Progress in Table Olive Debittering: Degradation *in vitro* of Oleuropein and Its Derivatives by *Lactobacillus plantarum*

V. Marsilio[a,*], B. Lanza[a], and N. Pozzi[b]

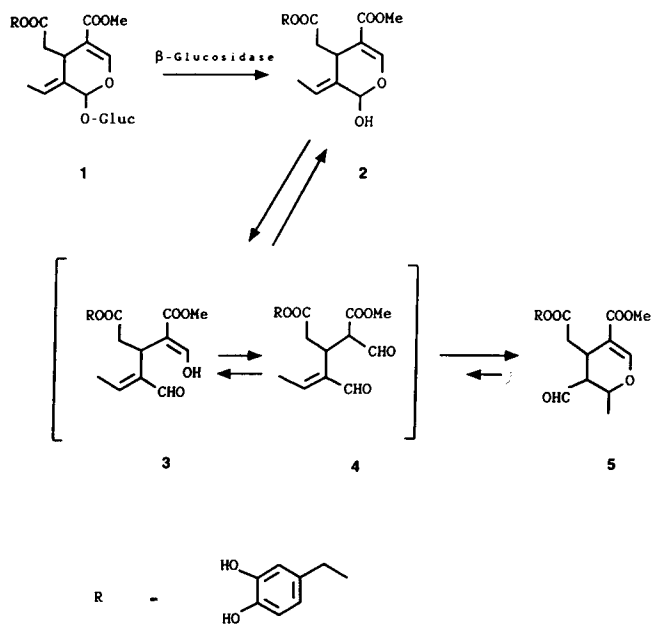
[a]Istituto Sperimentale per la Elaiotecnica and [b]Ispettorato Centrale Repressione Frodi, 65013 Città S.Angelo (PE), Italy

ABSTRACT: Oleuropein, a bitter-tasting secoiridoid glycoside present in olive leaves and fruit (*Olea europaea* L.), is hydrolyzed by oleuropeinolytic *Lactobacillus plantarum* strains. The work reports the results of a gas-chromatographic study of the oleuropein derivatives released by incubation with *L. plantarum* B21, isolated from table olive brines, and by *L. plantarum* ATCC 8014. Process kinetics indicate that the bacterial strains initially hydrolyze the oleuropein by means of β-glucosidase action with formation of an aglycone (the first observable intermediate compound). In a second step, this derivative, by means of esterase action, gives rise to 2-(3,4-dihydroxyphenyl) ethanol (identified) and elenolic acid (not identified).
JAOCS 73, 593–597 (1996).

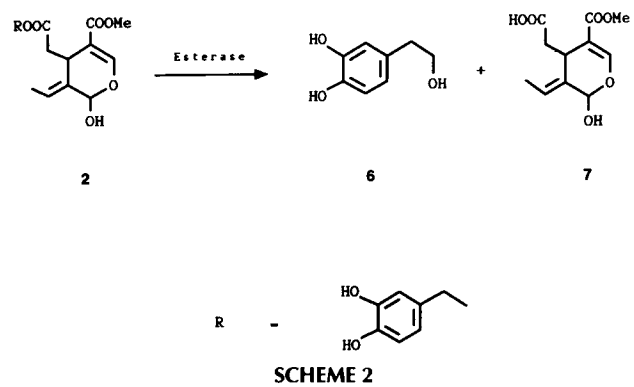
KEY WORDS: Kinetics of the debittering process, lactic acid bacteria, oleuropein, olive fruit.

For centuries, the fruits of the olive tree (*Olea europaea* L.), a plant that is typical of countries of the Mediterranean basin, have been used for direct consumption and for oil production. For table olive consumption, the fruits must first be transformed by means of technologies that provide some form of debittering. All of these technologies are based on delicate fermentations during which microbial transformation of sugars to secondary metabolites (lactic acid, acetic acid, and other minor compounds), which give the finished product a progressive acidification and a particular "flavor," is achieved (1,2). The pH reduction that follows inhibits undesired secondary fermentations and guarantees the product a longer shelf life.

Table olive fermentation is due to composite microflora, consisting mainly of lactic bacteria and yeasts (3–7). In green table olives, transformed by the Spanish or Sevillian system, wherein an alkaline treatment with dilute soda solutions (NaOH) is used to debitter the olives, lactic acid bacteria, in particular *Lactobacillus plantarum* (8), are the predominant microorganisms involved. On the contrary, in undebittered table olives, such microorganisms are almost always inhibited (9). Inhibition of *L. plantarum* in the brines of these olives is attributed to the presence of phenolic substances (10–12) and particularly oleuropein (**1** in Scheme 1) (13–14), an ester of elenolic acid (**7** in Scheme 2) and 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol) (**6** in Scheme 2). Scheme 1



SCHEME 1



SCHEME 2

*To whom correspondence should be addressed.

shows the degradation by *L. plantarum* strains for the hydrolysis of oleuropein (**1**); Scheme 2 shows the degradation of aglycone (**2**) to hydroxytyrosol (**6**) and elenolic acid (**7**). Although it undergoes a notable decrease during the course of fruit ripening, this compound is of considerable importance in process technologies of table olives due to its intense bitter taste and noteworthy bactericidal properties (15–18). This substance is enzymatically hydrolyzed by β-glucosidase, releasing glucose and aglycones (**2, 3, 4, 5** in Scheme 1) (the latter compounds are also bitter and have a distinctly inhibiting function toward *L. plantarum*). It appears to be completely degraded by the alkali treatment that is commonly used in the preparation of Spanish-style green table olives, yielding hydroxytyrosol (**6** in Scheme 2) and elenolic acid (**7** in Scheme 2), with a consequent sweetening of the fruits (19,20).

Recent research (21) has led to the isolation of *L. plantarum* strains, which have shown the *in vitro* capacity to hydrolyze not only oleuropein, but also the aglycone derivative. With such results, it is possible to carry out significant changes in the process technologies of table olives by means of a completely microbiological procedure based on the use of *L. plantarum* as both the debittering and the fermentation agent. Continuing the investigations on these strains, the present work reports the results of capillary gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses of the degradation of oleuropein in growth media inoculated with the oleuropeinolytic *L. plantarum* strain B21 (isolated from olive brines) and with *L. plantarum* ATCC 8014.

EXPERIMENTAL PROCEDURES

Microorganisms. Logarithmically grown bacterial strains *L. plantarum* B21 and ATCC 8014, with cell concentrations of 1.9 and 2.9×10^{10}, respectively, were inoculated in a ratio of 1% (vol/vol) into modified MRS broth (Man, Rogosa, and Sharpe medium) of the following composition (in grams per liter): peptone from soy meal (Merck, Darmstadt, Germany), 10; beef extract powder (Sigma Chemical, St. Louis, MO), 10; yeast extract (Difco, Detroit, MI), 5; sodium acetate (Carlo Erba, Milano, Italy), 5; di-potassium phosphate (Merck), 2; tri-ammonium citrate (Merck), 2; magnesium sulfate (Sigma), 0.2; manganese sulfate (Carlo Erba), 0.05; Tween 80 (Bio-Rad, Richmond, CA), 1 mL/L. Oleuropein (Extrasynthese, Genay, France) was added to concentrations of 0.5% and 1% (wt/vol) and the cultures were incubated at 30°C. Oleuropein hydrolysis and the formation of derivative compounds were followed by analyzing samples with GC/MS at intervals of 4 d.

Sample extraction. Aliquots (0.5 mL) of samples, after centrifugation at $12,000 \times g$ for 10 min, were extracted with 3×1.5 mL ethyl acetate (J.T. Baker, Deventer, The Netherlands), vortexed for 1 min, and centrifuged at $3,500 \times g$ for 5 min. The supernatant was evaporated to dryness under a stream of nitrogen and converted to trimethylsilyl derivatives with a silylation mixture made up of pyridine, hexamethyldisilazane, and trimethylchlorosilane (2:1:1) at 60°C for 30 min. The silanized extracts were dried, dissolved in isooctane, and further analyzed by GC.

GC analysis. A Carlo Erba HRGC 5160 Mega Series gas chromatograph, equipped with a flame-ionization detector (temperature 330°C) and a Nordion OV 1 capillary column (Helsinki, Finland) (10 m × 0.32 mm i.d.; 0.1–0.15 μm film thickness), was used with hydrogen as carrier gas (head pressure 20 kPa). Temperature was programmed from 100 to 250°C at 8°C/min and from 250 to 310°C at 10°C/min. Samples (0.4 μL) were injected by using the "on column" mode.

Mass-spectral analysis. The GC/MS analyses were performed with an HP model 5890A equipped with an HP 5970B mass-selective detector (Hewlett-Packard, Palo Alto, CA). The capillary column used (J&W Scientific DB-5, Folsom, CA; fused-silica 30 m × 0.32 mm i.d.; 0.25 μm film thickness) was directly introduced into the ion source, which was operating in electron impact mode (EI). Other conditions and parameters were as follows: interface at 320°C, and the column oven programs as most appropriate; helium was the carrier gas with a head pressure of 15 kPa; flow rate of 1.9 cm^3/min with 70 eV of ionizing electron energy; electron current of 0.3 mA; ion source 200°C, and the vacuum was 1.3 kPa. Samples (0.4 μL) were injected by the "on column" mode.

Mass spectra of compounds [*m/z* (rel. int.)]: (i) for 2-(3,4-dihydroxyphenyl) ethanol, [M^+] 370 (35), 267 (100), 113 (18), 73 (28), 45 (10); (ii) for oleuropein aglycones, in accordance with their products of rearrangement, [M^+] 594 (1), 280 (100), 193 (19), 73 (25) (see structures **2, 3** at equilibrium in Scheme 1) and [M^+] 522 (1), 280 (100), 193 (17), 73 (22) (see structures **4, 5** at equilibrium in Scheme 1), were recorded and compared with those of reference data (22). Calibration curves were prepared with pure oleuropein and aglycone derivatives at various concentrations. Aglycone was obtained by enzymatic hydrolysis of the oleuropein with β-glucosidase, while for hydroxytyrosol, the standard curve was prepared with pure tyrosol because hydroxytyrosol was not commercially available. The ranges of the curves were 0–0.9 mg/mL, 0–1 mg/mL, and 0–0.8 mg/mL for oleuropein, aglycone, and tyrosol, respectively. Triplicate analyses were performed, and the means of these determinations are presented.

Calculations. Data were processed on a PC 486 computer with Excel 5.0 spreadsheets from Microsoft (Redmond, WA). This approach enables the use of various computational and graphic facilities.

RESULTS AND DISCUSSION

Gas-chromatographic patterns obtained from the extracted broth samples, containing added oleuropein and inoculated with *L. plantarum* strains, showed a significant decrease of oleuropein content over time with a concomitant increase of aglycone derivatives (Scheme 1), which were further degraded to hydroxytyrosol (Scheme 2). Because of the instability of the

free hemiacetal moiety of the secoiridoid in aqueous solution, aglycone, the first compound of the enzymatic degradation of the oleuropein, rearranges to other aglycone structures before transforming into stable final compounds. Therefore, at various steps of the enzymatic reaction, different chemical structures were determined as deriving from: (i) the opening of the elenolic ring and the formation of unstable elenolic species, (ii) addition of water to the keto-enol double bond, and (iii) final rearrangement to secoiridoid compounds (22–27). In agreement with such isomerization, the GC analyses showed multiple peaks of slightly different retention times, of which MS fragmentation patterns exhibited the molecular ions *m/z* 594, 522 and the characteristic ion of the aromatic residue, *m/z* 280 (Fig. 1). Hydroxytyrosol was identified by comparison of its GC retention time and mass fragmentation, *m/z* 370 [M^+] and intensive fragment *m/z* 267 ([M^+] – CH_2OTMS), to an authentic sample (Fig. 2).

Tables 1 and 2 show the concentrations of oleuropein and its derivatives, obtained by analyzing extracts of samples at various times of incubation. Oleuropein hydrolysis took place, and a loss of more than 99% occurred after about 11 d. Oleuropein was progressively converted to aglycone, which reached the maximum level at approximately the same time, whereas the production of hydroxytyrosol began to increase significantly only after the complete disappearance of oleuropein and reached the maximum level after about 2 wk incubation.

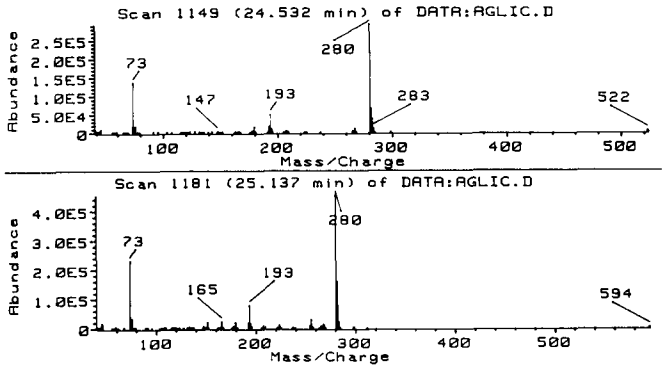


FIG. 1. Electron impact-mass spectra of the aglycones obtained from the hydrolysis of oleuropein by *Lactobacillus plantarum* strains.

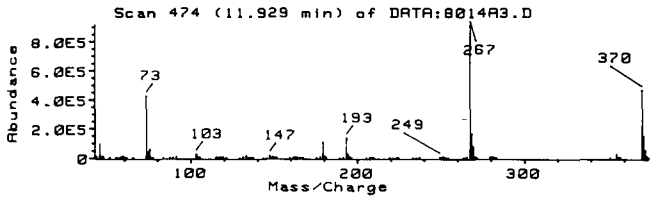


FIG. 2. Electron impact-mass spectrum of hydroxytyrosol obtained from the degradation of aglycone by *Lactobacillus plantarum* strains.

Results indicated that the tested *L. plantarum* strains possessed similar oleuropeinolytic activity because oleuropein

TABLE 1
Time-Dependent Changes in Oleuropein, Aglycone, and Hydroxytyrosol Contents in Samples Inoculated with *Lactobacillus plantarum* B 21

	0.5% Oleuropein[a]			1.0% Oleuropein		
Days	Oleuropein	Aglycone	Hydroxytyrosol	Oleuropein	Aglycone	Hydroxytyrosol
0	1.00×10^{-2}	0	0	2.00×10^{-2}	0	0
1	4.44×10^{-3}	8.00×10^{-5}	0	1.18×10^{-2}	1.77×10^{-3}	3.00×10^{-4}
4	1.91×10^{-3}	1.11×10^{-3}	8.20×10^{-4}	2.28×10^{-3}	5.67×10^{-3}	1.69×10^{-3}
7	1.30×10^{-4}	1.74×10^{-3}	1.81×10^{-3}	1.10×10^{-4}	4.49×10^{-3}	2.27×10^{-3}
11	2.00×10^{-5}	2.04×10^{-3}	2.75×10^{-3}	6.00×10^{-5}	5.32×10^{-3}	4.61×10^{-3}
14	1.80×10^{-5}	1.76×10^{-3}	3.57×10^{-3}	6.00×10^{-5}	4.55×10^{-3}	5.60×10^{-3}
21	0	6.20×10^{-4}	2.77×10^{-3}	0	6.80×10^{-4}	5.07×10^{-3}

[a]Data are expressed as mol/L. The means of three analyses are shown.

TABLE 2
Time-Dependent Changes in Oleuropein, Aglycone, and Hydroxytyrosol Contents in Samples Inoculated with *Lactobacillus plantarum* ATCC 8014

	0.5% Oleuropein[a]			1.0% Oleuropein		
Days	Oleuropein	Aglycone	Hydroxytyrosol	Oleuropein	Aglycone	Hydroxytyrosol
0	1.00×10^{-2}	0	0	2.00×10^{-2}	0	0
1	1.35×10^{-3}	1.07×10^{-3}	0	1.07×10^{-2}	2.66×10^{-3}	3.70×10^{-4}
4	3.40×10^{-4}	2.34×10^{-3}	8.60×10^{-4}	1.06×10^{-3}	5.14×10^{-3}	1.66×10^{-3}
7	1.00×10^{-4}	2.73×10^{-3}	1.93×10^{-3}	1.10×10^{-4}	6.20×10^{-3}	3.28×10^{-3}
11	4.00×10^{-5}	1.77×10^{-3}	2.48×10^{-3}	1.30×10^{-4}	4.79×10^{-3}	4.92×10^{-3}
14	3.00×10^{-5}	2.04×10^{-3}	3.30×10^{-3}	8.00×10^{-5}	4.39×10^{-3}	5.48×10^{-3}
21	0	7.40×10^{-4}	2.85×10^{-3}	0	9.90×10^{-4}	4.73×10^{-3}

[a]Data are expressed as mol/L. The means of three analyses are shown.

hydrolysis to derivative products ran the same way. The results of Tables 1 and 2 indicate that the degradation kinetics of oleuropein can be described by an exponential decay model:

$$y_t = y_0 \exp(-kx_t) \quad [1]$$

where y_t and y_0 are the oleuropein contents at times t and zero, respectively; k is the relative oleuropein degradation rate, and x_t the reaction time.

The yield of oleuropein derivatives as a function of time is best described by a second-order polynomial equation:

$$y_t = a(x_t^2) + b(x_t) + c \quad [2]$$

where y_t is the oleuropein derivatives content, x_t is the reaction time, and a, b and c are constants. Curves of the kind shown in Figure 3 can be described by models based on equations employed to describe the biochemical degradation of oleuropein to derivative compounds. There is good agreement between the calculated and experimental values, with R^2 (correlation coefficient) values for the degradation of 1% oleuropein as follows: 0.87–0.80, 0.85–0.83, and 0.96–0.98 for oleuropein, aglycone, and hydroxytyrosol, respectively. Results revealed that the enzymatic degradation of oleuropein followed first-order reaction kinetics. Linear relationships between the logarithm of the residual concentrations and time were calculated (Fig. 4).

A balance was achieved over the course of the whole reaction process by considering the oleuropein concentration and the sum of the concentrations of aglycone and hydroxytyrosol. The gradual fall in the concentration of oleuropein, together with the corresponding increase in aglycone and hydroxytyrosol, can be interpreted as representing the result of bacterial activity because the oleuropein content in uninoculated culture medium (control test) remains stable over time. On the other hand, because the balance of oleuropein plus derivatives does not show a stoichiometric relation, it can be supposed that fragments of secoiridoids were used as an energy source by *L. plantarum* strains or that other types of reactions can affect the oleuropein degradation for which intermediate compounds were not detected.

The results suggest that the bacterial oleuropeinolytic action involves a two-step process: (i) an enzymatic hydrolysis of the glycosidic linkage with β-glucosidase to release the aglycone, the first observable intermediate in the process; (ii) after the β-glycosidic linkage is completely hydrolyzed, as deduced from the disappearance of oleuropein, other compounds originate, probably due to esterase activity that hydrolyzes the ester to the acid and alcohol. However, caution should be paid in applying the results observed in these experiments that were conducted in culture medium, to those in real olive systems, which are much more complex. Further research is necessary to determine if the trends shown in this study are also manifested in olive systems during processing and storage.

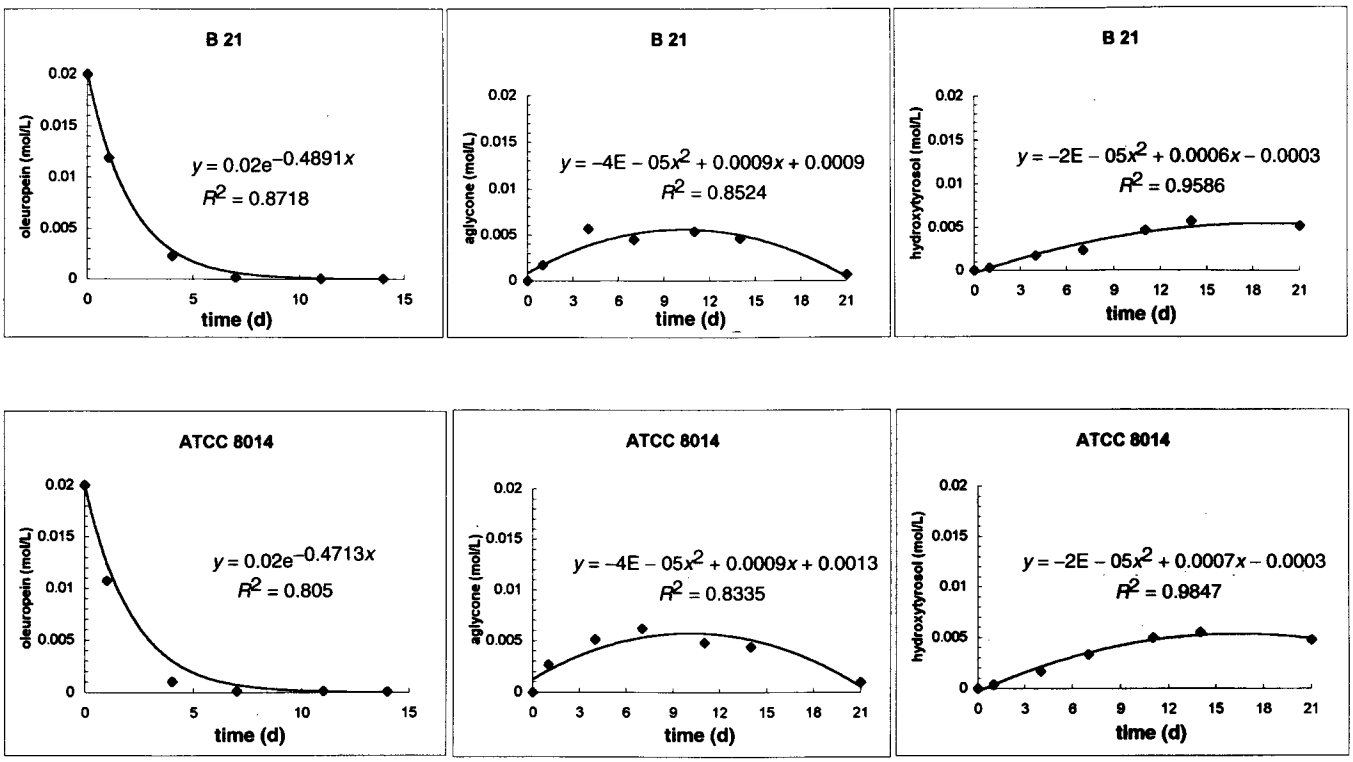


FIG. 3. Kinetic curves of oleuropein hydrolysis to derivative products by *Lactobacillus plantarum* B 21 and ATCC 8014 strains. *R* is the correlation coefficient.

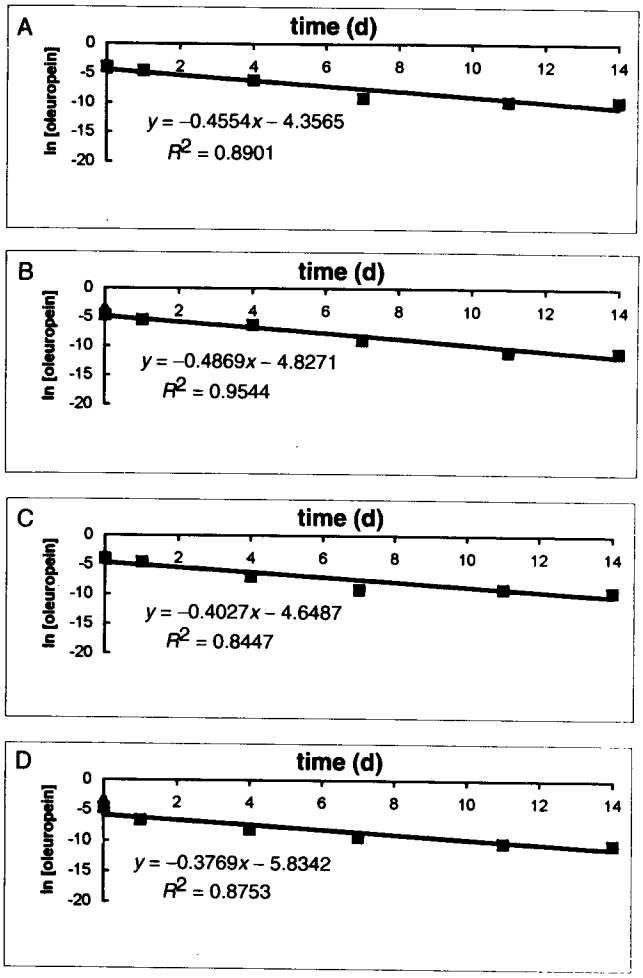


FIG. 4. Linear plot of first-order degradation's rate of oleuropein by *Lactobacillus plantarum* B 21 (A) 1%; (B) 0.5%, and ATCC 8014 (C) 1%; (D) 0.5% as a function of time. *R* is the correlation coefficient.

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[Received June 23, 1995; accepted January 17, 1996]