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Formation of Aroma Components from Nonvolatile Precursors in Passion Fruit

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Thermal treatment at native pH value during simultaneous distillation–extraction of passion fruit pulp significantly increased the concentration of a series of monoterpene hydrocarbons, alcohols, and oxides. GLC–MS investigation of a $CHCl_3$ extract led to the identification of 3,7-dimethylocta-1,5-diene-3,7-diol, 3,7-dimethylocta-1,7-diene-3,6-diol, 3,7-dimethyloct-1-ene-3,7-diol, and 3,7-dimethyloctene-3,6,7-triol. In model experiments the role of these nonvolatile constituents as precursors of a spectrum of volatile terpenoids in passion fruit could be demonstrated. Isolation of a glycosidic fraction on a C_{18} reversed-phase adsorbent and following enzymatic and acid hydrolysis revealed that linalool, nerol, geraniol, and α-terpineol are not present in passion fruits in the free form but rather are present in the bound, glycosidic form. Thermal acid-catalyzed treatment liberates these monoterpene alcohols and leads to transformation into a complex spectrum of passion fruit aroma components.

The flavor composition of yellow (*Passiflora edulis* f. *flavicarpa*) and purple (*Passiflora edulis* Sims) passion fruits has been investigated intensively in the past few years. Murray et al. (1972), Parliment (1972), and Winter and Klöti (1972) gave a first insight into the complex mixture of aroma components of this tropical fruit. Degradation products of carotenoids (Whitfield et al., 1973, 1977; Näf et al., 1977; Demole et al., 1979; Winter et al., 1979b), sulfur-containing components (Winter et al., 1976), and unusual aliphatic esters (Winter et al., 1979a) were reported to play important roles in the unique and delicate flavor.

The techniques of aroma isolation applied in these investigations ranged from vacuum steam distillation and extraction with organic solvent (Parliment, 1972) to collection from headspace and adsorption on Tenax GC (Chen et al., 1982).

In the present study we wanted to demonstrate the effects of isolation procedures on the flavor composition of passion fruits. To elucidate these influences we did not limit our investigations to the spectrum of volatiles but extended them to the field of nonvolatile components. This combination revealed the relationship between degradation of nonvolatile (glycosidic) precursors and the liberation of volatile aroma components.

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EXPERIMENTAL SECTION

Materials. Yellow passion fruits (*P. edulis* f. *flavicarpa*) were obtained in full ripe state by air freight from Brazil and were stored at 4 °C until analyzed.

Isolation of Volatiles. A total of 600 g of passion fruit pulp was homogenized with 1000 mL of distilled water for 30 s. The homogenate was cleared by filtration through a muslin cloth in a Hafico tincture press at 400 atm. This clarified juice, possessing a pH value of 3.0, was divided into two equal portions. The pH of 0.5 was adjusted to 7.0 by adding an aqueous solution of sodium hydroxide (1 N). The volatiles of both portions were isolated by means of simultaneous distillation–extraction at atmospheric pressure in a modified Likens-Nickerson apparatus using pentane–ether (1:1) for 2 h (Schultz et al., 1977). The aroma extracts were dried over Na_2SO_4, concentrated to a volume of 0.3 mL by using a Vigreux column, and investigated by capillary GLC–MS.

Isolation of Nonvolatile Components. A total of 1.5 kg of passion fruit pulp was homogenized with 500 g of NaCl. The slurry was filtrated through a muslin cloth in a Hafico tincture press at 400 atm and the clarified juice was liquid–liquid extracted with $CHCl_3$ for 24 h. After evaporation of the solvent the residue was taken up in H_2O (5 mL) and washed with pentane (3 × 10 mL). The

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aqueous solution was extracted again with $CHCl_3$, and after being concentrated to 0.5 mL, this extract was used for GLC–MS investigations.

Isolation of Glycosidic Components. A total of 800 g of passion fruit pulp was homogenized with 2 L of distilled water. The homogenate was pressed through a muslin cloth and the filtrate was centrifuged at 15000*g* for 20 min. The clear juice was pumped through a column (250 × 16 mm, Vertex column, Knauer, West Germany), filled with LiChroprep RP-18 (particle size 40–63 μm, Merck 13900). The column was washed with 5 L of water, and then the retained components were eluted with 250 mL of methanol. After evaporation of the solvent, the residue was taken up in H_2O (5 mL). This aqueous solution was washed with ether to remove all possibly interfering monoterpenes and was used for the following hydrolysis experiments.

Enzymatic Hydrolysis of Glycosidic Components. Half of the glycosidic fraction was diluted with potassium phosphate buffer solution (pH 5.25). A mixture of glycosidases (Röhm & Haas, West Germany) was added at 38 °C. After 16 h the solution was extracted with ether and the extract was investigated by GLC–MS.

Acid Hydrolysis of Glycosidic Components. Half of the glycosidic fraction was diluted with distilled water (500 mL). By addition of HCl (0.1 N) the pH value was adjusted to 3.0, and the solution was subjected to simultaneous distillation–extraction using pentane–ether (1:1) for 2 h. Isolated volatiles were investigated by GLC–MS.

Reference Compounds. 3-Methyl-1,3-butanediol (I) was synthesized by reaction of 3-methyl-2-buten-1-ol with an equimolar quantity of 3-chloroperbenzoic acid in dry ether at 0 °C over 24 h. The formed epoxide was reduced with $LiAlH_4$ in refluxing THF over 24 h. The complex $LiAlH_4$ alcoholates were hydrolyzed with sulfuric acid (10%), and the diol was obtained by extraction of the solution with ether: Kovats index (CW 20M, column A) 1670; MS *m/e* (rel intensity) 43 (100), 59 (73), 41 (39), 39 (25), 58 (23), 56 (20), 71 (18), 55 (17), 53 (12), 89 (10).

The synthesis of the hydroxylated linalool derivatives II–V and the conversion of triol III to its acetonide were carried out as described by Matsuura and Butsugan (1968) and Williams et al. (1980a).

Other reference samples were purchased if available or were gifts from Dragoco (Holzminden), Haarman & Reimer (Holzminden), or Firmenich (Geneva).

Model Experiments. In each case ca. 1 mg of the synthesized compounds I–V and of linalool was dissolved in 800 mL of water, adjusted to pH 3.0. These solutions were subjected to simultaneous distillation–extraction using pentane–ether (1:1) for 2 h. The isolated volatiles were investigated by GLC–MS.

Capillary Gas Chromatography–Mass Spectrometry. Capillary GLC was carried out by using a 50-m glass capillary column (0.32-mm i.d.) coated with Carbowax 20M in a Carlo Erba Fractovap 2900 with linear temperature program from 70 to 180 °C, at 2 °C/min (column A). For capillary GLC–mass spectrometry a 50-m glass capillary column (0.32-mm i.d.) coated with UCON (column B) in a Carlo Erba 2101 was connected to a double-focusing mass spectrometer, CH5-DF (Varian-MAT, West Germany). Conditions were as follows: column temperature 70–180 °C, 2 °C/min; ionization voltage 70 eV; ion source temperature 200 °C (10% valley).

RESULTS AND DISCUSSION

During our investigations of passion fruit aroma we observed large differences in the composition of volatiles, when using different isolation techniques. Simultaneous distillation–extraction at atmospheric pressure in a modified Likens-Nickerson apparatus (Schultz et al., 1977) led to an aroma spectrum much more various than that obtained by liquid–liquid extraction. Passion fruits possess high contents of organic acids (Chan et al., 1972), and therefore, a sample used for distillation–extraction, prepared by diluting pulp with distilled water, showed a pH value of 3.0. Considering these acidic conditions chemical transformations in flavor and aroma composition caused by thermal treatment during the distillation seemed possible. In addition to the isolation of aroma components at the native pH value, we therefore carried out a distillation–extraction after adjusting the pH with sodium hydroxide solution to the neutral value of 7.0. The large differences in the composition of volatiles obtained under these two conditions are demonstrated in the gas chromatograms in Figure 1. The aroma pattern obtained at the neutral pH value of 7.0 was similar to that obtained by liquid–liquid extraction. In contrast to that, distillation–extraction at the native pH of 3.0 led to a much broader spectrum of volatiles. Besides some components, biosynthesized in the fruit and not significantly influenced by pH differences during the isolation, e.g., ethyl butanoate, hexyl hexanoate, or butanol and hexanol, which can be seen as "internal standards", the concentrations of a series of unsaturated alcohols and monoterpenoids are greatly increased (Table I).

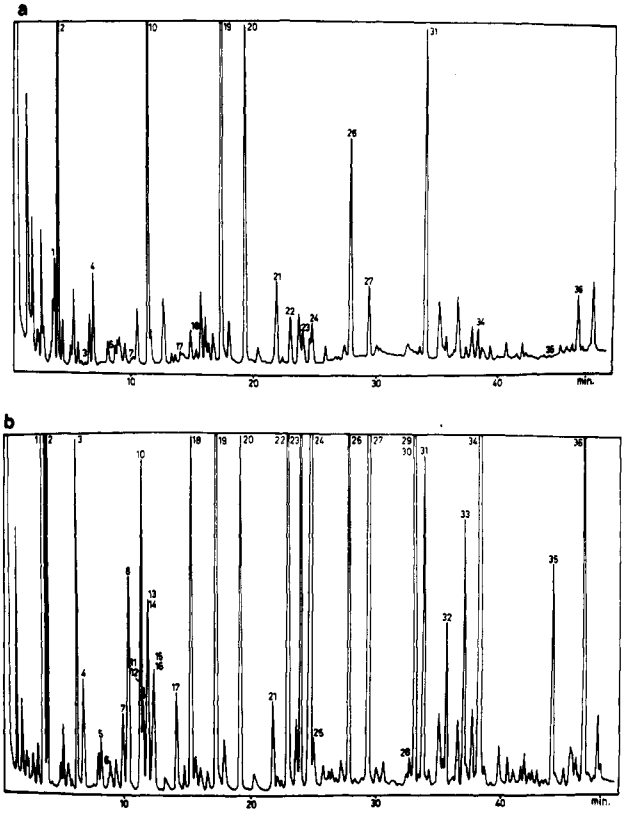

Figure 1. Capillary gas chromatograms (column A) of volatiles obtained from passion fruits by distillation–extraction at pH 7.0 (a) and pH 3.0 (b). Peak numbers correspond to those in Table I.

The three unsaturated C_5-alcohols 2-methyl-3-buten-2-ol, 3-methyl-3-buten-1-ol, and 3-methyl-2-buten-1-ol were determined only as minor constituents in the pulp, adjusted to the neutral point, but they became main components (up to 5.9 ppm) when being isolated under acidic conditions. We supposed a common nonvolatile precursor for these structurally related alcohols. Synthesized 3-methyl-1,3-butanediol (I) was a compound that in model

Table I. Volatiles Obtained from Passion Fruit Pulp by Distillation-Extraction at Different pH Values

peak no.	components	IK,[c] CW 20M	μg/kg pH 7.0	μg/kg pH 3.0	identification
1	2-methyl-3-buten-2-ol	1016	100	5900	a
12	3-methyl-3-buten-1-ol	1232	10	70	a
18	3-methyl-2-buten-1-ol	1302	10	550	a
7	limonene	1202	5	60	a
5	myrcene	1159	5	90	a
6	α-terpinene	1181	d	10	a, b
11	(Z)-ocimene	1232	d	30	a
16	(E)-ocimene	1248	d	140	a
15	γ-terpinene	1246	d	10	a, b
17	terpinolene	1282	5	120	a
3	2,6,6-trimethyl-2-vinyltetrahydropyran	1109	10	520	a, b
8	(E)-anhydrolinalool oxide A	1209	d	250	a
13	(Z)-anhydrolinalool oxide B	1237	d	200	a
14	2,2-dimethyl-5-(1-methylpropenyl)tetrahydrofuran	1237	d	50	a, b
22	(E)-linalool oxide A	1424	60	1500	a
24	(Z)-linalool oxide B	1452	30	1000	a
25	nerol oxide	1458	d	50	a, b
27	linalool	1526	90	4800	a
28	4-terpineol	1581	d	25	a
29	hotrienol	1586	d	500	a, b
30	myrcenol	1586	d	200	a, b
32	(Z)-ocimenol	1627	d	200	a, b
33	(E)-ocimenol	1650	d	400	a, b
34	α-terpineol	1668	40	2000	a
35	nerol	1773	5	300	a, b
36	geraniol	1818	70	1000	a
23	furfuraldehyde	1441	30	700	a
26	benzaldehyde	1500	300	900	a
2	ethyl butanoate	1028	700	650	a
10	ethyl hexanoate	1227	300	250	a
21	hexyl butanoate	1407	100	100	a
31	hexyl hexanoate	1598	350	300	a
4	butanol	1123	100	100	a
19	hexanol	1334	700	700	a
20	(Z)-3-hexenol	1365	550	550	a

[a] Comparison of retention time and mass spectrum with that of authentic sample. [b] Identified for the first time as passion fruit constituents. [c] Kovats-GLC index on column A. [d] Concentration less than 5 μg/kg.

experiments showed the expected behavior: no recovery by distillation–extraction from neutral aqueous solutions and acid-catalyzed decomposition into the three unsaturated alcohols by distillation–extraction at pH 3.0 in a ratio comparable to that determined in the passion fruit system. We could not detect 3-methyl-1,3-butanediol in extracts obtained from passion fruits by liquid–liquid extraction with ether or $CHCl_3$, but according to its behavior we tentatively want to postulate this compound as a natural precursor in the fruit. Probably it might be present in a bound, glycosidic form and therefore might not be accessible to simple extraction with organic solvents.

Another decisive difference between the two distillations–extractions are the significantly different patterns of terpenoid components. After thermal treatment under acidic conditions the concentrations of a series of monoterpene hydrocarbons, alcohols, and oxides, of which some possess very strong sensory properties, are changed from trace to ppm level. Linalool (27) could be detected only as a minor component in the neutrally adjusted pulp, but its concentration increased more than 50-fold during isolation at the low pH value. Similar behavior could be observed for other monoterpene alcohols like α-terpineol (34), nerol (35), geraniol (36), terpinen-4-ol (28), hotrienol (29), and myrcenol (30) and the two isomeric ocimenols (32, 33). The concentrations of the terpenoid oxides 2,6,6-trimethyl-2-vinyltetrahydropyran (3), 2,2-dimethyl-5-(1-methylpropenyl)tetrahydrofuran (14), and nerol oxide (25), the isomeric anhydrolinalool oxides A and B (8, 13), and the two linalool oxides (22, 24) were enhanced considerably by acid-catalyzed thermal treatment. Comparable pH dependency of the detected amounts was also observed for the hydrocarbons limonene (7), myrcene (5), α-terpinene (6), (Z)-ocimene (11), (E)-ocimene (16), γ-terpinene (15), and terpinolene (17). This spectrum of terpenoid components obtained by thermal treatment during the distillation–extraction of passion fruit pulp at the native pH value of 3.0 is very similar to that obtained by Williams et al. (1980b), when investigating the headspace composition of heated juice of muscat grapes.

To find precursor components of this series of terpenoids we carried out a liquid–liquid extraction of passion fruit pulp using $CHCl_3$ as the solvent. In the water-soluble fraction of this extract we could identify 3,7-dimethylocta-1,5-diene-3,7-diol (II), 3,7-dimethylocta-1,7-diene-3,6-diol (III), and 3,7-dimethyloct-1-ene-3,7-diol (V) directly and 3,7-dimethyl-1-ene-3,6,7-triol (IV) after the conversion to its acetonide for the first time as passion fruit constituents. Retention times and mass spectra of the identified components were identical with those of synthesized samples. Terpenediols II and III were reported by Takaoka and Hiroi (1976), Rapp and Knipser (1979), and Rapp et al. (1980) as constituents of *Cinnamomum camphora* and *Vitis vinifera* grapes, respectively. Additionally to these two diols compounds IV and V were characterized in muscat grapes by Williams et al. (1980a).

To investigate their role as precursors we carried out distillations–extractions at pH 3.0 with synthesized samples of these polyols. This thermal acid-catalyzed treatment, analogous to our isolation procedure, led to the complete spectrum of terpenoids as liberated in the fruit. Figure 2 presents the percentage distribution of volatiles formed from the nonvolatile precursors in these model experiments. The determined ratios correspond to those

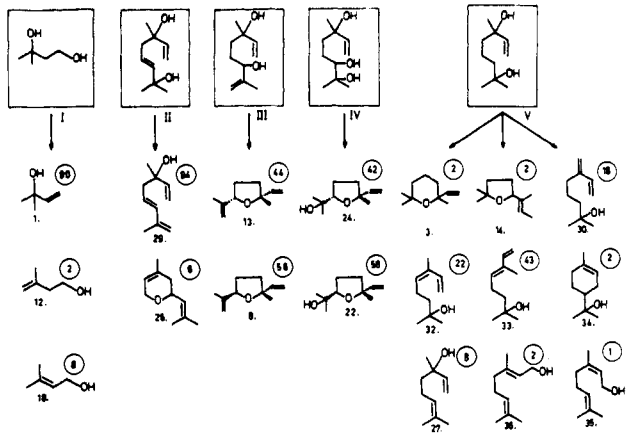

Figure 2. Novolatile precursors and their degradation products in passion fruit. The encircled numbers represent the percentage distribution in model experiments with synthesized samples.

in the natural system, and the spectrum of components is comparable to the headspace compositions, obtained by Williams et al. (1980b), when heating model solutions of these polyols for 15 min at 70 °C at pH 3.2.

Williams et al. (1982b) demonstrated that some of the nonvolatile precursors of monoterpenes in muscat grapes are not present in the free form but are present in the bound, glycosidic form. Therefore we applied a method to isolate glycosidic derivatives of monoterpenes by selective retention on a C_{18}-bonded reversed-phase adsorbent, developed and successfully used for grape juices and wines by Williams et al. (1982a), to passion fruit pulp. This method yielded a fraction, free of volatile monoterpenes, in which under the hydrolytic influence of a commercial mixture of glycosidase linalool, α-terpineol, geraniol, and nerol were liberated in a ratio of 91:4:5:1.

When the glycosidic fraction was subjected to distillation–extraction at pH 3.0, a complex mixture of terpenoid hydrocarbons, alcohols, and oxides could be isolated. The percentage distribution of components in this mixture, containing linalool (27), nerol (35), geraniol (36), and α-terpineol (34) as main constituents, is demonstrated in Figure 3. It qualitatively corresponds to aroma patterns, which we obtained in model experiments with aqueous solutions of linalool at pH 3.0, and it is comparable to the spectrum of components isolated by Williams et al. (1982c), when heating synthetic β-D-glucopyranosides of linalool, nerol, geraniol, and α-terpineol at pH 3.2. The structures of the sugar moieties in the isolated monoterpene alcohol glycosides were not determined within this study, but further investigations in this field are in progress. The fact that monoterpene alcohols are present in passion fruits not in the free but in glycosidic form may explain the decreasing concentrations of linalool and α-terpineol in the fruit during the development from the immature green to full ripe state as observed by Casimir et al. (1977–1978). Possibly the transformation of biosynthesized linalool and α-terpineol from the free into the glycosidic form takes place within this period.

CONCLUSIONS

The investigations in this study indicate that in passion fruits in addition to the spectrum of biosynthesized volatiles there exists a pool of nonvolatile polar precursor compounds, especially glycosides of monoterpene alcohols and hydroxylated linalool derivatives, which can be transformed into important aroma components by chemical or enzymatic reactions. The degree of liberation and degradation decisively determines the spectrum of isolated

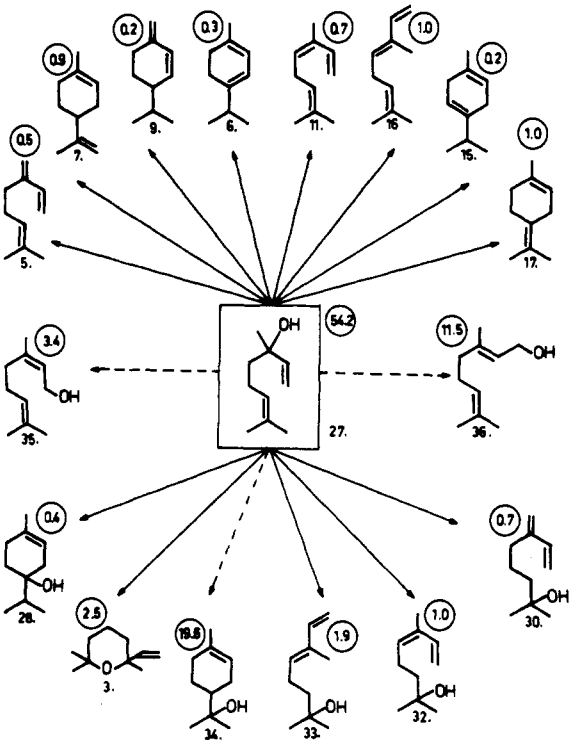

Figure 3. Percentage distribution of aroma components obtained from monoterpene glycosides in passion fruit.

volatiles. This may explain the difficulties of comparing the results of quantitative determinations of passion fruit aroma components in extracts obtained by different isolation techniques.

Transformations as described in this study could also take place during technological processing of passion fruits. The ratio of nonvolatile precursors to volatile aroma components might be influenced by thermal treatments (concentration, pasteurization, canning) at native pH value and determine the flavor spectrum in the final passion fruit product.

Registry No. I, 2568-33-4; II, 13741-21-4; III, 51276-33-6; IV, 73815-21-1; V, 29210-77-3; 3-chloroperbenzoic acid, 937-14-4; 2-methyl-3-buten-2-ol, 115-18-4; 3-methyl-3-buten-1-ol, 763-32-6; 3-methyl-2-buten-1-ol, 556-82-1; limonene, 138-86-3; myrcene, 123-35-3; α-terpinene, 99-86-5; (*Z*)-ocimene, 3338-55-4; (*E*)-ocimene, 3779-61-1; γ-terpinene, 99-85-4; terpinolene, 586-62-9; 2,6,6-trimethyl-2-vinyltetrahydropyran, 7392-19-0; (*E*)-anhydrolinalool oxide A, 54750-70-8; (*Z*)-anhydrolinalool oxide B, 54750-69-5; 2,2-dimethyl-5-(1-methylpropenyl)tetrahydrofuran, 7416-35-5; (*E*)-linalool oxide A, 34995-77-2; (*Z*)-linalool oxide B, 5989-33-3; nerol oxide, 1786-08-9; linalool, 78-70-6; 4-terpineol, 562-74-3; hotrienol, 20053-88-7; myrcenol, 543-39-5; (*Z*)-ocimenol, 7643-59-6; (*E*)-ocimenol, 7643-60-9; α-terpineol, 98-55-5; nerol, 106-25-2; geraniol, 106-24-1; furfuraldehyde, 98-01-1; benzaldehyde, 100-52-7; ethyl butanoate, 105-54-4; ethyl hexanoate, 123-66-0; hexyl butanoate, 2639-63-6; hexyl hexanoate, 6378-65-0; butanol, 71-36-3; hexanol, 111-27-3; (*Z*)-3-hexenol, 928-96-1.

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Metabolism of Limonoids by *Arthrobacter globiformis* II: Basis for a Practical Means of Reducing the Limonin Content of Orange Juice by Immobilized Cells

Shin Hasegawa,* Vickie A. Pelton, and Raymond D. Bennett

Arthrobacter globiformis II, a newly isolated bacterium from soil, metabolized bitter limonin of citrus juice to a nonbitter metabolite, limonol, when the juice was treated on a column packed with immobilized cells. On the basis of this information, a biological process that uses immobilized cells of this bacterium in acrylamide gel was developed for reduction of the limonin bitterness of citrus juice sera. The activity and operational stability of the process were demonstrated.

Bitterness due to limonin in certain citrus juices is one of the major problems of the citrus industry worldwide and has significant economic impact.

Substantial progress has been made in studies of the biochemistry of the bacterial degradation of limonoids during the past 10 years. The occurrence of two metabolic pathways of limonoids in bacteria has been firmly established: one via deoxylimonoids such as deoxylimonin (Hasegawa et al., 1972a) and the other via 17-dehydrolimonoids such as 17-dehydrolimonate A-ring lactone (Hasegawa et al., 1972b). Enzymes involved in the pathways have been isolated and characterized (Hasegawa et al., 1972b, 1974b,c; Hasegawa, 1976).

A continuing study of limonoid-metabolizing microorganisms, metabolic pathways of limonoids, and enzymes involved in the pathways resulted in the finding of a new metabolic pathway of limonoids in bacteria. This paper shows that immobilized cells of a newly isolated bacterium, *Arthrobacter globiformis* II, metabolized bitter limonin of citrus juice to nonbitter limonol.

Recently, biological processes using immobilized bacterial cells have been developed for reduction of the limoin bitterness of citrus juices. The process using *Acinetobacter* cells entrapped in a dialysis sac reduced the limonin content of navel orange juice by converting it to nonbitter deoxylimonin and deoxylimonic acid (Vaks and Lifshitz, 1981). The process using immobilized cells of *Arthrobacter globiformis* in acrylamide gel converted limonin to nonbitter 17-dehydrolimonoate A-ring lactone (Hasegawa et al., 1982). Both processes require multienzyme catalytic systems. In the newly found pathway, the conversion of limonin to limonol is apparently catalyzed by the action of a single enzyme. This paper also shows the results of studies on the reduction of the limonin content of navel orange juice serum using *A. globiformis* II cells immobilized in acrylamide gel.

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MATERIALS AND METHODS

Limonol was synthesized from limonin by the procedure of Melera et al. (1957). Navel oranges were purchased from a local market. The juices were extracted with a Sunkist juicer, and the sera were obtained from the juices by centrifugation at 2500*g* for 10 min. Limonin was added to the sera to obtain convenient working concentrations.

Growth of Cells. The substrate, 500 mL of a mineral salt (Hasegawa et al., 1972b), 0.2% nutrient broth (Difco Laboratory, Detroit, MI), and 0.2% limonate (both A and D rings of limonin open), was placed in a 2.8-L Fernbach flask and incubated with 20 mL of 72-h culture of *Arthrobacter globiformis* II. Incubation was at 25 °C on a shaker. After 48-h incubation, cells were harvested by centrifugation, washed with 0.5 M potassium phosphate buffer at pH 7.0, and frozen until used for immobilization.

Immobilization of Cells in Acrylamide Gel. Cells were immobilized in acrylamide gel by the method of Tosa et al. (1974). The resulting gel was then gently ground with a Polytron and packed in a column.

Metabolism of Limonin with Immobilized Cells. Since limonin is almost insoluble in aqueous solution, 20% navel orange juice serum was used as a carrier solution. A total of 45 mg of limonin was dissolved in 1000 mL of