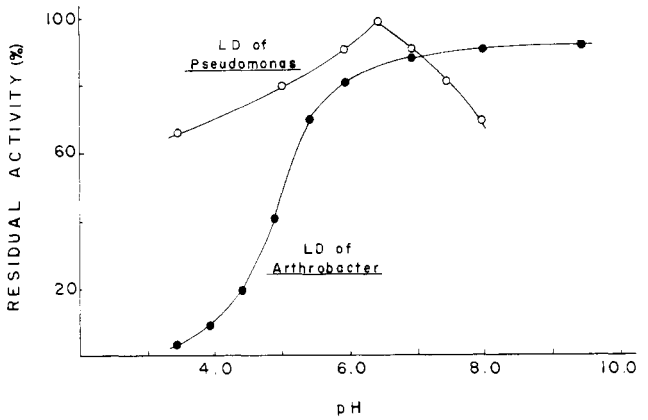

Figure 2. pH stability of limonoate dehydrogenase (LD) enzymes. Enzyme incubated 5 min at 23° C.

centration. At substrate concentrations that were not limiting, the activity of LD-Ps was roughly 15 times that of LD-Ag.

Figure 2 shows that the stabilities of the two enzymes over the pH range 3.5–9.0 differed widely. The differences between the stabilities of the two enzymes at pH values lower than 5.5 could explain the wide difference in their activities in orange juice. At 23°C, the stability of LD-Ps was maximum at pH 6.5 and decreased above and below that pH. LD-Ps retained substantial activity after 5 min at pH 3.5, 23°C. LD-Ag was stable at pH 7.0–9.5 but its stability fell off sharply below pH 6. Very little activity remained after 5 min at pH 3.5.

The limonoate dehydrogenases of *Arthrobacter* and of *Pseudomonas* functioned both in freshly prepared navel orange juice and in hydrolyzed reconstituted navel orange juice concentrate. The LD of *Pseudomonas* was the more stable at low pH and therefore the most effective in reducing the eventual limonin content of the juice. Its effectiveness increased with increasing pH (up to pH 6.5), and with cofactor and enzyme concentration.

With regard to the potential use of limonoate dehydrogenase in citrus juice systems, it should be noted that both a limonoate dehydrogenase enzyme (Hasegawa et al., 1974b) and its reaction product 17-dehydrolimonoate A-ring lactone (Hsu et al., 1973) occur naturally in citrus fruits.

Work is underway to further explore the potential utility of the limonoate dehydrogenase of *Pseudomonas*-sp. 321-18. We are now investigating the growth of the organism and its production of limonoate dehydrogenase (Hasegawa et al., 1974a). Of special interest is whether a sufficiently high yield of enzyme per unit volume of growth media can be attained to make feasible commercial production of the enzyme. The search for new limonoid-degrading microorganisms and new enzymes is continuing. Several new microorganisms which metabolize limonoids have recently been isolated and are being studied.

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Metabolism of Limonoids. Limonin D-Ring Lactone Hydrolase Activity in *Pseudomonas*

Shin Hasegawa

Pseudomonas sp. 321-18 possesses a limonin D-ring lactone hydrolase similar to that isolated from grapefruit seeds. The two enzymes have similar pH optima for both hydrolysis and lactonization, and also have similar substrate specificities. However, they differ greatly in heat resistance.

Limonin (I) is the intensely bitter triterpenoid dilactone present in citrus seeds (Arigoni et al., 1960; Barton et al., 1961). Because of bitterness in certain citrus juices and other processed products, the metabolic pathways of limonoids in citrus and microorganisms have been intensively investigated (Chandler, 1971; Flavian and Levi, 1970; Hasegawa et al., 1972a,b, 1974a–c; Hsu et al., 1973; Nomura, 1966).

We have found that there are at least two limonoid metabolic pathways in bacteria: one through 17-dehydrolimonoate A-ring lactone (IV) and the other through deoxylimonin (III) (Hasegawa et al., 1972b, 1974b). In plants, the 17-dehydrolimonoate pathway has been shown to be present in navel orange tissues (Hasegawa et al., 1974a), and a metabolite of that pathway, 17-

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dehydrolimonoate A-ring lactone, has been found in various tissues of citrus fruit (Hsu et al., 1973).

Previously, Maier et al. (1969) isolated from grapefruit seeds limonin D-ring lactone hydrolase, which catalyzes reversibly the hydrolysis of the D-ring lactone of limonin. This hydrolase is the only limonoid-metabolizing enzyme isolated from citrus.

Recently, we found limonin D-ring lactone hydrolase activity in *Pseudomonas* sp. 321-18. This paper compares some characteristics of the bacterial enzyme with those of the citrus hydrolase. Its possible biological role is also discussed.

EXPERIMENTAL SECTION

Materials. Limonin D-ring lactone hydrolase of citrus was isolated from grapefruit seeds as described previously (Maier et al., 1969). 19-Deoxylimonoic acid D-ring lactone (commonly referred to as "isoobacunoic acid") and its 3-methyl-^{14}C ester were provided by Dr. R. D. Bennett of this laboratory. Limonin was extracted from grapefruit seed meals with acetone and crystallized from dichloromethane–2-propanol.

Assay Methods. Hydrolyzing activity, the hydrolysis of limonin (I) to limonoate A-ring lactone (II), was the basis for routine assay of hydrolase activity. The reaction mixture consisted of 5×10^{-3} *M* limonin, 0.1 *M* Tris buffer (pH 8.0), 20% acetonitrile, and an appropriate amount of enzyme in 0.3 ml. After 5 min of incubation at 30°C, 5 μl portions of the reaction mixture were spotted on a silica gel thin-layer chromatography (TLC) plate. The plate was developed with benzene–ethyl acetate–water–acetic acid (200:47:15:1, upper layer) (Maier et al., 1969), and limonoids were revealed by spraying the plates with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965). The sizes and intensities of the unknowns relative to those of standards indicated the amounts of products formed. Under the conditions used, one unit of hydrolase activity was defined as "the amount which catalyzes the production of 1 μmol of limonoate A-ring lactone per 1 min".

For studies of temperature and hydrogen ion effects on activity of enzyme, its substrate was 19-deoxylimonoic acid D-ring lactone 3-methyl-^{14}C ester. Radioactive peaks were located by a Vanguard Automatic Chromatogram Scanner. Then, both the substrate and product zones were removed from the plate and were counted by a Beckman liquid scintillation spectrometer.

Lactonizing activity was assayed at pH 6.0, with sodium limonoate or 19-deoxylimonoate 3-methyl-^{14}C ester as substrate.

Growth of Cells. In a 2-l. Erlenmeyer flask, 400 ml of 0.3% limonoate–mineral salt medium was inoculated with 1% of a 48-hr culture of *Pseudomonas* sp. 321-18 (Hasegawa et al., 1972a). The mixture was incubated on a shaker at 25°C for 72 hr, then centrifuged at 20000*g* for 10 min. The cells were washed with 0.1 *M* phosphate buffer (pH 7.0) and frozen until used.

Enzyme Preparation. Cells were suspended in about 10 vol of 0.1 *M* phosphate buffer (pH 7.0) and ruptured in an ice bath with a Branson sonifier, J-22. The suspension was centrifuged at 20000*g* for 10 min, and the supernatant was brought to 0.9 saturation with solid $(NH_4)_2SO_4$. The mixture was centrifuged at 20000*g* for 15 min; the recovered precipitate was dissolved in a minimum of 0.01 *M* phosphate buffer (pH 7.0), and dialyzed against the same buffer for 3 hr.

RESULTS

The bacterial enzyme optimally catalyzed the hydrolysis of limonin to limonoate A-ring lactone and the reverse reaction, lactonization, at pH 8.0 and 6.0, respectively.

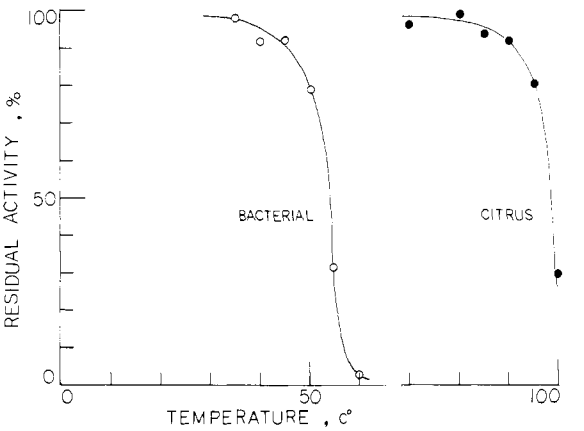

Figure 1. Heat stability of bacterial and citrus limonin D-ring lactone hydrolase. Enzyme solutions containing about 0.05 unit/ml of 0.05 *M* phosphate buffer (pH 5.0) were heated for 5 min at various temperatures. Residual activity was assayed with 19-deoxylimonoic acid D-ring lactone 3-methyl-^{14}C ester by the method described in the text.

These results were very similar to those for citrus hydrolase (Maier et al., 1969).

Reaction products were identified by the TLC method used for study of the citrus hydrolase (Maier et al., 1969). Limonoate A-ring lactone was identified as the hydrolysis product of limonin at pH 8.0, and limonoate D-ring lactone, as the product of sodium limonoate at pH 6.0.

At rates similar to the rate for limonin, both citrus and bacterial enzymes also catalyzed the hydrolyses of compounds such as obacunone, nomilin, and ichangin, which differ from limonin structurally in the vicinity of the A- and A'-rings. However, both were inactive toward deoxylimonin.

The two enzymes were significantly different with respect to heat resistance. Citrus hydrolase was markedly heat resistant (Figure 1), retaining about 30% of its activity after 5 min at 100°C. It required about 15 min of boiling for complete inactivation. On the other hand, the bacterial hydrolase was inactivated almost completely at 60°C for 5 min, and about 50% at 53°C for 5 min.

DISCUSSION

Cell-free extracts of *Pseudomonas* sp. 321-18 possessed limonin D-ring lactone hydrolase, which apparently differs from that isolated from grapefruit seeds (Figure 1). The catalytic functions of both were, however, very similar as shown by studies of substrate specificity and pH optimal.

Pseudomonas sp. 321-18 metabolizes both limonin and limonoate via two pathways: one through deoxylimonin and the other through 17-dehydrolimonoate or 17-dehydrolimonoate A-ring lactone (Figure 2) (Hasegawa et al., 1972b, 1974b). Two enzymes involved in the pathways, limonoate dehydrogenase and deoxylimonin hydrolase, have been isolated and characterized (Hasegawa et al., 1972b, 1974b,c).

The enzyme that catalyzes the formation of deoxylimonin has not been identified. However, limonin rather than limonoate A-ring lactone has been suggested as the immediate precursor of deoxylimonin (Hasegawa et al., 1974b). The presence of limonin D-ring lactone hydrolase and its reversible catalytic nature strongly support this suggestion. During growth on limonoate media, the *Pseudomonas* produced deoxylimonin and deoxylimonic acid extracellularly (Hasegawa et al., 1974c). Probably, the limonoate was converted by the hydrolase to limonin, which, in turn, was metabolized to deoxylimonin. On the other hand, when grown on limonin media, *Arthrobacter*

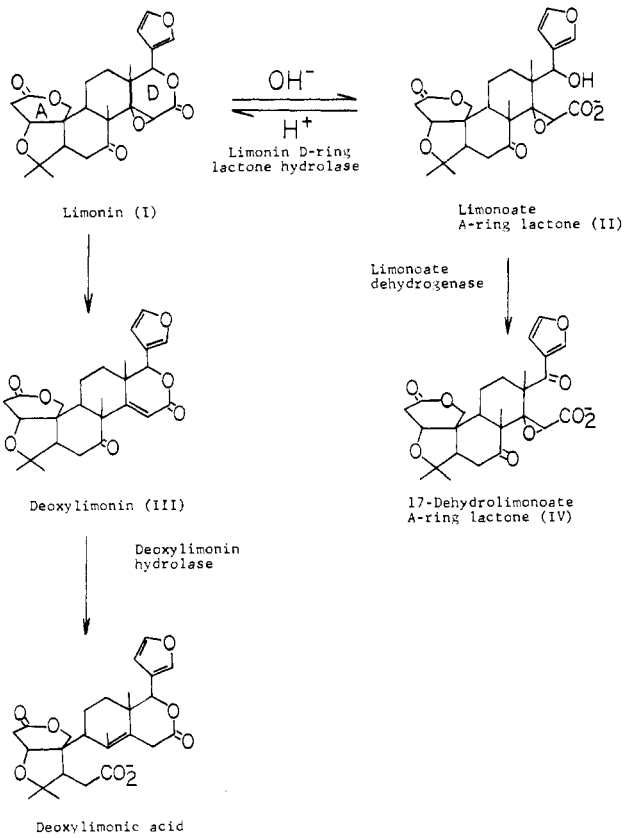

Figure 2. Metabolic pathways of limonoids in *Pseudomonas* sp. 321-18.

globiformis produced 17-dehydrolimonoate A-ring lactone as the major metabolite (Hasegawa et al., 1972b). Seemingly, the hydrolase was also present in *Arthrobacter globiformis* and catalyzed the conversion of limonin to limonoate A-ring lactone. The latter would then have been dehydrogenated by limonoate dehydrogenase, which has specificity for the open D-ring of limonoids (Hasegawa et al., 1972b).

Maier et al. (1969) questioned the biological functions of limonin D-ring lactone hydrolase, specifically whether it is involved in the synthesis of limonoids, their catabolism, or both. Our findings clearly show that the hydrolase was involved in the degradation of limonoids. Whether the enzyme also plays a role in their biosynthesis remains to be determined.

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Specific Oxidation of Methionine to Methionine Sulfoxide by Dimethyl Sulfoxide

Samuel H. Lipton* and C. E. Bodwell

In the presence of hydrochloric acid or other hydrogen halides, the dipolar aprotic solvent and selective oxidant dimethyl sulfoxide (Me_2SO) oxidizes methionine to methionine sulfoxide and is concomitantly reduced to the hydrophobic and low-boiling dimethyl sulfide (Me_2S). The reaction is reversed (i.e., methionine sulfoxide is reduced to methionine) by an excess of Me_2S in a sealed tube. This oxygen exchange reaction occurs under relatively mild conditions, without interference from or oxidation of other common amino acids (except for oxidation of cysteine, for which a correction can be made). The reaction has been investigated as the basis of a methionine assay by measurement of the evolved Me_2S in a gas chromatograph equipped with a highly sensitive sulfur-specific flame photometric detector. This approach has potential for measuring methionine and/or nutritionally available methionine by a rapid chemical method which does not require conventional protein hydrolysis.

Dimethyl sulfoxide (Me_2SO) was used (Searles and Hays, 1958) for selective oxidation of organic sulfides to sulfoxides without formation of sulfones, although relatively severe conditions were required (8–12 hr at 160–175°C). More recently Me_2SO was used (Spencer and Wold, 1969) for the purpose of oxidizing cysteine and cystine to cysteic acid during the hydrolysis of proteins by HCl. The sulfur amino acids, methionine and cystine, as well as the amino acid tyrosine were reported (Lipton and Bodwell, 1973) to be oxidized by the presence of traces of Me_2SO under these same severe conditions. During efforts to develop a chemical method for estimation of nutritionally available methionine (Lipton et al., 1974), the

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