Occurrence of an Inhibitor of Lactic Acid Bacteria in Green Olives[1]

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Green olives were found to contain an inhibitor(s) of several species of lactic acid bacteria usually associated with the Spanish-type brined olive fermentation. The inhibitor was demonstrated by the presence of inhibition zones surrounding tissue which had been cut from frozen olives and implanted in a seeded nutritive agar medium. Relative potencies of aqueous extracts of frozen olives were determined by a paper disc assay method. The Mission variety of olive contained the most inhibitor, and the Manzanillo and Ascolano, about 50 and 40% as much as the Mission variety, respectively. Sevillano and Barouni varieties contained comparatively little inhibitor. Effects of the inhibitor on growth rates of lactic acid bacteria were determined by adding various amounts of a concentrated aqueous extract of olives to a nutritive broth medium contained in screw-capped tubes. Of the four species of lactic acid bacteria tested, *Leuconostoc mesenteroides* was the most sensitive, and *Lactobacillus plantarum* was the least sensitive; *Pediococcus cerevisiae* and *Lactobacillus brevis* were intermediate in sensitivity. Extracts possessed a bactericidal property, as evidenced by their effect on *L. mesenteroides*. Sodium chloride, especially at concentrations of about 5% and higher, greatly increased the effectiveness of the inhibitor. The inhibitor was ethyl alcohol-soluble and was stable when heated at 100 C in aqueous solution. Potencies of extracts were reduced greatly by adjustment to *p*H 10, but no appreciable effect was noted by adjustment to *p*H 0.8.

The fermentation of Spanish-type green olives characteristically proceeds at a slower rate than does the fermentation of either cucumbers or cabbage. Occasionally, vats or casks of olives fail to attain a normal fermentation, as evidenced by the failure of lactic acid bacteria to proliferate, utilize fermentable sugars, and produce preservative levels of lactic acid. Consequently, microbial spoilage due to yeasts, clostridia, and other microorganisms may ensue (13, 14). Attempts to establish the cause of anomalous olive fermentations are complicated by the treatments that the olives receive prior to brining for fermentation. First, they are treated with alkali to remove bitterness, and then are washed to remove most of the alkali. Thus, the natural bacterial population is greatly lowered as is the concentration of growth factors and fermentable carbohydrates. Several factors, including those above, have been suggested as being responsible for variability in the fermentation of olives. The possibility that olives may contain antimicrobial agents has been considered (13), but little scientific evidence has been reported regarding the effect that such compounds may have on the lactic acid fermentation of brined olives.

This laboratory has been successful with the fermentation of cucumbers and other vegetables by pure cultures of lactic acid bacteria (4). Recently, efforts have been directed toward applying the technique to the fermentation of olives (3). Obvious differences in the fermentation rates of cucumbers and olives (3) prompted us to explore possible explanations for this observation. The present study was undertaken to determine whether olives contain water-soluble substances which are inhibitory to lactic acid bacteria.

Materials and Methods

Stock cultures of bacteria used in this study were carried as stabs in screw-capped tubes containing 10 ml of Orange Serum Agar (Difco). These organisms included *Lactobacillus plantarum* (culture no. FBB-12, -15, -68, and L-442), *L. brevis* (culture no. FBB-50 and -70), *Pediococcus cerevisiae* (culture no. FBB-39 and -61), *Leuconostoc mesenteroides* (culture no. FBB-42,

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-43, -71, and -73), and an isolate from a commercial olive brine designated as WSO (tentatively, *L. plantarum*). Further identification of these cultures was provided earlier (3, 4). The cultures were activated prior to study by inoculating 10 ml of cucumber juice broth (CJB) in screw-capped tubes and transferring daily in this medium for 4 days or more, allowing 16 to 18 hr for growth before refrigerating and holding until the appropriate time for transferring. All cultures were incubated at 30 C.

Cucumber juice media were prepared from frozen Model variety cucumbers [1 to 1¼ inches (2.5 to 3.2 cm) in diameter]. The cucumbers were thawed and then macerated in a blender; the juice was squeezed through cheese cloth. The juice was heated to boiling, cooled, and centrifuged; the supernatant fluid was filtered through filter paper to remove any suspended particles. The broth medium (CJB) was prepared by dispensing 10 ml of the extract into 16-mm screw-capped tubes and autoclaving for 10 min at 121 C. This clear, light amber-colored medium was excellent for growth of the cultures under study. Cucumber juice agar (CJA) was prepared by diluting the filtered extract with an equal volume of distilled water and adding 1.5% agar and 0.0075% bromocresol green. After heating to melt the agar, the medium was dispensed into bottles and autoclaved at 121 C for 15 min.

Growth of the bacteria was followed by incubating cultures in 10 ml of CJB and reading the optical density at 650 mμ with a Bausch & Lomb Spectronic-20 colorimeter at specified time intervals. Appropriate blanks were used to correct for color differences in the broth, a particularly variable factor when olive extracts were added.

The olives used in this work were shipped, air freight, by the Lindsay Ripe Olive Co., Lindsay, Calif. Upon receipt, the olives were sorted to remove damaged fruit and foreign material, washed, and stored at −15 C in plastic freezer bags. The varieties used and the condition of the samples are given in Table 1.

In preparing extracts, olives were removed from the freezer, allowed to thaw partially at room temperature for a few minutes, and then pitted with a hand-operated pitting machine. The pitted olives were added to an equal weight of boiling water contained in a foil-covered beaker, and the mixture was heated for 30 min while being stirred. After being cooled to room temperature, the mixture was blended; the slurry was adjusted to a standard volume and then centrifuged for 20 min at 10,000 × *g*. The aqueous phase was suction-filtered through Whatman no. 7 paper. After removing 10% of this solution, designated herein as the aqueous extract, the remaining portion was concentrated in vacuo at 70 C to one-fifth volume. To this concentrate was added five volumes of 95% ethyl alchol, and the mixture was held overnight at 5 C. The liquid was decanted from the gelatinous precipitate and was concentrated to about one-twentieth volume. Ten volumes of 95% ethyl alcohol was added to this second concentrate, and the mixture was again held overnight at 5 C. The liquid was decanted from a dark-brown sticky residue.

TABLE 1. *Characteristics of the experimental olives*

Variety	Condition	Approximate size (count/kg)
Ascolano	All green, a few bruises	101
Barouni	All green, no bruises	132
Manzanillo	All green, no bruises	172
Mission	Mostly light purple, no bruises	295
Sevillano	All green, a few bruises	103

This liquid was concentrated to one-fifth volume and is designated herein as the ethyl alcohol-soluble fraction.

Bacterial inhibition zones were determined for olive tissue and extracts. These assays were made in disposable petri dishes (100 by 15 mm; Falcon Plastic, Los Angeles, Calif.) which contained 20 ml of CJA seeded with one drop of a 16-hr culture of the test organism. Tissue samples were placed in the medium before it solidified and were positioned so as to expose both the pit and skin sides laterally in the dish. Samples of extracts were pipetted onto 13-mm diameter paper discs (Schleicher & Schuell no. 740-E) and were allowed to dry partially before the discs were placed on the surface of the seeded agar. Assay plates were placed in incubation at 30 C immediately after addition of the disc samples.

Total phenolic content of extracts was determined by the Folin-Denis method (12). Assays were expressed on the basis of catechol as the colorimetric standard. Absorbance was determined at 725 mμ with a Bausch & Lomb Spectronic-20 colorimeter.

RESULTS

Preliminary studies showed that aqueous extracts of frozen Manzanillo olives were not fermented by the lactic acid bacteria customarily used by this laboratory for the pure culture fermentation of cucumbers. Also, the fermentation rate of cucumber extract was lowered by the addition of olive extracts. Zones of inhibition produced by the disc method for extracts and also by implanting tissue sections in seeded CJA demonstrated conclusively the presence of a bacterial inhibitor in olives. It was found that tissue sections from olives that had been frozen exhibited a much larger zone of inhibition than did tissue from the unfrozen fruit. Also, aqueous extracts of frozen olives appeared to be much more inhibitory. Thus, all extracts reported herein were made from olives that were frozen and held at −15 C.

Inhibitor contents of five varieties of olives. L. mesenteroides FBB-42 was one of the most sensitive cultures to olive extracts and was chosen as the test organism for determining relative potencies of preparations. Olive tissue implants

in seeded CJA plates resulted in measurable inhibition zones, although the zone boundaries frequently were unsymmetrical. Inhibition zones were measured at the widest point on the pit side of the tissue, since these zones were usually larger than on the skin side. Average zone sizes of triplicate assays were 11.0, 4.7, 5.3, 10.7, and 10.7 mm for the Ascolano, Barouni, Sevillano, Manzanillo, and Mission varieties, respectively. This same relationship was found when *L. plantarum* L-442, *P. cerevisiae* FBB-39, and *L. brevis* FBB-50 were the test organisms, except that the Barouni and Sevillano varieties produced no zones of inhibition when any of the last three bacteria was used.

Aqueous extracts of the five olive varieties were prepared to relate the inhibitor contents on a quantitative basis. The paper disc assay method was used to determine the relative potencies of these extracts. Figure 1 illustrates a plate with typical inhibition zones resulting from four different amounts of inhibitor. The ethyl alcohol-soluble fraction from an extract of Manzanillo olives served as the inhibitor standard with which a standard curve was prepared (Fig. 2). This solution contained 20 mg of total phenols per ml on the basis of catechol as the colimetric standard. In preparing the standard curve, one unit of inhibitor was defined as the zone diameter (millimeters) produced with a disc containing 0.05 ml of the above solution. Appropriate dilutions were made so that 0.05 ml/disc provided the units of inhibitor indicated. The test medium consisted of 20 ml of CJA seeded with one drop of a 16-hr culture of *L. mesenteroides* FBB-42. Solutions to be assayed were diluted to allow application of 0.1 to 0.8 unit of inhibitor per disc, as the semilogarithmic plot was linear in this range (Fig. 2). All assays, including the standard curve, were performed in triplicate.

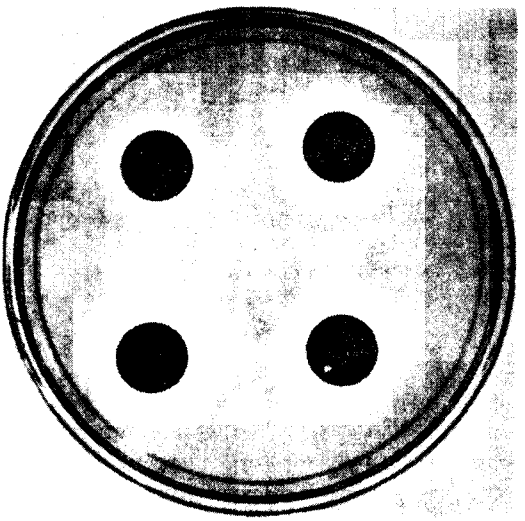

FIG. 1. *Inhibition zones of Leuconostoc mesenteroides FBB-42 in CJA plates produced by an ethyl alcohol extract of Manzanillo olives. The zones demonstrate inhibitory responses over a fourfold range in amount of inhibitor applied to the discs.*

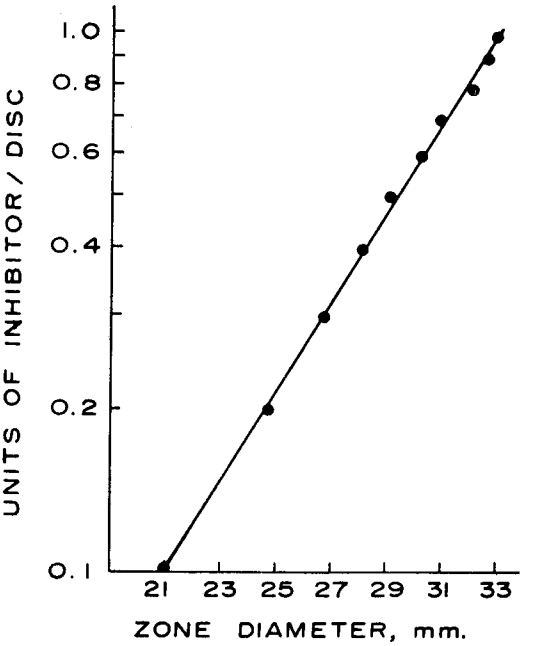

FIG. 2. *Standard curve of inhibition zones of Leuconostoc mesenteroides FBB-42 obtained by disc assay of a Manzanillo olive ethyl alcohol extract. One unit of inhibition was defined as the zone, in millimeters, resulting from the application of 0.05 ml per disc of this standard solution, which contained 20 mg total phenols per ml (catechol basis).*

Aqueous extracts of the Mission variety of olives contained the largest amount of inhibitor of the five varieties analyzed (Table 2). Manzanillo extracts contained about 54% and Ascolano 41% of the potency of the extract from the Mission olive variety. The Barouni and Sevillano extracts gave no inhibition zones at the levels tested, but they may contain small amounts of inhibitor since tissue sections gave small inhibition zones when *L. mesenteroides*, the most sensitive organism, was used to inoculate the assay medium. The inhibitory principle was quite soluble in ethyl alcohol, as over 80% of the potency was recovered in ethyl alcohol extracts of the concentrated aqueous solutions. The precipitate formed upon addition of ethyl alcohol to the aqueous concentrates contained no detectable inhibitor.

TABLE 2. *Inhibitor and total phenolic content of extracts from five varieties of olives*[a]

Olive variety	Inhibitor content per 100 g of olives	Inhibitor recovery in ethyl alcohol	Total phenols per 100 g of olives	Total phenols recovered in ethyl alcohol
	units	%	*mg*	%
Ascolano	196	83	380	74
Barouni	—	—	218	64
Sevillano	—	—	184	65
Manzanillo	257	98	389	78
Mission	478	84	510	77

[a] Inhibitor and total phenolic contents represent the quantities present in aqueous extracts, prepared as described in Materials and Methods, and are expressed on the basis of 100 g of pitted green olives.

Relative sensitivity of selected cultures of lactic acid bacteria. Thirteen cultures of lactic acid bacteria were tested for their sensitivity to the inhibitory principle in extracts of Manzanillo olives. An ethyl alcohol-soluble fraction of desired potency was diluted with 5 volumes of distilled water and then concentrated in a flash evaporator to remove the ethyl alcohol. This solution was centrifuged to remove a dark gummy material that usually formed. The solution was then filter-sterilized by filtration through a 0.22-μ filter (Millipore Corp., Bedford, Mass.). Appropriate dilutions of the solution were made and then added aseptically to 16-mm screw-capped culture tubes containing 10 ml of CJB. Table 3 summarizes the effects of inhibitor on growth of the test cultures. *L. mesenteroides* was the most sensitive, and *L. plantarum* the least sensitive; *P. cerevisiae* and *L. brevis* were intermediate in sensitivity among the four species of bacteria tested. There were variations in sensitivity among different cultures of the same species. A study of the growth curves of the cultures indicated that the inhibitor had a greater effect on delaying the onset of growth than on retarding the rate of growth subsequent to this lag period. This effect is typified in Fig. 3, with *L. plantarum* FBB-15 as the fermenting organism.

Synergistic effect of NaCl and inhibitor. Since the NaCl concentration in naturally fermented green olives is quite high (13), it seemed desirable to test its effect in combination with various levels of the inhibitory material. *L. plantarum* FBB-15 was selected to demonstrate the effect of NaCl because of its comparatively high resistance to the inhibitor. This organism grew at concentrations of NaCl as high as 9% in the absence of inhibitor, although the growth rate was lowered and the lag period was extended at higher concentrations (Fig. 4A). Results given earlier affirm the relatively high resistance of *L. plantarum* FBB-15 to the inhibitor (Table 3 and Fig. 3). In combination, however, the effects of NaCl and inhibitor on growth were synergistic (Table 4).

TABLE 3. *Effect of inhibitor level on growth of cultures of lactic acid bacteria*[a]

Culture	Units of inhibitor/ml				
	0	0.5	1.0	1.5	2.5
Lactobacillus plantarum					
FBB-12	+	+	+	−	−
FBB-15	+	+	+	+	+
FBB-68	+	+	+	−	−
L-442	+	+	+	+	−
WSO	+	+	+	+	−
Pediococcus cerevisiae					
FBB-39	+	+	+	−	−
FBB-61	+	+	+	−	−
L. brevis					
FBB-50	+	+	+	+	−
FBB-70	+	+	+	−	−
Leuconostoc mesenteroides					
FBB-42	+	+	−	−	−
FBB-43	+	+	−	−	−
FBB-71	+	+	−	−	−
FBB-73	+	+	−	−	−

[a] Visual turbidity of inoculated CJB tubes containing the inhibitor was used as an indication of growth. Growth within 5 days (+), no growth (−).

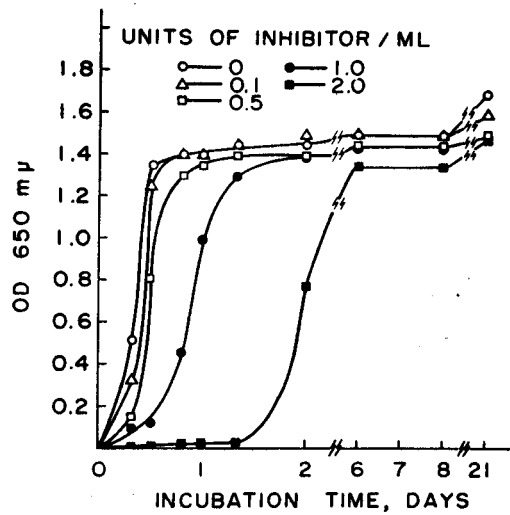

FIG. 3. *Growth curves of Lactobacillus plantarum FBB-15 in CJB containing various concentrations of inhibitor. The broth tubes, 16 × 150 mm screw-capped tubes containing 10 ml of medium, were periodically shaken and the optical density was determined.*

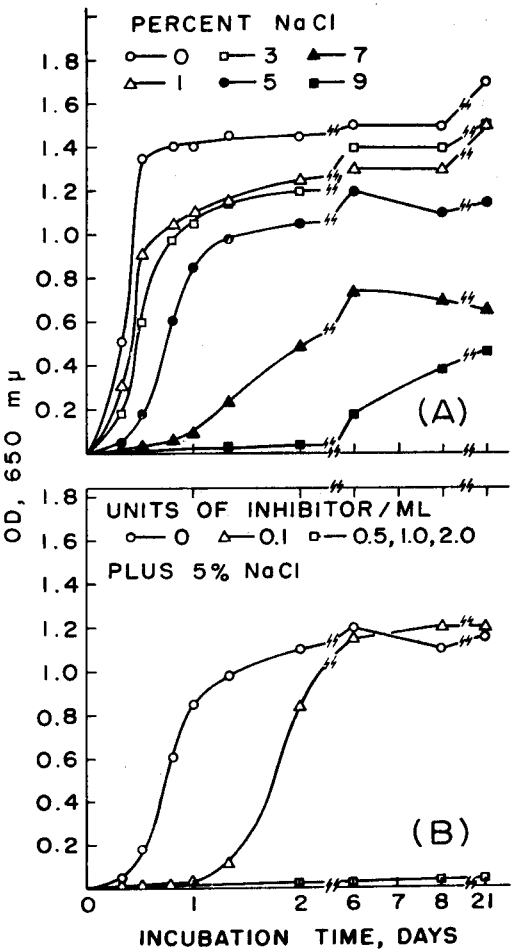

FIG. 4. *Growth curves of Lactobacillus plantarum FBB-15 in CJB containing NaCl with and without inhibitor. (A) Various concentrations of NaCl in the absence of inhibitor; (B) 5% NaCl in the presence of various concentrations of inhibitor. The broth tubes, 16 × 150 mm screw-capped tubes containing 10 ml of medium, were periodically shaken and the optical density was determined.*

L. plantarum FBB-15 failed to grow with 5% NaCl when as little as 0.5 unit of inhibitor per ml was present. The growth curves shown in Fig. 4A represent typical effects of NaCl on the growth of *L. plantarum*, and the effect of various levels of inhibitor with 5% NaCl is shown in Fig. 4B. Again, the lag in onset of growth seemed to be influenced more by the inhibitor than did the growth rate when growth occurred (Fig. 4B).

Bactericidal effects. It was of interest to learn whether the inhibitor possessed bactericidal properties. *L. mesenteroides* FBB-42 was used as a test organism to answer this question. The experiment was performed by incubating 6×10^7 cells/ml at room temperature in a screw-capped tube containing 10 ml of CJB and 0.5 unit of inhibitor per ml. The cells were added to the solution of CJB and inhibitor, and the tube was then shaken. Samples (1 ml) were removed at specified times and pipetted into dilution bottles containing 99 ml of sterile 0.85% NaCl. Further dilutions were made and plated in CJA. There was

TABLE 4. *Effect of levels of inhibitor and salt on growth of Lactobacillus plantarum FBB-15*[a]

NaCl	Units of inhibitor/ml				
	0	0.1	0.5	1.0	2.0
%					
0	+	+	+	+	+
1	+	+	+	+	+
3	+	+	+	+	+
5	+	+	−	−	−
7	+	+	−	−	−
9	+	−	−	−	−

[a] Visual turbidity of inoculated CJB tubes containing the inhibitor was used as an indication of growth. Growth within 5 days (+), no growth (−).

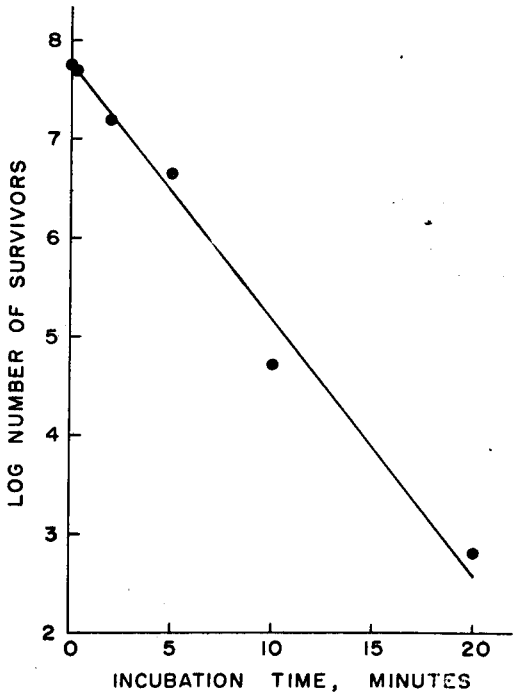

FIG. 5. *Survivor curve of Leuconostoc mesenteroides FBB-42 in CJB containing 0.5 unit of inhibitor/ml.*

approximately a 99.999% reduction in viable cells within 20 min, as illustrated in the survivor curve (Fig. 5). At an inhibitor concentration of 5 units/ml, no viable cells were detected after 10 min of incubation, whereas at 0.1 unit/ml no apparent reduction in viable cells resulted after cells were in contact with the inhibitor for 1 hr.

Effect of pH and temperature on stability of the inhibitor. The alkali treatment and subsequent washings that Spanish-type green olives undergo could have a significant influence on the stability and content of inhibitory compounds which were originally present in the olives. The following study was designed to establish the effect of *p*H on the stability of the inhibitor. Six samples of 2.5 ml, each containing about 20 units of inhibitor/ml, were pipetted into tubes (18 × 80 mm). The samples were divided into two sets of three each and were purged with either air or nitrogen for 1 min before the *p*H was adjusted. The samples were then adjusted with 6 N NaOH or 6 N HCl to the *p*H indicated in Table 5. The gas was allowed to bubble through the solution for 10 min before the *p*H was readjusted to 4.7, which was the same as that of the control. All solutions were then adjusted to the same NaCl concentration (5%), diluted to 10-ml volume, and filter-sterilized through a 0.22-μ filter (Millipore Corp.). The potencies of these solutions were assayed by adding portions to CJB tubes, inoculating with *L. mesenteroides* FBB-42, and observing growth (Table 5). Both assay methods indicated about a fourfold reduction in potency due to the alkali treatment. The basic treatment was effective under an anaerobic as well as an aerobic atmosphere, and in both instances the bitterness was eliminated. Low *p*H had no observable effect on the potency of the inhibitor, and the solution remained bitter.

TABLE 5. *Effect of pH on the stability of the inhibitor*

Purging gas	*p*H adjustment[a]	Growth within 5 days with indicated units of inhibitor/ml[b]				Recovery of inhibitor by disc assay[c]
		0.8	0.4	0.2	0.1	
						%
Air	0.8	−	−	−	+	85
	10.8	−	+	+	+	15
	4.7[d]	−	−	−	+	98
Nitrogen	0.8	−	−	+	+	83
	10.0	−	+	+	+	25
	4.7[d]	−	−	−	+	100

[a] The *p*H was adjusted with 6 N NaOH or HCl to that indicated, held for 10 min while purging with air or nitrogen, and then readjusted to that of the control (*p*H 4.7).

[b] The inhibitor solutions were filter-sterilized after *p*H adjustments, and samples were added to 10 ml of CJB in screw-capped tubes. The units of inhibitor per milliliter are expressed on the basis of activity prior to adjustments.

[c] Recovery was based on activity relative to the nitrogen control.

[d] Control.

The inhibitor was stable at elevated temperatures. No appreciable decrease in potency was observed by the disc assay method when aqueous solutions of the inhibitor were heated at 100 C for 30 min or after autoclaving at 121 C for 5 min.

DISCUSSION

The occurrence of antimicrobial agents in plants has been widely reported and has been the object of surveys (2). Theories have been proposed which attribute various defense mechanisms of plants to such substances (5, 6). Food products are derived from some of these inhibitor-containing plants. Although the occurrence of such substances in foods that are preserved by many processes may be only incidental, inhibitory compounds may be very important in regulating the type of fermentation and the resulting product when foods are fermented.

On the basis of the present findings, it seems plausible that inhibitory compounds may exert a significant influence on the survival and subsequent activity of competing microbial groups that populate olive brines, as was suggested by Etchells et al. (3). The levels of inhibitor in extracts of the five varieties of olives assayed were in approximately the same order as their relative rates of fermentation, which were reported by Vaughn (13). Vaughn noted that the Manzanillo and Mission varieties ferment more slowly than the Ascolano and Sevillano varieties. He stated that the first two varieties contain more of the bitter principle, oleuropein, and that this compound "may eventually be shown to suppress the activity of the lactic acid bacteria." However, as Vaughn indicated, the Manzanillo and Mission varieties are usually brined at higher levels of salt, which may account in part for their slower rates of fermentation.

Inhibitory properties of olive tissue and extracts were much more obvious when the olives had been frozen. It is not clear whether freezing caused a release of the inhibitor due to physical changes in the olive or whether it resulted in a subsequent chemical alteration. In either case, the inhibitor or its precursor is subjected to rather drastic conditions in the processing of Spanish-type green olives. In the debittering treatment prior to brining, alkali is allowed to penetrate the olive about three-fourths or more

of the way to the pit and then the alkali is removed by several washings (13). The inhibitor should be reduced greatly in this treatment, as indicated by its destruction when extracts were adjusted to *p*H 10. However, the effects of any residual inhibitor might be enhanced significantly by the presence of 4 to 7% NaCl, which is the normal brine strength for green olives. It was shown that the effectiveness of the inhibitor is much greater in the presence of NaCl.

The inhibitory principle of olives remains to be identified. A phenolic-containing compound is an obvious possibility. Varietal differences in water-soluble phenolic content were correlated with relative amounts of the inhibitor in the five varieties of olives assayed. Many plants have been reported to contain phenolic antimicrobial compounds, either preformed in the tissue or formed as a physiological response to injury or disease (5, 6). Among such compounds are mono- and dihydric phenols, phenolic glycosides, flavonoids, anthocyanins, aromatic amino acids, and coumarin derivatives. Anthocyanins typify the occurrence of such compounds in food products. The anthocyanins of certain grapes and wines have been reported to be bactericidal (7). These pigments and related compounds from several other plants possess varying degrees of antibacterial activity (1, 10).

Oleuropein, the bitter principle of olives, has been identified as a glucoside containing an *o*-dihydroxy phenol (8). This compound reportedly possesses a hypotensive effect (8) and presumably is related to the therapeutic properties long known to be present in leaves and other parts of the olive plant (9). The color of black ripe olives has been attributed at least partially to oxidation products of oleuropein (11). Oleuropein, or compounds related to it, may possess antibacterial activity. Although possibly coincidental, both bitterness and inhibitory properties of olive extracts were reduced greatly by adjustment to *p*H 10.

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