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.

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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

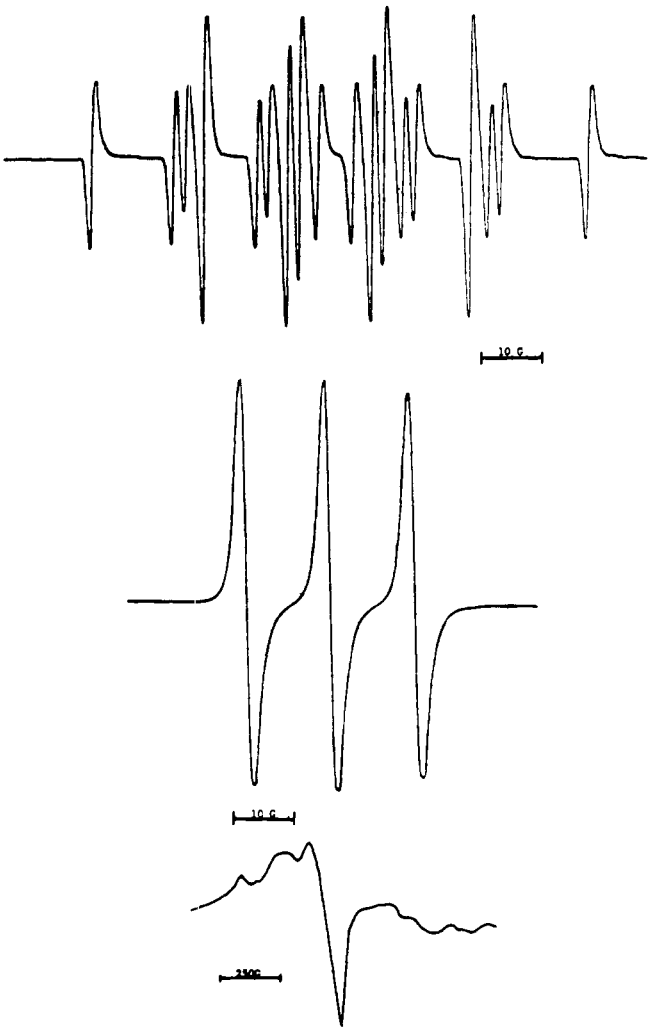

Figure 1. Epr spectra of solutions of proline nitroxide in water (top), in squalene (center), and in the solid state (bottom).

Table I. Coupling Constants of Proline Nitroxide

	A_N	$A_{\beta\text{-}1^H}$	$A_{\beta\text{-}2^H}$
This paper	14.5	16.3	19.5
Previous preparation[a]	15.5	17.7	21.1
Literature[b]	14.82	16.88	20.02, 20.32

[a] Van der Veen *et al.* (1970). [b] Janzen (1971); three additional smaller splitting constants were assigned to γ protons. J. R. Windle observed that in dilute alkaline solution our preparation exhibited the same coupling constants as those reported by Janzen (1971).

more *tert*-butyl hydroperoxide. The sequence was repeated until there remained no methanol-insoluble solid. The combined methanol extracts were taken to dryness, and the residue was dissolved in methanol and crystallized by the addition of acetone. The yield of light yellow crystals was 35%. The product was homogeneous by thin-layer chromatography (Tom, 1973; Weil, 1968a,b).

CHARACTERIZATION

Proline nitroxide was easily soluble in water and methanol, but solubilized with difficulty in ethanol or isooctane. It decomposed with change of color from light yellow to orange-red at 115–117° and with further heating to 158–160°, with evolution of reddish fumes. *Anal.* Calcd for $C_5H_8NO_3$: C, 46.2; H, 6.2; N, 10.8. Found: C, 46.1; H, 6.60; N, 10.8.

The epr spectra (Varian E-3) of proline nitroxide in water, in squalene, and in the solid state are shown in Figure 1. The 18-line spectrum in water is consistent with that previously published (Van der Veen *et al.*, 1970) but the coupling constants (Table I) previously measured were larger, possibly due to the lower temperature of the measurement and the presence of hydrogen peroxide and tungstate in the alkaline medium. The three split lines in squalene had a coupling constant of A_N = 14.0 G; proton involvement was not visualized. The signal in the solid state indicates that the free radical had not dimerized during crystallization.

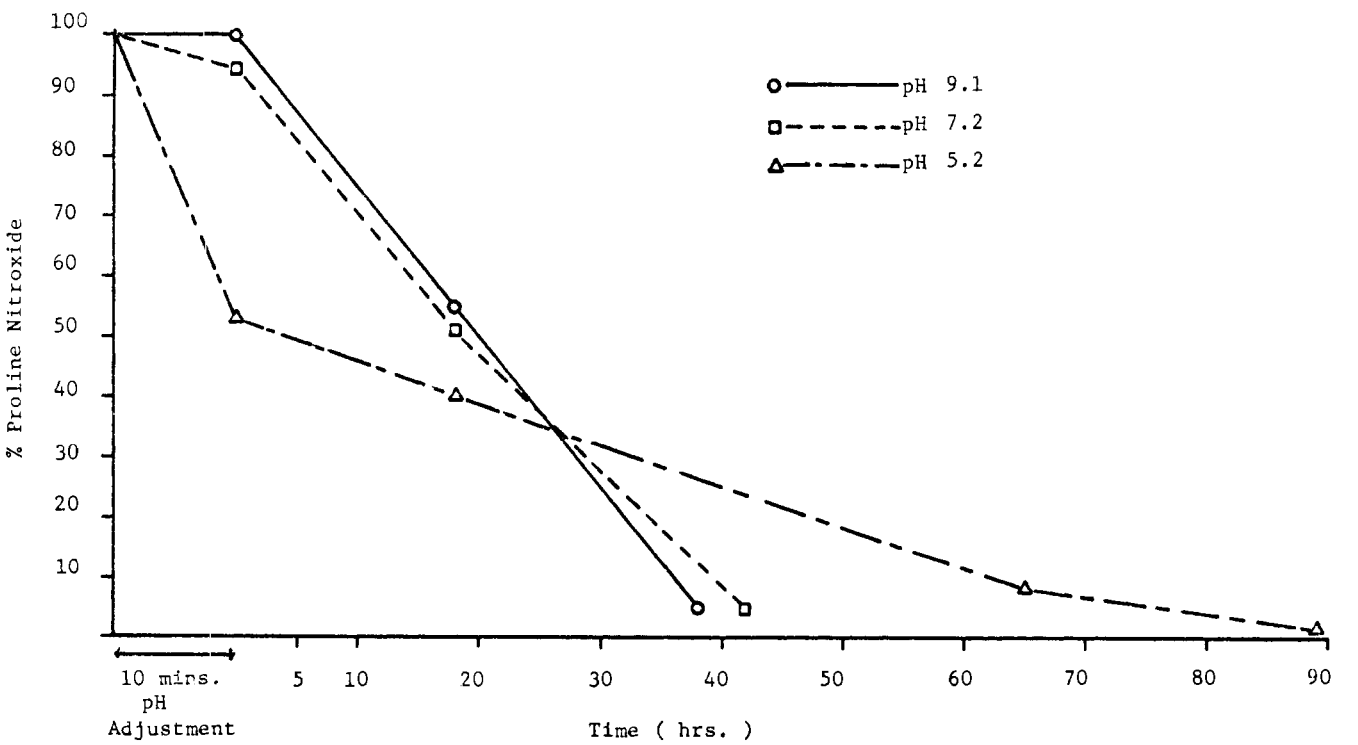

Figure 2. Effect of pH on stability of proline nitroxide (4.6×10^{-3} *M*) in 0.1 *M* phosphate buffer solutions at 24°.

Proline nitroxide had an absorption band in the ultraviolet at 235 nm with extinction coefficient $\epsilon = 1363$ characteristic of nitroxides (Forrester *et al.*, 1968). *N*-Hydroxyproline has no band in this region. The infrared spectrum, in accord with the known structure, had a triplet at 1350 to 1370 cm^{-1}, characteristic of a nitroxide NO vibration (Forrester *et al.*, 1968). An nmr spectrum (Tom, 1973) also showed no unexpected bands.

STABILITY

Proline nitroxide was unexpectedly stable. It appeared to keep indefinitely in the solid state; in solution the stability depended on temperature and pH. In phosphate buffer (0.1 *M*) at pH 7, the signal of a 6.9×10^{-3} *M* solution of proline nitroxide disappeared after 80, 40, and 20 hr at 24°, 37°, and 50°, yielding first-order rate constants of 7.0×10^{-4}, 1.5×10^{-3}, and 4.3×10^{-3} min^{-1}, respectively (Tom, 1973).

The strength of the epr signal decreased upon acidification but thereafter disappeared more slowly than from alkaline media (Figure 2). The 50% loss of signal strength seen when the pH was adjusted to pH 5.2 from pH 9.7 was regained if the solution was immediately adjusted back to the original pH.

Proline nitroxide is an effective lipid antioxidant despite its lack of ready oil solubility. By a weight gain method (Olcott and Einset, 1958), samples of purified squalene and menhaden oil containing 0.065% (1 μmol/200 mg) proline nitroxide had induction periods of 2 months and 1 month, respectively, at 24° compared to controls which were rancid in 1 day.

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A Microcolumn Apparatus for Rapid Cleanup of 2,4-Dinitrophenylmethylamine Extracts for Carbamate Pesticide Analysis

A microcolumn apparatus has been devised and tested for cleanup of the reaction product of methomyl (an *N*-methyl carbamate insecticide) and 1-fluoro-2,4-dinitrobenzene before gas chromatography. Microcolumns packed with silica gel G-HR gave consistently good cleanup and quantitative recovery of 2,4-dinitrophenylmethylamine. Details of the apparatus and the cleanup procedure are described.

Microcolumn chromatography has been studied for cleanup and separation of some organophosphorus and organochlorine pesticide residues before gas–liquid chromatography (glc) (Kadoum, 1967, 1968a,b; Law and Goerlitz, 1970; Leoni, 1971). The types of columns used were disposable pasteur pipets (Kadoum, 1967); chromaflex columns of 6, 7, and 9 mm i.d. and a macrochromatographic column of 350 mm × 22 mm i.d. (Kadoum, 1968b); "disPo" disposable transfer pipets (Law and Goerlitz, 1970); and 30 cm × 4.2 mm i.d. columns (Leoni, 1971). The columns were packed with silica gel 950 (Kadoum, 1967, 1968a; Leoni, 1971), silica gel 923 (Kadoum, 1968b), and silica gel or deactivated alumina (Holden and Marsden, 1969; Law and Goerlitz, 1970). Recoveries reported ranged from above 90 to 100%.

Microcolumn cleanup was also used in a method developed for methomyl determination (Mendoza and Shields, 1974). The method consisted of alkaline hydrolysis of methomyl to produce methylamine and reaction of this product with 1-fluoro-2,4-dinitrobenzene (DNFB). The reaction extracts gave a large glc peak which interfered with that of 2,4-dinitrophenylmethylamine (DNPMA), a reaction product of DNFB and methylamine. We found the silica gel G-HR microcolumn satisfactory for cleaning up the DNPMA extracts before the glc analysis. However, elution was slow and time consuming.

We therefore devised a microcolumn apparatus attached to a vacuum source and using disposable pasteur pipets. The microcolumn apparatus was found to be a time-saving and efficient device. The design and use of this apparatus are described in this report.

MATERIALS AND METHODS

Preparation of Microcolumns. A 9-in. pasteur pipet was plugged tightly with a wad of glass wool. It was then filled with MN silica gel G-HR powder (Macherey Nagel and Co., Düren, West Germany) up to a height of 5 cm. (This gel is normally used in thin-layer chromatography.)