Enzyme-Mediated Aldehyde Change in Orange Juice

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Flavor stability of unheated orange juice was assessed by following enzymically the change of volatile aldehyde content of freshly extracted orange juice incubated at 30°. The aldehyde content increased linearly for 4 hr to 130% of that in the initial juice. Sodium pyruvate stimulated accumulation of aldehyde about 20-fold. Radioactivity was recovered in acetaldehyde fraction from gc of headspace of juice incubated with sodium pyruvate-^{14}C. Freshly extracted juice contains active pyruvic decarboxylase (E.C. 4.1.1.1), which is still about 20% active after 1 hr incubation. If pyruvic decarboxylation were the only source of the aldehyde increase, about 1.4 ppm of acetaldehyde would accumulate in 4 hr at 30°.

Citrus fruits contain numerous hydrolytic and oxidative enzymes (Kefford and Chandler, 1970). When fruit is extracted for juice, most of the enzymes are rapidly inactivated by the acid released from the vacuole. However, several enzymes retain detectable activity in fresh unprocessed citrus juices: acetylesterase (Jansen *et al.*, 1947), alcohol dehydrogenase (Roe, 1972), pectinesterase (MacDonnell *et al.*, 1945), peroxidase (Davis, 1942), and phosphatase (Axelrod, 1947). Only pectinesterase is known to produce adverse quality changes in citrus juice products. It destabilizes the colloidal system of suspended particles, resulting in juice clarification.

Acetylesterase and alcohol dehydrogenase react with important citrus flavor components, namely esters, aldehydes, and alcohols, but the significance of their activity on juice quality has not been determined. We observed that the aldehyde content increased when fresh orange juice was held for several hours at 30°. Identity of the aldehydes that change and the mechanism of their formation were sought to ascertain whether these or other enzymes are involved, and to evaluate the flavor significance of the change. This paper reports the results of that investigation.

MATERIALS AND METHODS

Commercially mature Valencia oranges were obtained from a local grove. They were hand reamed the same day or after storage at 4° for up to 2 weeks. Juice was passed through two layers of cheesecloth to remove seeds and heavy pulp and was used immediately for experiments.

Alcohol and aldehyde contents were estimated enzymically with yeast alcohol dehydrogenase (ADH) (Bergmeyer, 1965) using clear serum, devoid of endogenous ADH, obtained by centrifuging juice at 15,000 × *g* for 20 min at 4°. Pyruvic acid was determined enzymically (Bergmeyer, 1965); furfural (Gutterman *et al.*, 1951) and diacetyl (Murdock, 1966) were determined chemically.

Pyruvic decarboxylase (E.C. 4.1.1.1) was assayed by a modification of a spectrophotometric method (Bergmeyer, 1965). The enzyme extract was prepared from neutralized fresh juice (pH 6.5). The juice was strained through four layers of cheesecloth and centrifuged at 15,000 × *g* for 20 min to obtain a clear extract. The reaction was followed at 340 nm with a Beckman DU spectrophotometer. In a 3-ml cuvette, a 0.5-ml portion of the enzyme extract was added to 2.5 ml of 0.02 *M* phosphate buffer, pH 6.5 (Na_2HPO_4–NaH_2PO_4), containing 0.55 μmol of NADH and 30 μmol of sodium pyruvate or salt of other 2-keto acid. After being equilibrated at 30° for 10 min, the reaction was initiated by addition of 0.005 ml of ADH (7.5×10^3 units/ml). One pyruvic decarboxylase unit is defined as the oxidation of 1 mol of NADH/min at 30°.

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Ethanol-^{14}C Experiment. To 10 ml of freshly reamed orange juice was added 1.5 mol of ethanol-*1*-^{14}C (sp act., 2.4 mCi/mmol) in 0.1 ml of glass distilled water. A 4-ml aliquot was transferred to a 25-ml lyophilizing vial and immediately film frozen with liquid N_2. Another 4-ml aliquot was incubated at 30° for 4 hr and then film frozen. From each frozen sample about 1 ml of liquid was distilled under reduced pressure (100 μ) into liquid N_2-cooled traps.

Pyruvate-^{14}C Experiment. Two 65-ml serum bottles, one containing freshly extracted orange juice and the other containing juice heated to 100° for 3 min, were sealed with rubber stoppers and placed in a 30° shaking water bath. After 5 min, 1 ml of 0.2 *M* sodium pyruvate-*UL*-^{14}C (sp act., 0.19 mCi/mmol) was added to each bottle through the rubber septum. One milliliter of headspace gas was withdrawn from each bottle 1, 30, and 60 min after the substrate was added, as described by Davis and Chace (1969), and was injected into a Hewlett-Packard 7620 A gas chromatograph.

Radioactivity Measurement. Radioactivities of juice (2 μl), distillate (2 μl), and chromatographic cuts were measured in 20 ml of scintillation mixture [5 g of diphenyloxazole and 0.1 g of 1,4-bis(2-[4-methyl-5-phenyloxazolyl])-benzene/l. of toluene] with a Packard Tri-Carb Liquid Scintillation Spectrophotometer Model 4322 or Beckman Model LS 100. The chromatographic cuts were collected by immersing the gas exit needle into the scintillation mixture.

Gas Chromatographic Separation. Juice distillates were chromatographed on Chromosorb 101 (⅛ in. × 3 ft stainless steel) at 63° in a Perkin-Elmer Model 154 Vapor Fractometer with a flame ionization detector. A 10-μl aqueous sample was injected using helium carrier gas at 25 ml/min. Sample equivalent standards of acetaldehyde and ethanol gave retention times of 2 and 5 min. In the ethanol-^{14}C experiment, the carrier gas exit port was fitted with a no. 22 syringe needle submerged in the scintillation mixture. Acetaldehyde and ethanol cuts were collected for 2 min, 1 and 4 min after injection.

Headspace gas samples were chromatographed on Porapak Q 50–80 mesh (⅛ in. × 5 ft stainless steel) at 110° in a Hewlett-Packard 7620 A Chromatograph with a flame ionization detector. A 1-ml gas sample was injected using helium carrier gas at 40 ml/min. Sample equivalent standards of acetaldehyde and ethanol gave retention times of 2.2 and 3.5 min, respectively. Acetaldehyde and ethanol concentrations in juice were calculated as follows: μmol/ml of juice = peak area of juice/peak area of standard × μmol/ml of standard.

In the sodium pyruvate-^{14}C experiment, the carrier gas exit port was fitted with a no. 22 syringe needle. Exit gas was collected in 1-min fractions into scintillation mixture.

Yeast alcohol dehydrogenase, NAD, and NADH were obtained from Boehringer Mannheim Corp. and thiamine pyrophosphate (TPP) was obtained from Sigma Chemical

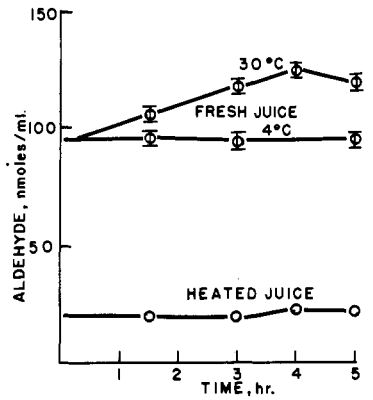

Figure 1. Change in aldehyde concentration of fresh orange juice at 30° and at 4°, and change in aldehyde concentration of heated juice. Mean ± SE from four experiments using enzyme assay; concentration expressed in nmol of acetaldehyde/ml.

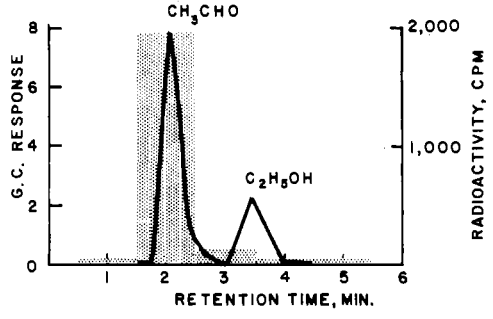

Figure 2. Recovery of acetaldehyde-^{14}C from orange juice containing sodium pyruvate-^{14}C after 1 hr at 30°. Gc chromatograph of 1 ml aliquot of headspace. One-minute samples were collected on the ½ min for radioactivity measurements. Total radioactivities of samples are shown as bar graphs.

Co. Palmitic, oleic, and linoleic acids were obtained from Hormal Institute and ethanol-*1*-^{14}C and sodium pyruvate-*UL*-^{14}C were obtained from New England Nuclear Corp. Other chemicals were obtained from Fisher Scientific Co.

RESULTS AND DISCUSSION

When freshly extracted orange juice was incubated at 30° for 4 hr, ADH assayed aldehyde increased about 30% before it declined slightly during the next hour (Figure 1). Heating juice for 2 min at 100° destroyed the aldehyde accumulating system and volatilized most of the ADH-assayed aldehyde. No aldehyde accumulated in fresh juice at 4°.

The ADH-assay is specific for aldehydes and ketones but their reactivities depend on molecular weight, with acetaldehyde the most reactive. It is 10× more reactive than butanal and 100× more reactive than either furfural or diacetyl (Roe, 1972). These latter two compounds were not detected in fresh or incubated juice. Because acetaldehyde is the major volatile aldehyde in juice (Kirchner and Miller, 1957), it is probably the major component in the aldehyde increase.

Ethanol was thought to be the source of the increased aldehyde because it is in enzymic equilibrium with acetaldehyde in the intact fruit (Bruemmer and Roe, 1971) and the enzyme, citrus alcohol dehydrogenase, was detected in fresh orange juice (Roe, 1972). However, the concentration ratio of ethanol to acetaldehyde is about 100 to 1, so that 30% increase in acetaldehyde would correspond to 0.3% decrease in ethanol. This small change was not detected in juice or juice distillates by the enzyme assay for ethanol.

Conversion of ethanol to acetaldehyde in orange juice was examined by using ethanol-*1*-^{14}C (Table I). Label in

Table I. Recovery of Ethanol-*1*-^{14}C from Orange Juice after 4-Hr Incubation at 30°

Sample	Radioactivity, cpm[a] Before	After
Juice	143,274	158,342
Distillate	521,820	564,096
Ethanol cut	471,371	496,188
Acetaldehyde cut	2,708	2,740

[a] These values represent activities in 2-μl portions of juice and distillate, and in ethanol and acetaldehyde cuts from chromatograph of 2 μl of distillate. The ethanol cuts represent 82 and 80% recovery of ethanol-^{14}C added to juice.

Table II. Effect of Various Metabolites on Aldehyde Formation

Metabolite[a]	Aldehyde, nmol/ml[b]
None	33
Pyruvic acid	380
Palmitic acid	30
Oleic acid	29
Linoleic acid	47
Glutamic acid	0
Alanine	0
Serine	0
2-Ketoglutaric acid	0

[a] Metabolites (10 μmol/ml) were added to fresh juice as aqueous solutions or homogenized aqueous suspensions (palmitic, oleic, and linoleic acids) and the juice incubated at 30° for 4 hr. [b] Aldehyde increase, determined enzymically, expressed as acetaldehyde.

the acetaldehyde cut was not increased after the juice was incubated with ethanol-*1*-^{14}C for 4 hr at 30° even though the ADH assayed aldehyde increased 30%. These data indicate that ethanol is not the major source of increased acetaldehyde in orange juice.

Pyruvic acid stimulated aldehyde accumulation in fresh unheated orange juice and is probably the native source of acetaldehyde in the juice (Table II). All compounds listed in Table II are sources of enzymically formed aldehydes through oxidation, reduction, deamination, and decarboxylation. However, only pyruvic acid and, to a slight extent, linoleic acid, were stimulatory. Orange juice sacs contain a linoleic oxidase (oxygenase) which probably hydroperoxidizes linoleic acid (Roe, 1972). Hexanal and octanal are degradation products of linoleic hydroperoxide (Keeney, 1962). Glutamic acid, alanine, serine, and 2-ketoglutaric acid suppressed aldehyde accumulation. Heated juice accumulated no aldehyde with pyruvic or linoleic acids.

Heat labile decarboxylation to acetaldehyde of added sodium pyruvate suggests that the reaction is mediated by the enzyme pyruvic decarboxylase (E.C. 4.1.1.1). The decarboxylase had been shown to be as active as alcohol dehydrogenase in neutralized extracts from juice vesicles of fresh and stored oranges and grapefruit (Davis *et al.*, 1973), but it had not been detected previously in juice.

Confirmation that acetaldehyde is the major aldehyde in pyruvic acid-added orange juice was obtained by using sodium pyruvate-^{14}C. Radioactivity in the headspace sample from orange juice incubated with sodium pyruvate-^{14}C was localized in the collection sample corresponding to the acetaldehyde peak (Figure 2). No activity was found in headspace samples from heated juice. The peak height for acetaldehyde in the headspace sample from unheated juice increased about 10× during the 1 hr incubation; the acetaldehyde peak of heated juice and the ethanol peak of heated and unheated juice did not change.

Stimulation of acetaldehyde accumulation by added so-

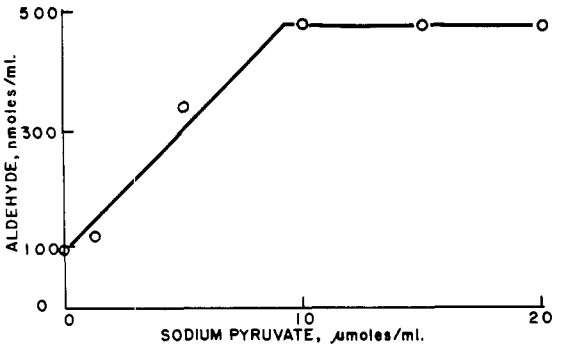

Figure 3. Pyruvic acid dependent accumulation of aldehyde. Fresh orange juice was incubated with sodium pyruvate at 30° for 2 hr. Enzyme assayed aldehyde is expressed as acetaldehyde.

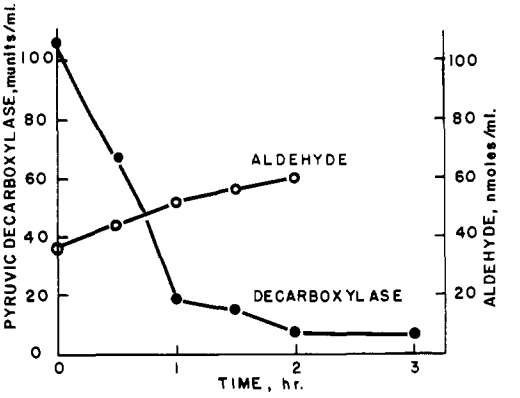

Figure 4. Decline of pyruvic decarboxylase activity in orange juice (pH 3.4) at 30°. Enzyme assayed aldehyde is expressed as acetaldehyde.

dium pyruvate suggests that native pyruvic acid (0.04 μmol/ml) is below the enzyme saturation level for maximum decarboxylation. Figure 3 shows that the saturation level for 2 hr accumulation was about 10 μmol of sodium pyruvate/ml. Above this level accumulation was constant; below this level accumulation was dependent upon sodium pyruvate concentration.

Because the level of native pyruvic acid in fresh juice is much lower than the enzyme saturation level required for maximum decarboxylation, much of the pyruvic decarboxylase in fresh juice is not substrate activated and is not involved in the reaction. Thus, the rapid decline in total pyruvic decarboxylase (assayed at saturation level of sodium pyruvate) has very little effect on the accumulation of aldehyde (Figure 4).

Pyruvic decarboxylation of native pyruvic acid (0.04 μmol/ml) could account for the 4 hr accumulation of 0.03 μmol of aldehyde in fresh juice. However, orange juice decarboxylates other 2-keto acids (Table III). If these keto acids are present in orange juice, aldehydes formed from them would also contribute to the ADH-assayed aldehyde content.

Orange juice pyruvic decarboxylase requires Mg^{2+} and thiamine pyrophosphate (TPP) for maximum activity (Table IV). Dialysis of neutralized juice against distilled water for 16 hr decreased activity about 30%. Activity was restored by adding both $MgCl_2$ and TPP. Activity responses to sulfhydryl poisons (Table V) are similar to responses reported for yeast pyruvic decarboxylase (Singer, 1955). The metal chelator, EDTA, decreased activity probably by decreasing Mg^{2+} availability.

Table III. Substrate Specificity of Orange Juice 2-Ketodecarboxylase

Substrate[a]	Relative reactivity, %
Pyruvic acid	100
2-Ketobutyric acid	34
2-Ketovaleric acid	18
2-Ketoisovaleric acid	18
2-Keto-3-methylvaleric acid	18
2-Ketocaproic acid	15
2-Ketoisocaproic acid	15

[a] Substrates were added as sodium salts at 10 μmol/ml.

Table IV. Cofactors for Orange Juice Pyruvic Decarboxylase[a]

Sample	Pyruvic decarboxylase, mU/ml
Enzyme extract	90
Dialyzed enzyme extract	58
Dialyzed enzyme extract Plus 1×10^{-2} *M* $MgCl_2$	68
Dialyzed enzyme extract Plus 1×10^{-4} *M* TPP	68
Dialyzed enzyme extract Plus 1×10^{-2} *M* $MgCl_2$ Plus 1×10^{-4} *M* TPP	97
Undialyzed enzyme extract Plus 1×10^{-2} *M* $MgCl_2$ Plus 1×10^{-4} *M* TPP	102

[a] Cofactors were added to buffer and equilibrated with extract for 5 min before reaction was initiated with ADH.

Table V. Inhibitors of Orange Juice Pyruvic Decarboxylase[a]

	Concn, *M*	% inhibition
Iodoacetate	2×10^{-4}	46
$CuSO_4$	1×10^{-5}	62
$HgCl_2$	1×10^{-5}	77
p-Chloromercuribenzoate	1×10^{-5}	89
EDTA	1×10^{-4}	20

[a] Inhibitors were equilibrated with enzyme reaction mixture for 5 min before reaction was initiated with ADH.

CONCLUSION

If acetaldehyde were the only aldehyde formed, the 33 nmol that accumulated in orange juice in 4 hr at 30° would represent an increase of 1.4 ppm. This increase would probably not be flavor detected because fresh orange juice already contains about 5 ppm.

Acetaldehyde accumulation in fresh orange juice probably contributes, however, to flavor change after canning and storage. Acetaldehyde is a precursor of acetoin and diacetyl in frozen stored vegetables (Buck and Joslyn, 1956; David and Joslyn, 1953) and canned stored vegetables (Ralls, 1959). Similar reactions probably occur in canned juice. Kirchner and Miller (1957) reported that during storage of canned orange juice at room temperature (80–90°), acetaldehyde declined and diacetyl increased. Because diacetyl has a low flavor threshold in orange juice (0.25 ppm) (Beisel *et al.*, 1954), even a small conversion from acetaldehyde during storage may be detected.

Diacetyl is a common metabolite of microbial degradation of orange juice and is responsible for specific off-flavors (Beisel *et al.*, 1954; Hill *et al.*, 1954). To retard microbial degradation before pasteurization, processors should hold the juice in refrigerated tanks while quality tests are being run. A special benefit from this practice would appear to be depression of enzymic decarboxylation and therefore less acetaldehyde for subsequent formation of diacetyl in storage.

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Physicochemical Appraisal of Changes in Egg White during Storage

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Polyacrylamide gel electrophoresis was used to separate egg white proteins of eggs stored 5, 10, or 18 weeks at 0°, 13°, or 22°. Three fractions appearing to undergo greatest change were recovered and compared by physicochemical means to similar fractions from fresh eggs. Fraction 1 was shown to be conalbumin and fraction 2 to be ovoglobulins; fraction 3 was also ovoglobulin. Increased electrophoretic mobilities of all components resulted from increase in negative charge of the proteins during storage. Electrophoretic bands and densitometric tracings were least sharp for fractions 1 and 2. Immunological and sedimentation studies and molecular weight determinations indicated that alteration such as spatial unfolding (denaturation) had occurred in fraction 1, while in fraction 2 a new, closely related but smaller molecule had split off the principal fraction. Fraction 3 was only slightly changed. Higher storage temperature had a greater effect in causing these changes in egg white proteins than did longer storage at lower temperatures.

Storage of shell eggs causes alterations in the gel structure of egg white proteins. A series of long-term storage studies was undertaken to gain information about the location and types of changes occurring.

The second of these studies (Koehler and Jacobson, 1972) evaluated changes during storage in terms of flavor deterioration. Flavor changes in yolks and whites were judged organoleptically after long-term storage and, although the yolk developed musty and rancid off-flavors, the principal deteriorative changes occurred in the flavor of the white.

In the experiments reported here, electrophoresis was used to separate the protein components of egg white. Three of the separated components were selected to be recovered for further investigation. Densitometer scanning of electrophoretic bands, immunological and sedimentation studies, and molecular weight determinations were carried out in attempts to clarify the nature of changes occurring during storage.

Techniques of electrophoresis used to separate proteins of egg white have been reviewed by Chang *et al.* (1970). They used polyacrylamide gel and published electrophoretic patterns and densitometric tracings of native and heat-treated egg white.

Electrophoresis has been used to study egg white deterioration resulting from various treatments (Baker and Manwell, 1962; Croizier and Sauveur, 1967; Donovan *et al.*, 1970, 1972; Evans and Bandemer, 1956; Evans *et al.*, 1958; Feeney *et al.*, 1963; Kloos and Schmidt, 1967). None of these investigations involved extraction and testing of separated protein components, although two different hypotheses were advanced (Donovan *et al.*, 1972; Feeney *et al.*, 1964) as to causes of the thinning of egg white. Study of separated components may help to understand the nature of changes undergone by individual proteins.

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EXPERIMENTAL PROCEDURE

Eggs used were laid by two white Leghorn hens of the same strain selected from the flocks of the Washington State University Department of Animal Sciences. Eggs were stored at 0°, 13°, or 22° and were tested fresh and after 5, 10, or 18 weeks of storage; these temperatures were chosen for comparison with a previous study in this laboratory. Two whites representing the same treatment were composited and strained through four thicknesses of cheesecloth to obtain outer thin white for all samples (Baker and Manwell, 1962). It is recognized that although study of the fresh white involved only outer thin white, after storage sampled thin white included part of what had previously been thick white. Measurement was made of pH of each composite.

Electrophoresis. Polyacrylamide gel was employed to facilitate recovery of protein components. An adaptation was devised for using the large vertical Büchler (starch-gel) mold to enable recovery of larger quantities of components. The procedure of DeVillez (1964, 1971) was used, modifying the buffers to pH 8.5 as follows: Tris-citrate stock solution, 62 g of Tris and 16 g of citric acid in 1000 ml of water; borate stock solution, 118 g of boric acid and 12 g of lithium hydroxide in 1000 ml of water; gel buffer,