Nitrite-Reacting Substances in Japanese Radish Juice and Their Inhibition of Nitrosamine Formation

Tsutao Kurechi, Kiyomi Kikugawa,* and Sachiko Fukuda

Japanese radish (*Raphanus sativus*) juice effectively decreased nitrite at acidic ranges. Nitrite-consuming activity was due to dialyzable, negatively charged, and unstable substances showing characteristic ultraviolet absorption maxima at 270 and 332 nm (pH 12). The major nitrite-reacting substance(s), obtained from partial purification by dialysis and subsequent column chromatography through a diethylaminoethylcellulose column, was (were) assumed to be a phenolic compound(s). The nitrite-reacting substances were transformed into other compounds showing absorption maximum at 288 nm (pH 12) by reaction with nitrite. Japanese radish juice effectively inhibited the formation of N-nitrosodimethylamine and N-nitrosodiethylamine from reaction between nitrite and the corresponding secondary amines.

Nitrite readily produces nitrosamines by reaction with secondary amines at gastric pH (Sander and Seif, 1969; Mirvish, 1970), and these compounds are potential carcinogens (Druckrey et al., 1967). Nitrite may be readily derived by bacterial reduction (Ayanaba and Alexander, 1973) and salivary reduction (Ishiwata et al., 1975; Speigelhadler et al., 1976; Tannenbaum et al., 1976) from nitrate present in a wide variety of vegetables (Yanagihara et al., 1963; Walker, 1975). Nitrite and nitrate are also synthesized endogenously by nitrifying bacteria in the human body (Tannenbaum et al., 1978). Several substances that are endogenous or may be added to foodstuffs are known to react with nitrite and exert effects on the formation of nitrosamines under gastric conditions. As for the substances that exert inhibitory effects on the nitrosation, ascorbic acid (Mirvish et al., 1972; Fan and Tannenbaum, 1973), sorbic acid (Tanaka et al., 1978), α-tocopherol (Pensabene et al., 1978), and butylated hydroxyanisole (Kurechi et al., 1980) are known. There are several contradictory effects on nitrosamine formation with phenols such as gallic acid (Gray and Dugan, 1975; Walker et al., 1975; Yamada et al., 1978), chlorogenic acid (Challis and Bartlett, 1975), sesamol (Kurechi et al., 1978), and p-cresol (Davies and McWeeny, 1977), which exerted effects of inhibition or stimulation depending upon the workers and the conditions.

It will be worthwhile to investigate the effects of foodstuffs consisting of several sorts of materials on the nitrosamine formation under gastric conditions. The previous study with lipid (unsaturated fatty acid)-containing foods such as cow's milk revealed that they effectively prevented the nitrosamine formation (Kurechi and Kikugawa, 1979). It is conceivable that a variety of vegetables containing substances such as ascorbic acid and polyphenolics will exert some effects on the nitrosamine formation. The study now reported was undertaken to investigate the reactions of nitrite and the effects on nitrosamine formation with several vegetables including Japanese radish (*Raphanus sativus*), one of the most common vegetables in Japan.

MATERIALS AND METHODS

Materials and Reagents. Carrot, cucumber, potato, Japanese radish, and apple were available in all seasons and purchased from local markets in Tokyo (Japan). The

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vegetables were pared, grated, and pressed to be separated into fibers and juice. The juice was subsequently filtered through cotton wool. L-Ascorbic acid is a product of Wako Pure Chemical Industries, Ltd. Diethylaminoethyl (DEAE)-cellulose SH (capacity 0.85 mequiv/g) and carboxymethyl (CM)-cellulose (capacity 0.77 mequiv/g) are products of Serva in Germany. Griess reagent was prepared by mixing an equal volume of 1.0% w/v sulfanilic acid in 30% glacial acetic acid and 1.0% w/v 1-naphthylamine in 30% glacial acetic acid just before use. Citrate buffer was prepared by adjusting 0.2 M sodium citrate solution to the required pH with concentrated hydrochloric acid.

Analysis. Absorbances were measured by use of a Hitachi 101 spectrophotometer. Ultraviolet (UV) absorption spectrum was measured with a Shimadzu UV-200S double-beam spectrophotometer. Spectra were taken in 0.1 N HCl, 0.1 M acetate buffer (pH 5.0), 0.1 M phosphate buffer (pH 8.0), and 0.1 M glycine–sodium hydroxide buffer (pH 12). Unless otherwise mentioned, absorbances of the radish juice were expressed as those at 332 nm in the alkaline medium (pH 12). The peaks containing UV-absorbing substances from the DEAE-cellulose columns were monitored by a mini UV monitor, type I (254 nm) (Ichibishi Co., Ltd.).

Paper chromatography of the substance(s) in radish juice was performed on Toyo filter paper, No. 51 (Toyo Roshi Kaisha, Ltd.), by using the solvents (a) water or (b) 1-butanol saturated with water. The chromatogram was developed during the short time of 30–60 min with an ascending technique in order to avoid the degradation of the substance(s) in the juice. The spots of the juice were located by UV-ray (254 nm). When the chromatogram was sprayed by 0.1 M sodium nitrite and by Griess reagent after a 3–5-min interval, the spots corresponding to the nitrite-reacting substance(s) remained uncolored against the pink-colored background. The spots corresponding to the phenolic substance(s) turned brown when sprayed with 1% 2,6-dichloroquinone monochloroimide solution in ethanol.

Nitrosamines were detected by a Yanaco gas chromatograph and a glass column (3 mm i.d. × 2 m) of poly(ethylene glycol) 6000 (25%) on 80–100 mesh Chromosorb W AW. The chromatograph was operated isothermally at 120 °C (column temperature) and 140 °C (injection temperature) with a carrier nitrogen gas flow of 25 mL/min. The amount of the N-nitrosamine was determined by comparing the peak area of the samples with that of each authentic standard (N-nitrosodimethylamine and

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N-nitrosodiethylamine; Wako Pure Chemical Industries, Ltd.) solution in chloroform (5 μL of 0.40 mg/mL).

Determination of Ascorbic Acid. Ascorbic acid content in vegetable juice was determined by titration with 2,6-dichlorophenolindophenol solution. Ascorbic acid contents in vegetable juice were 0.065 (carrot), 0.098 (cucumber), 0.065 (potato), and 0.13 mg/mL (Japanese radish).

Determination of Dried Matter. Radish juice (10–100 mL) was evaporated and coevaporated with ethanol in vacuo to dryness at below 40 °C. Weights of the residue were measured.

Nitrite Loss by Vegetable Juice. An indicated volume of each vegetable juice or 20 mM L-ascorbic acid solution was made up to 19.0 mL with 0.2 M citrate buffer (pH 3.0, 4.0, and 5.0). To this was added 1.0 mL of 2.0 mM sodium nitrite. The mixture was incubated at 37 °C for up to 2 h in a 50-mL stoppered flask. The pH of the reaction mixture was checked both at the start and at the finish of incubation and was generally unchanged. Portions of 1.0 mL were transferred into a mixture of 5.0 mL of 2% glacial acetic acid and 0.40 mL of Griess reagent, and the mixture was allowed to stand at room temperature for 15 min. Absorbance at 520 nm was measured. Control experiments of 0.1 mM sodium nitrite in the buffers (pH 3.0, 4.0, and 5.0) showed the absorbances 0.400–0.420 during the period of 2 h (Figure 1).

Determination of Nitrite-Consuming Activity of Radish Juice. Potency of nitrite-consuming substances in the juice was defined as 1.0 activity unit when 0.02 μmol of nitrite was lost at pH 3.0 during 60 min. Specific activity was defined as activity per gram weight of the dried matter in the juice. Thus, an indicated volume of radish juice or purified juice was made up to 19.0 mL with 0.2 M citrate buffer (pH 3.0), and to this was added 1.0 mL of 2.0 mM sodium nitrite. The mixture was incubated at 37 °C for 60 min, and the loss of nitrite was determined as described. Percent decrease in nitrite concentration was calculated based on the control experiment. When the percent decrease was plotted vs. volume of juice, a linear relationship was observed. If 1.0% of nitrite in the solution was lost under the conditions, there was 1.0 activity unit of nitrite-consuming substances in the indicated volume of the juice.

Dialysis of Radish Juice. Radish juice (450 mL) in eight portions was dialyzed against 1.2 L of distilled water by use of seamless cellulose tubings (24/32) (Visking Co., Ltd.) in a cold room overnight. Clear outer solutions of dialysis were pooled for subsequent experiments: measurement of UV-absorption spectra (Figure 2), stability tests, adsorption to anion exchangers (Figure 3), and effects on nitrosamine formation (Table II).

Column Chromatography of Radish Juice. The outer solution of dialysate (20 mL) was applied on to a column (1 cm i.d. × 5 cm) of Dowex 1-X8 (chloride form, 100–200 mesh) anion exchanger. Neither nitrite-consuming nor UV-absorbing materials could be eluted from the column by any means. The solution from the dialysate (20 mL) was applied on to a column of DEAE-cellulose anion exchanger, and nitrite-consuming and UV-absorbing substances were eluted by triethylamine bicarbonate as illustrated in Figure 3.

Effect of Radish Juice on Nitrosamine Formation. To 35 mL of radish juice, dialyzed and condensed radish juice, or water was added 1.47 g of sodium citrate dihydrate. To the mixture were added 5.0 mL of 2 M sodium nitrite and 2.5 mL of 1 M dimethylamine or diethylamine hydrochloride. The solution was adjusted to the required
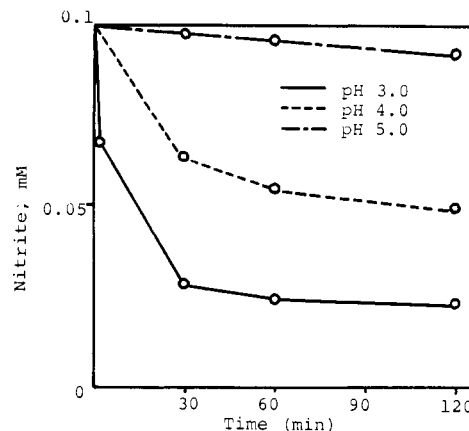

Figure 1. Time course of the decrease in nitrite concentration by Japanese radish juice. Nitrite (0.10 mM) was treated with 1.0 mL of juice in 20 mL of citrate buffer at 37 °C.

pH with concentrated hydrochloric acid and made up to 50 mL with water. The mixtures were placed in stoppered 100-mL flasks and kept at 20 °C for 4.5 h. The pH of the reaction mixture was generally unchanged when it was checked after the reaction. Since Yamamoto et al. (1979) described that the amount of the nitrosamine in certain reaction mixtures was altered and increased by alkalization before extraction, extraction of the nitrosamines in the present study was performed in two ways: one is the direct extraction of the acidic mixture (extraction 1) and the other is the extraction of the mixture after alkalization (extraction 2). Thus, a 10-mL portion of each of the reaction mixtures was added to a mixture of 2.0 g of sodium chloride and 40 mL of chloroform in the presence and absence of 5.0 mL of 5 N NaOH, and the mixtures were shaken for 5 min to obtain chloroform extracts, to which most of the nitrosamines were transferred. The amount of the nitrosamines in the extract was determined by gas chromatography as soon as possible.

Warning. *N*-Nitrosodimethylamine, *N*-nitrosodiethylamine, and 1-naphthylamine, all of which are carcinogens, should be handled with great caution.

RESULTS

Percent decrease in nitrite concentration by vegetable juices was 3, 7, 5, 79, and 15 when 20 mL of 0.10 mM nitrite was incubated with 1.0 mL of the juice from carrot, cucumber, potato, Japanese radish, and apple, respectively, at pH 3.0 and 37 °C for 1 h. Juice from Japanese radish most effectively reduced nitrite under the mild acidic conditions. Time courses of the loss of nitrite by 1.0 mL of Japanese radish juice at different pH values were followed (Figure 1), and a maximal decrease in nitrite concentration was achieved at pH 3.0 in a 1-h incubation. The activity of the nitrite consumption of Japanese radish juice, defined as 1.0 activity unit for a 0.02 μmol loss of nitrite, varied in a rather wide range (40–150 units/mL; 0.8–3 μmol loss of nitrite/mL) from radish to radish, and the radish harvested in winter appeared to have the highest potency.

Since ascorbic acid has been shown to react with nitrite (Dahn et al., 1960) and thus inhibit nitrosamine formation (Mirvish et al., 1972), the nitrite-consuming activity of the radish juice might be ascribed to the acid as its content in Japanese radish juice was higher than those in other vegetables tested. The potency of nitrite loss by the radish juice was compared with that by pure ascorbic acid. While 7.0 and 10.5 mg of ascorbic acid showed 65 and 85 activity units of nitrite loss, respectively, 1.0 mL of juice containing only 0.13 mg of ascorbic acid exhibited 90 activity units, in a 1-h incubation with 0.1 mM nitrite at pH 3.0. The

Table I. Partial Purification of Nitrite-Consuming Substances of Japanese Radish Juice

	vol, mL	nitrite-consuming act., units	total absorbance (332 nm, pH 12)	dried matter, g	sp act.	total absorbance per g wt
dialysis[a]						
before	450	58 500	25 000	22.5	2600	1110
outer soln of dialysate	1200	41 500	16 300	14.6	2860	1130
DEAE-cellulose separation[b]						
before	20	660	264	0.26	2540	1015
f_1	200	0	23	0.20		
f_2	75	0	0			
f_3	35	198	82			
f_4	20	273	116	0.031	8800	3730
f_5	30	0	0			

[a] Radish juice was dialyzed against 2.7-fold volumes of distilled water in a cold room overnight. [b] The outer solution of dialysate was applied onto a DEAE-cellulose column as shown in Figure 3.
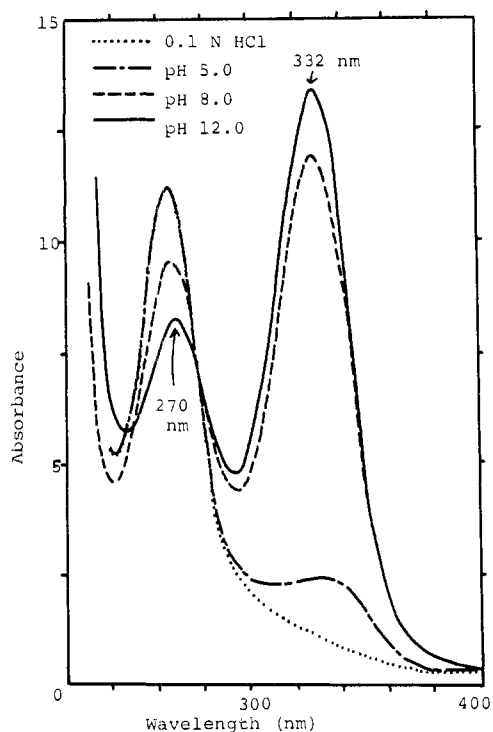

Figure 2. Ultraviolet absorption spectra of the outer solution of Japanese radish juice dialysate.
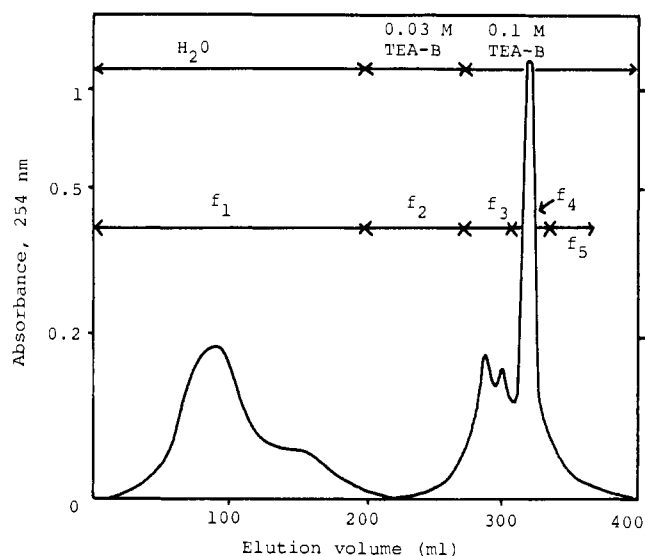

Figure 3. Purification by DEAE-cellulose of substances obtained by Japanese radish juice dialysis. The outer solution of dialysate (20 mL) was applied onto a column (1.8 cm i.d. × 10 cm) of DEAE-cellulose (bicarbonate), and the column was eluted by triethylamine bicarbonate, pH 9.0 (TEA-B). The fraction, f_4, was passed through a column (2 cm i.d. × 3 cm) of CM-cellulose (H^+) to remove triethylamine bicarbonate, and the eluate was evaporated to dryness for subsequent characterization.

results showed that the ascorbic acid content in Japanese radish juice was too small to reduce nitrite to such an extent and that nitrite loss by the juice must by due to substances other than ascorbic acid.

The radish juice was dialyzed through a cellulose membrane against distilled water in a cold room. UV-absorption spectra (Figure 2) of the outer solution, close to those of the original juice, showed the absorption maxima at 268 nm (pH 1) and 270 and 332 nm (pH 12) with a characteristic bathochromic shift of the maxima at the alkaline pH. By dialysis, 71% of activity, 65% of total absorbance (at pH 12 and 332 nm), and 65% of dried matter were recovered in the outer solution (Table I). When the outer solution was kept at 4–10 °C for 5 days, both activity and absorbance were significantly reduced.

The principle of using a water-insoluble polymer to remove phenolic substances from solution has been employed by Lam and Shaw (1970), who treated homogenized plant material with a strong anion-exchange resin such as Dowex 1-X8 (chloride). The outer solution of dialysate was passed through a column of Dowex 1-X8 (chloride), while the nitrite-consuming and UV-absorbing substances adsorbed

to the column too tightly to be eluted by any means. When the solution was applied into a column of DEAE-cellulose (bicarbonate) and chromatographed by a stepwise elution with triethylamine bicarbonate (Figure 3), several UV-absorbing peaks appeared. Recoveries of nitrite-consuming activity, absorbance, and dried matter in the fractions f_1–f_5 are summarized in Table I. The fraction f_1, eluted by water and having negligible activity, contained only 9% of total absorbance and 77% of dried matter. Most of the activity and absorbance were recovered in the fractions f_3 and f_4, eluted by 0.1 M triethylamine bicarbonate. While the fraction f_3 was comprised of at least two UV-absorbing substances, the fraction f_4 appeared as a single UV-absorbing peak in the chromatography. The fraction f_4 contained 41% of activity, 44% of total absorbance, and 12% of dried matter, and its specific activity and total absorbance per unit weight of dried matter were 3 times as high as those before fractionation. UV-absorption spectra of f_3 and f_4 (Figure 4A) indicate the maxima of f_3 appeared at 278 and 338 nm (pH 12) and the maxima of f_4 appeared at 269 and 327 nm (pH 12); both the spectra were close to but different from those before fractionation. They showed a characteristic bathochromic shift of ab-
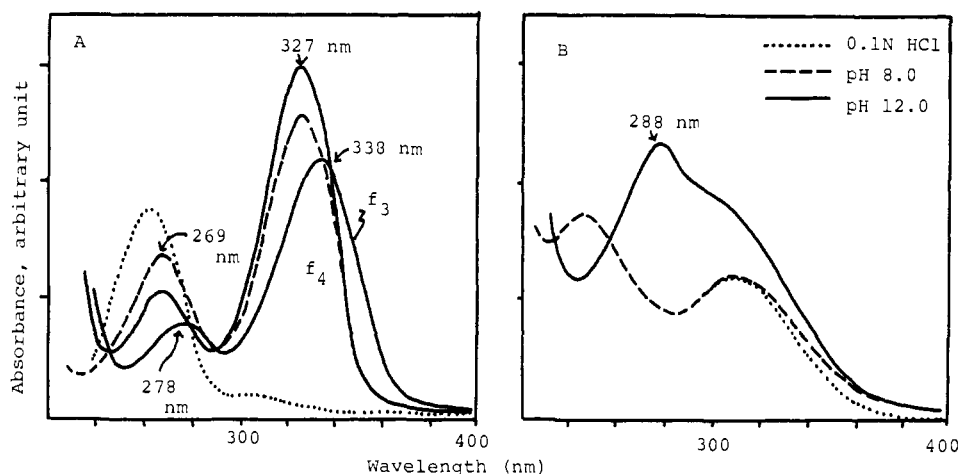
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Figure 4. Ultraviolet absorption spectra of the fractions separated by the DEAE-cellulose column. (A) Fractions f_3 and f_4 in Figure 3. (B) Fraction f_2' in Figure 5.
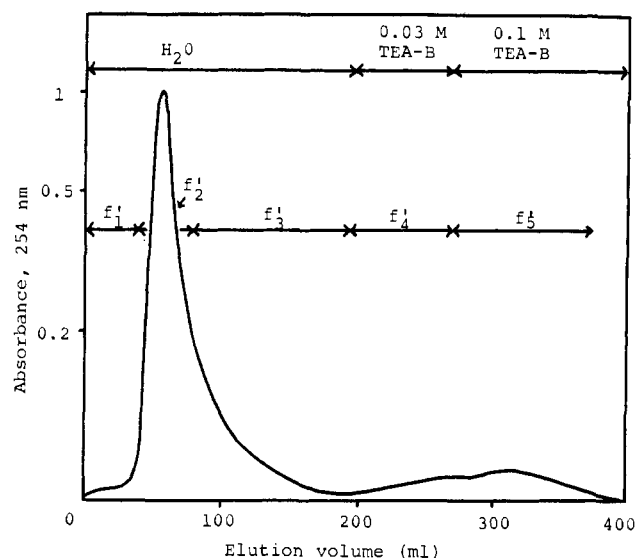

Figure 5. Separation by DEAE-cellulose of substances in Japanese radish juice treated with nitrite. The outer solution of dialysis (23.5 mL) was condensed to 1.0 mL (activity, 700 units; total absorbance, 305), and it was treated with 0.5 mL of 40 mM nitrite at pH 3 and 37 °C for 1 h. The neutralized reaction mixture was applied onto a column of DEAE-cellulose as described in the legend of Figure 3. Total absorbance at 288 nm (pH 12) of f_2' was 100.

sorption maxima in an alkaline medium and different from those of ascorbate showing maxima at 245 (pH 0.6), 218 and 265 (pH 8.3), and 300 nm (pH 13.5) (Dahn and Hauth, 1957).

Paper chromatography of f_4 was performed with two solvent systems (a and b). The spots located by UV-ray overlapped those by spraying nitrite and Griess reagent and appeared as long tailing single spots at the different R_f values (0.60 with solvent a; 0.85 with solvent b) from those of ascorbic acid (0.95 with solvent a; 0.15 with solvent b). The spots from f_4 was colored brown by 2,6-dichloro-quinone monochloroimide, while they were not colored by other phenolic reagents such as ferric chloride and diazotized p-nitroaniline. The nitrite-reacting substances were unstable, dialyzable through cellulsoe membrane, negatively charged to adsorb to the anion exchangers such as Dowex 1 and DEAE-cellulose, and exhibiting characteristic bathochromic shift of UV-absorption maxima at an alkaline pH. The major substance(s) obtained by DEAE-cellulose column chromatography was (were) colored by the chloroimide reagent specific to phenolic compounds.

Table II. Effects of Japanese Radish Juice on N-Nitrosoamine Formation from 0.2 M Nitrite and 0.05 M Dimethylamine (or Diethylamine) in 4.5-h Incubation at 20 °C (50 mL)

	pH	extrac-tion[a]	N-nitroso-dimethylamine mg/mL	N-nitroso-dimethylamine % inhibn	N-nitroso-diethylamine mg/mL	N-nitroso-diethylamine % inhibn
control	3.6	1	1.24		0.20	
		2	1.36			
radish juice[b]	3.6	1	0.92	25.8[d]	0.16	22.0
		2	1.09	19.9[d]		
control	4.1	1	0.66			
		2	0.86			
radish juice[b]	4.1	1	0.45	31.8[d]		
		2	0.52	21.2[d]		
control	3.0	1	1.44			
dialyzed and condensed fraction[c]	3.0	1	0.64	55.6		

[a] See Materials and Methods. [b] Radish juice, 35 mL (activity, 3000–4500 units; total absorbance, 1700–1960). [c] Dialyzed and condensed fraction, 35 mL (activity, 15 500 units; total absorbance, 7260). [d] Mean values of three experiments.

These results may suggest that the nitrite-reacting substances were phenolics.

The outer solution of dialysate was treated with 1.4-equiv amount of nitrite at pH 3.0 and 37 °C for 1 h, and the reaction mixture was applied onto a DEAE-cellulose column (Figure 5). Elution profiles were quite different from those of the nontreated; the peaks f_3 and f_4 completely disappeared and the major UV-absorbing peak appeared in the water-eluted fraction f_2'. The spectra of f_2' (Figure 4B) exhibited the maximum at 288 nm (pH 12), which reflected that UV-absorbing substances in f_3 and f_4 were completely transformed into another compounds in f_2' by reaction with nitrite.

Effects of Japanese radish juice and the outer solution of dialysate on N-nitrosodimethylamine and N-nitroso-diethylamine formation by reaction between 0.2 M nitrite and 0.05 M dimethylamine and diethylamine in a 4.5-h treatment at 20 °C were investigated (Table II). Nitrosamines in the reaction mixture were extracted with chloroform by two methods and determined by gas chromatography. Japanese radish juice in 70% concentration inhibited N-nitrosodimethylamine formation by 19–32% at pH 3.6 and 4.1 and N-nitrosodiethylamine formation by 22% at pH 3.6. Inhibitory effects of the condensed outer solution of dialysate, having about fourfold activity

and total absorbance of the original radish juice, were more striking, and N-nitrosodimethylamine formation was inhibited by 56% at pH 3.0.

DISCUSSION

Japanese radish (R. sativus) is one of the most common vegetables in Japan, and its juice lost more nitrite than other vegetable juices. While the major nitrite-reacting components in the radish juice have not yet been identified, it may be labile phenolic-like substances judging from strong adsorption to anion exchangers, characteristic bathochromic shift of UV-absorption spectrum at an alkaline pH, and coloration by chloroimide reagent. In other sorts of radish, phenolic substances such as p-coumaric acid, caffeic acid, and ferulic acid have been found by extraction under rather severe conditions (Stohr and Herrmann, 1975). UV-absorption spectra of the unstable substances in Japanese radish obtained in the present study were not identical with those of any commonly known plant phenolics.

Nitrite is produced under variety conditions: by salivary (Ishiwata et al., 1975; Speigelhadler et al., 1976; Tannenbaum et al., 1976) and by bacterial (Ayanaba and Alexander, 1973) reduction of nitrate present in vegetables and endogenously by nitrifying bacteria in the body (Tannenbaum et al., 1978), which is then available for carcinogenic nitrosamine formation under gastric conditions. Ascorbic acid (Mirvish et al., 1972; Fan and Tannenbaum, 1973) and phenolics (Gray and Dugan, 1975; Yamada et al., 1978), which distribute in vegetables, have been shown to reduce nitrite and thus inhibit the nitrosamine formation, although the latter have contradictory effects depending upon the conditions (Challis and Bartlett, 1975; Walker et al., 1975; Davies and McWeeny, 1977; Kurechi et al., 1979). Japanese radish juice inhibited nitrosamine formation by reaction between nitrite and secondary amines under acidic conditions. This inhibition of nitrosamine formation may be due to the nitrite-reacting phenolic substances rich in the juice rather than to ascorbic acid.

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Tissue Distribution and Disposition of Hymenoxon

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Hymenoxon reacts with endogenous constituents of blood, but the sulfhydryl group is not the primary reactive site. Hymenoxon is excreted as glucuronides via urine and bile.

Hymenoxys odorata bitterweed), Helenium hoopsii (sneezeweed), and Baileya multiradiata (Desert baileya) are toxic range plants that belong to the Composite family. These plants are experimentally poisonous to sheep, goats, and rabbits, but field cases of poisoning are observed mainly in sheep. Sheep intoxicated with bitterweed, sneezeweed or baileya show signs of anorexia, emaciation, depression, vomition, and muscle tremors. In some cases

of bitterweed poisoning, convulsions with opisthotonus have been observed (Sperry et al., 1964; Rowe et al., 1973; Lewis and Dollahite, 1960; Kingsbury, 1964).

Hymenoxon, a toxic sequiterpene lactone (Figure 1), has been isolated and characterized from bitterweed by Kim et al. (1974, 1975) and Ivie et al. (1975). Hymenoxon has also been identified in seenzeweed (Hill et al., 1977; Ivie et al., 1976) and baileya (Hill et al., 1977). The ip LD_{50} of the crystalline compound in sheep is ca. 7 mg/kg. The iv LD_{50} and LD_{90} of partially purified preparations of the lactone for the dog are 30 and 50 mg/kg, respectively. Hymenoxon intoxication in dogs receiving an acute iv in-

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