, were negative with Hugh and Leifson's medium (1953).

The bacterium grew slowly in a mineral salts–glucose medium and in tubed nutrient broth, but grew more rapidly in shake culture of nutrient broth. It also grew on citrate as the sole carbon source.

Nitrate was not reduced and no change was observed in litmus milk after 3 days of incubation at 28°. The organism produced indole but not H_2S. It also had oxidase and catalase activity. These results indicated that our organism fairly closely matches the genus *Xanthomonas* (Hayward, 1966). The yellow pigment from culture of our bacterium, however, did not have absorption peaks at 418, 437, and 463 mμ in petroleum ether as described by Starr and Stephens (1964). Also, our organism produced indole and oxidase in contrast to the above description of *Xanthomonas*. Based on DNA homology experiments, Deley *et al.* (1966) suggested that the genus *Xanthomonas* be eliminated and that organisms so designated be incorporated into the genus *Pseudomonas*. Because of the highly aerobic nature of our organism and its divergence from the description of the genus *Xanthomonas*, it was assigned to the genus *Pseudomonas*. We, therefore, designated our organism as "*Pseudomonas*-sp. 321-18."

Growth Characteristics of *Pseudomonas* on Limonoid Media. Figure 1 shows the relation among growth curves of *Pseudomonas*-sp. 321-18, the decrease in substrate (limonoate) concentration, and the formation of metabolites in the medium. The organism multiplied and passed through a succession of typical bacterial growth phases with a simultaneous decrease in substrate concentration.

Analyses of metabolites in the medium showed that deoxylimonin appeared first at the early stage of the growth (0.125 mg/ml after 23 hr of incubation) but never accumulated in substantial quantity. Deoxylimonate (VI), on the other hand, appeared after 30 hr of incubation and its concentration increased steadily thereafter. After 60 hr of incubation, growth ceased and the total amounts of limonoids remained constant thereafter, but the limonoate was continuously converted to deoxylimonate. Appearance of deoxylimonin before deoxylimonate is reasonable evidence that deoxylimonin is the first metabolite of limonoate produced by *Pseudomonas*-sp. 321-18.

Because growth ceased after 60 hr even though substantial amounts of the substrate remained undigested, the next experiment was designed to test whether the accumulation of deoxylimonate inhibited the growth of the organism and also to find whether or not the organism utilized deoxylimonate. The organism was inoculated on

Table I. Purification of New Limonoate Dehydrogenase

Treatments	Total vol, ml	Total activity, munits	Total protein, mg	Specific activity, munits/mg pr	Purification	Recovery, %
Crude extract	68.0	332	297.5	1.11	1	100
$(NH_4)_2SO_4$ ppt after dialysis	16.5	325	129.0	2.52	2.3	98
1st DEAE cellulose column	20.0	313	15.2	20.6	18.6	94
2nd DEAE cellulose column	30.0	155	2.2	70.1	63.2	46

three different media, 25 ppm of limonoate, 25 ppm of deoxylimonate, and 25 ppm each of limonoate and deoxylimonate, and incubated at room temperature on a shaker. Deoxylimonate appeared to have no inhibitory action on the growth of the organism since the rate of growth on the limonoate–deoxylimonate medium was similar to that on the limonoate medium (Figure 2). The organism utilized deoxylimonate as an energy source although the rate of growth was slower than on limonoate. These results, coupled with the fact that this organism demands high oxygen tension to grow on any sort of media, suggest that oxygen is one of the growth-limiting factors.

Characterization of Metabolites. Deoxylimonin and deoxylimonate were isolated from bacterial growth media and characterized (Hasegawa *et al.*, 1972a). Tlc analyses with four solvent systems and nmr spectra were identical to those of the authentic compounds.

Purification of Limonoate Dehydrogenase. Although no trace of 17-dehydrolimonoate was found in the bacterial growth medium, analyses of cell-free extracts of this organism showed the presence of considerable limonoate dehydrogenase activity. Unlike the dehydrogenase of *A. globiformis*, the dehydrogenase of *Pseudomonas*-sp. 321-18 was poorly retained on DEAE cellulose columns and eluted at a very early stage of fractionation (Figure 3). The overall purification during the preparative procedure represented a 63-fold increase in activity over that of the crude extract and recovery was 46% of the original activity (Table I).

Characterization of Limonoate Dehydrogenase of *Pseudomonas*-sp. 321-18. 17-Dehydrolimonoate A-ring lactone was isolated from enzymic reaction mixtures (Hasegawa *et al.*, 1972b). The mixture consisted of 10 mg of limonoate, 1 mmol of NADP, 0.1 *M* phosphate buffer at pH 8.0, and 0.5 unit of purified dehydrogenase in 15 ml. After 17 hr of incubation at 30°, the conversion was nearly completed. The isolate had R_f's identical to those of authentic 17-dehydrolimonoate A-ring lactone when developed with each of four solvent systems. The nmr spectrum of the methyl ester of the isolate was identical with that of authentic methyl 17-dehydrolimonoate A-ring lactone.

The new dehydrogenase differed markedly from the dehydrogenase of *A. globiformis* in three major characteristics.

The dehydrogenase of *A. globiformis* used only NAD as a cofactor, but the new dehydrogenase used both NAD and NADP, with the latter being twice as effective (Table II).

The second difference between the two enzymes was their behavior on a DEAE cellulose column (Figure 3). A mixture of two enzymes was transferred onto the top of a DEAE cellulose column (2.5 × 20 cm) which had previously been equilibrated to 0.01 *M* phosphate buffer at pH 7.0. The column then was eluted with a linear gradient consisting of 150 ml of 0.01 *M* phosphate buffer at pH 7.0 and 150 ml of the same buffer containing 0.8 *M* NaCl. The effluent was collected in 5-ml fractions. The new dehydrogenase was poorly retained by the column and eluted in fractions 18–22, whereas the dehydrogenase of *A. globiformis* was well retained and eluted at a relatively late stage.

The third difference was the effect of hydrogen ion concentration on their activities. For the dehydrogenase of *A. globiformis*, activity was optimal at pH 9.5 with low activity below pH 6.0. For the new dehydrogenase, activity was optimal at pH 8.0 and the pH activity range was much broader, with substantial activity in the low pH region (Figure 4).

Table II. Cofactor Requirements[a]

Cofactors	Activity, ΔOD/min: Dehydrogenase of *Pseudomonas*-sp. 321–18	Activity, ΔOD/min: Dehydrogenase of *A. globiformis*
NAD	0.58×10^{-2}	5.34×10^{-2}
NADP	1.25×10^{-2}	0

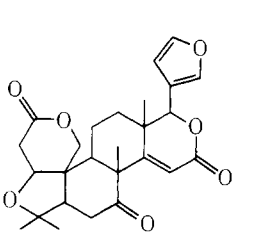
[a]Reaction mixtures consisted of 10 m*M* limonoate, 70 m*M* Tris buffer, enzyme, and 0.5 m*M* NAD or NADP in 1 ml. The dehydrogenase of *Pseudomonas* was assayed at pH 8.0 with 2 munits of enzyme, whereas the dehydrogenase of *A. globiformis* was assayed at pH 9.5 with 8.5 munits of enzyme.

$ZnCl_2$ (10^{-3} *M*) increased the activity 60%, but Ca, Mg, and Mn ions had no effect on the activity. $HgCl_2$ and *p*-chloromercuribenzoate were found to be potent inhibitors of the enzyme. 10^{-3} *M* of $HgCl_2$ inhibited the activity 100%, whereas 10^{-3} *M* of PCMB inhibited 72%.

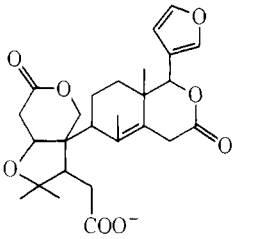
DISCUSSION

The work reported here confirms the previous report (Hasegawa *et al.*, 1972b) that limonoate is metabolized in bacteria by at least two different pathways, one *via* 17-dehydrolimonoate and the other *via* deoxylimonin.

When grown on limonoate, *Pseudomonas*-sp. 321-18 produced exocellularly a trace of deoxylimonin and substantial amounts of deoxylimonate as metabolites. This indicated that the organism metabolized limonoate through the deoxylimonin pathway. The presence of considerable dehydrogenase activity, however, suggested an alternative pathway of limonoate metabolism, *via* 17-dehydrolimonoate. In the case of *A. globiformis*, limonoate appeared to be metabolized through only the 17-dehydrolimonoate pathway.



V
Deoxylimonin



VI
Deoxylimonate

We have thus far shown that there are at least two types of limonoate dehydrogenase in bacteria, limonoate–NAD oxidoreductase (*A. globiformis*) and limonoate–NAD(P) oxidoreductase. These enzymes are different proteins with different cofactor requirements and pH optima, even though both catalyze the same reaction and require

Zn ions and sulfhydryl for activity. Such cases are very common.

Limonoids appeared to be metabolized also by molds, but neither metabolites nor enzymes were demonstrated (Nomura, 1966). In citrus fruits, limonoid content decreases with advancing maturity (Higby, 1938; Maier *et al.*, 1973; Scott, 1970). For many years attempts to demonstrate limonoid-degrading systems in citrus fruits have been unsuccessful. Recently, however, we isolated 17-dehydrolimonoate A-ring lactone from orange peel, orange juice, and lemon seedlings (Hsu *et al.*, 1973). Our bacterial work here further supports the previous suggestion that 17-dehydrolimonoate A-ring lactone is an immediate metabolite of limonate A-ring lactone in citrus fruits. Thus, it appears that limonoate dehydrogenase is an initial enzyme responsible for the disappearance of limonoids during maturation of citrus fruits.

We are studying the use of these dehydrogenase enzymes to prevent the development of limonin bitterness in navel orange juice; initial results are very encouraging. The dehydrogenases attack the limonoate A-ring lactone of freshly prepared juice and convert it to nonbitter 17-dehydrolimonoate A-ring lactone before the limonoate A-ring lactone is converted to the intensely bitter limonin by juice acids and native limonin hydrolase. Using this new dehydrogenase, which has a lower pH optimum, we have shown that in fresh navel orange juice sufficient limonoate A-ring lactone was converted to nonbitter 17-dehydrolimonoate A-ring lactone to reduce the ultimate limonin concentration of the juice to below the bitterness threshold. Details of this work will be reported elsewhere.

ACKNOWLEDGMENT

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COMMUNICATIONS

Proline Nitroxide

Proline nitroxide, a relatively stable free radical, was synthesized by the oxidation of *N*-hydroxyproline. Some chemical and physical characteristics are described. Proline nitroxide, like some other stable free radicals (Weil *et al.*, 1968), has antioxidant activity in unsaturated lipids.

The antioxidant activity of proline in unsaturated lipids was noted by Olcott and Kuta (1959). Subsequent observations indicated that aliphatic secondary amines could be oxidized to substituted hydroxylamines in oxidizing lipid system (Harris and Olcott, 1966) and that substituted hydroxylamines (Van der Veen *et al.*, 1970) and nitroxides (Weil *et al.*, 1968) had antioxidant activity. These combined observations suggested that the antioxidant activity of proline might be accounted for by the formation of the hitherto undescribed free radical proline nitroxide in the oxidizing system (Van der Veen *et al.*, 1970), similar to the formation of the free radical diphenyl nitroxide in an oxidizing system when diphenylamine was added as an oxidation inhibitor (Thomas, 1960).

Although a definitive epr signal indicated that proline nitroxide was present in a solution containing proline, 30% hydrogen peroxide, and catalytic amounts of sodium tungstate (Van der Veen *et al.*, 1970), we were unable to isolate the product at the time that observation was made. More recently Nagasawa *et al.* (1972) described a method for synthesizing *N*-hydroxyproline. Oxidation of this compound has now yielded proline nitroxide in amounts sufficient for isolation, purification, and characterization. In his review of the stereochemistry of nitroxides, Janzen (1971) refers to epr coupling constants of proline nitroxide and their interpretation but no details of its preparation or other properties have been published.

PREPARATION OF L-PROLINE NITROXIDE

N-Hydroxy-L-proline was synthesized by the method of Nagasawa *et al.* (1972). A mixture of 0.1 g of *N*-hydroxy-L-proline in 5 ml of water and 0.05 ml of *tert*-butyl hydroperoxide (K & K Laboratory) in 4 ml of ethanol was shaken gently for 2 min at room temperature and then extracted three times with equal volumes of isooctane to remove residual hydroperoxide. The isooctane extracts were extracted with water to recover some proline nitroxide and then discarded. The combined water fractions were taken to incipient dryness in a rotary vacuum evaporator at 35°, and proline nitroxide was extracted from the solid residue with 5 ml of methanol. The methanol-insoluble solids were unreacted *N*-hydroxyproline which was then treated with