Redox State of Nicotinamide–Adenine Dinucleotides in Citrus Fruit

J. H. Bruemmer

Juice vesicles from mature and immature orange and grapefruit were examined for distribution of the nicotinamide–adenine dinucleotides as the oxidized and reduced forms: NAD, NADP, NADH, and NADPH. Enzymic assay of these oxidoreductase coenzymes in extracts from the juice vesicles gave the following average values in nanomoles per 10 grams of vesicles; NAD, 60 to 118; NADH, 8.1 to 25; NADP, 7.9 to 12; and NADPH, 12 to 18. These values are comparable to concentrations of the coenzymes in apple and pear pulp, and are in general agreement with concentrations reported for metabolically active plant and animal tissues. The results suggest that citrus fruit has coenzyme capability to support synthesis of oxygenated compounds which contribute to flavor. The NADH-NAD ratio in orange and grapefruit juice increased with fruit maturity and was higher in freeze-thawed grapefruit. This increase with maturity probably reflects changes in oxidoreductase activities accompanying the Brix-acid ratio increase.

Recent studies on the composition of orange juice (Wolford *et al.*, 1963) and orange essence (Wolford and Attaway, 1967) support the earlier contention of Stanley (1958) that oxygenated aliphatic and terpenoid components are the major contributors to flavor and aroma of citrus products. Search for the origin of these components in the citrus plant led Attaway *et al.* (1967) to analyze volatile oils from leaves, petals, pistils, and stamens. These compositional studies provided no evidence of the anatomical or biochemical origin of the flavor components.

Results of several studies have failed to show that oxygenated terpenoid and aliphatic compounds are actually formed in citrus fruit, and are not transported there from leaves or some other site. Attaway and Metcalf (1966) were unable to stimulate formation of these compounds in orange juice by adding subcellular fractions of orange juice cells. Potty (1969) was unable to find hydroxymethyl-glutaryl-CoA synthase (EC. # 4.1.3.5) activity in extracts from peel, albedo, or juice vesicles. This enzyme catalyzes a key reaction in the usual synthesis of terpenoids in plant and animal tissues (Richards and Hendrickson, 1964).

As the first step toward determining whether citrus fruit can synthesize the flavor components, orange and grapefruit were examined for capability to support the type of oxidation-reduction reactions that are generally involved in the synthesis of oxygenated compounds. The pyridine dinucleotide-dependent oxidoreductases constitute the largest group of enzymes that function in this type reaction (Smith, 1967). Demonstration that citrus contain the coenzymes of these reactions would be considered evidence that the fruit can support them.

This paper contains results of analyses of orange and grapefruit juice vesicles for the coenzymes, nicotinamide–adenine dinucleotide, oxidized (NAD) and reduced (NADH), and nicotinamide–adenine dinucleotide phosphate, oxidized (NADP) and reduced (NADPH). These analyses were carried out on both mature and immature fruit. Freeze-thawed grapefruit were also analyzed for these coenzymes.

METHODS AND MATERIALS

Extraction. The reduced forms of the coenzymes, NADH and NADPH, are destroyed under acidic conditions and the oxidized forms, NAD and NADP, are destroyed in alkali. These effects of the coenzymes were used in obtaining acid extracts to assay the oxidized forms, and alkaline extracts to assay the reduced forms. Because the high acidity of citrus juices might destroy the reduced forms before they could be studied, a modification of the method of Ville (1962) was required for extraction of the coenzymes. Peeled fruit were frozen quickly in liquid nitrogen and the frozen chunks were further fractured into individual juice vesicles. The frozen vesicles were separated from seeds and section membranes and were mixed with a sufficient amount of 1*M* Tris [Tris-(hydroxymethyl) aminomethane] in frozen-bead form to neutralize the fruit acids. This mixture was powdered in a micromill (Chemical Rubber Co.) precooled with liquid nitrogen and operated at 20,000 r.p.m. Two 10-gram portions of the neutralized powder were warmed to 0°C in 25-ml. beakers. To one was added 2 ml. of 1*N* NaOH and to the other, 2 ml. of 1*N* HCl. The ice-cold beakers were transferred to magnet-stirred hot plates set at 170°C., heated for 2.5 minutes to bring the contents to 80°C, cooled immediately to 0°C. and adjusted to pH 7.0. A clear supernatant was obtained by centrifuging this heat-treated juice at 110,000 × G for 1 hour at 0°C.

The effectiveness of the extraction procedure was tested by fortifying 10-gram samples of neutralized, powdered, frozen juice vesicles with 100 μg. each of NADH and NADPH. Half the samples were treated with 1.0*N* HCl and the other half with 1.0*N* NaOH and extracted as described above. The neutralized extracts were assayed for NADH and NADPH, and after correction for sample blank, the values were expressed as % recovery of added coenzyme. In a second experiment, to study effects on the oxidized forms, 100 μg. each of NAD and NADP were used.

Enzyme Assay. The enzyme assay is based on the principle that, when the other reaction components are in excess, the rate of reduction of the coenzyme by a specific dehydrogenase is proportional to the concentration of the coenzyme. This reduction is coupled to the reduction of dichlorophenol indophenol (DCPIP) by the nonenzymic redox dye, phenazine methosulfate (PMS). The rate of reduction of DCPIP which is proportional to the rate of reduction of the coenzyme is measured in the assay. Since the coenzymes in effect "cycle" between oxidized and reduced form, both forms are assayed by the same system.

The assays described by Ville (1962) were modified for the assay of NADP and NADPH as follows: sodium isocitrate and NADP-specific isocitrate dehydrogenase (EC. # 1.1.1.42)

U. S. Fruit and Vegetable Products Laboratory, Winter Haven, Florida 33880

replaced glucose-6-phosphate dehydrogenase (EC. # 1.1.1.49) and its substrate. In addition, crystalline bovine serum albumin (BSA) was used to stabilize the dehydrogenases in both assays. Disodium ethylenediamine tetraacetic acid (EDTA) increased the sensitivity of the NADP assay so it was included in that system.

To assay the extracts for NAD and NADH, 0.02 to 0.10 ml of the extract, tinted to a pale blue with DCPIP, was added to a cuvet containing 20 μmoles of Tris at pH 7.4, 2000 μmoles of ethanol, 0.12 μmoles of DCPIP, and 330 μmoles of PMS in triple distilled water to make a total volume of 2.98 ml. The rate of change in absorbance at 600 nm and 30°C. was recorded with a Beckman DB spectrophotometer. Then was added 2.3 U of alcohol dehydrogenase (EC. # 1.1.1.1, E.C. defined as amount which reduces 1 μmole NAD per min.) in 0.02 ml. of 1.0% BSA in 0.001*M* phosphate buffer at pH 7.4. The difference in rate before and after addition of the dehydrogenase was transposed into nanograms of NAD or NADH from a standard curve prepared daily from recorded rates for concentrations from 10 to 100 ng. of NAD per cuvet.

The assay for NADP was performed similarly. Two units of isocitric dehydrogenase (E.C. defined as amount which reduce 1 μmole NADP per min.) were added to a cuvet containing 40 μmoles of Tris pH 7.4, 0.5 mg. crystalline BSA, 1 μmole EDTA, 10 μmoles of isocitrate, 1 μmole of $MgCl_2$, 330 μmoles PMS, 0.12 μmoles DCPIP and the extract to make a final volume of 3.0 ml.

Freezing and Thawing Whole Grapefruit. Eight mature grapefruit (Brix/acid = 12) were cooled from 20°C. to −7°C. (core temperature) in 16 hours at −15°C. Two were then quickly cooled to liquid nitrogen temperature and the vesicles were extracted and assayed as described. The other six were thawed by transferring them to a 20°C. room. Two fruit were removed from this room after 24, 72, and 144 hours, at which times they were peeled and quickly frozen with liquid nitrogen. The vesicles were extracted and assayed for pyridine dinucleotide coenzymes.

Titratable Acidity and Brix. Acidity was calculated from volume of standard NaOH to the end point, pH 8.2, and was expressed as % citric acid. Brix was calculated from refractometer measurements.

Fruit. *Citrus sinensis* (L.) Osbeck var. Valencia and *C. paradisi* Macf. var. Duncan were harvested from a local grove and cooled to 4°C. and held there until used. Fruit was used within 21 days.

Enzymes. Alcohol dehydrogenase (Stock No. 340-26) and isocitric dehydrogenase (type IV) were obtained from Sigma Chemical Company.

Chemicals. Tris (hydroxymethyl)aminomethane, crystallized bovine serum albumin, phenazine methosulfate, and 2,6-dichlorophenol-indophenol, grade 1, were obtained from Sigma Chemical Company (St. Louis, Mo.). Oxidized and reduced nicotinamide–adenine dinucleotide, and oxidized and reduced nicotinamide–adenine dinucleotide phosphate were obtained from Calbiochem Company (Los Angeles, Calif.).

Table I. Recovery of Dinucleotides with Heat Extraction[a]

	Per cent Recovery in	
	N NaOH	*N* HCl
Reduced forms	88% NADH 84% NADPH	5% NADH 5% NADPH
Oxidized forms	5% NAD 5% NADP	96% NAD 106% NADP

[a] Frozen orange-vesicle neutralized powder was fortified at the level of 100 μg. of dinucleotide per 10 grams of powder and extracted with hot acid or base.

RESULTS AND DISCUSSION

Selective Extraction of Dinucleotides. In first experiments the reduced forms of the dinucleotides were not detected using the method of Ville (1962). Failure to recover NADH and NADPH added to the fruit sections before homogenization suggested that the dinucleotides were lost during homogenization. The frozen-powder technique was developed to permit more intimate contact between the fruit acids and the neutralizing solution, thereby preventing loss of the acid-labile reduced forms. The technique was effective. About 98% of the NADPH and 93% of the NADH that was added to the powder (100 μg. each) was recovered in 1*N* NaOH extracts at 0°C. However, most of the oxidized form of the coenzymes was also recovered under these conditions. Since the enzyme assay measures total dinucleotide, oxidized and reduced, the NaOH extracts had to be heated to destroy the oxidized form. Heat treatment for 2.5 minutes was required. Table I summarizes the results of experiments to study effectiveness of the heat treatment in selective recovery of coenzymes in oxidized or reduced forms. Recovery of the reduced forms in 1*N* NaOH was incomplete. Over 96% of oxidized forms were recovered in acid.

Concentration of Dinucleotide Coenzymes in Citrus. Results of analyses for dinucleotides in orange and grapefruit juice vesicles are compared in Table II to other fruit. Values for the reduced forms in citrus have not been corrected for incomplete recovery. The concentrations of dinucleotides in orange and grapefruit vesicles are very similar to those in apple and pear pulp. These values for NAD and NADH are comparable to values reported for various plant leaves (Greenbaum *et al.*, 1965). Activities of a number of oxidoreductases have been reported for apple pulp (Hulme *et al.*, 1964, 1967) and their occurrence in plant leaves is well documented (Dixon and Webb, 1964). This close similarity in the concentration of the coenzymes between citrus fruit and other metabolically active plant tissues indicates that citrus fruit has the capacity to support reactions of the type shown in

Table II. Dinucleotides in Citrus Compared to Other Fruit

	Nanomoles per 10 Grams Fresh Weight						
	NAD	NADH	NADH/NAD	NADP	NADPH	NADPH/NADP	Reference
Orange, mature[a]	118 ± 5	25 ± 4	0.21	12 ± 2	16 ± 2	1.3	Present work
Grapefruit, mature[a]	60 ± 5	7.7 ± 2	0.13	9.3 ± 2	12 ± 2	1.3	Present work
Apple pulp	100	10	0.10	10	11	1.1	Rhodes and Wooltorton (1968)
Pear pulp	120	15	0.13	41	61	1.5	Rhodes and Wooltorton (1968)

[a] Each value ± standard error of the mean represents the mean of six samples.

Table III. Effect of Maturation on Redox Ratios of NAD and NADP in Juice Vesicles[a]

	% Citric acid	Brix / Acid	NADH / NAD	NADPH / NADP	(NADP + NADPH) / (NAD + NADH)
Immature grapefruit	1.6	7.5	0.07	1.5	0.31
Mature grapefruit	1.0	12	0.13	1.3	0.31
Immature orange	1.6	6	0.06	1.5	0.21
Mature orange	0.6	20	0.21	1.3	0.20

[a] Each value represents the ratio of the mean of six samples.

these other tissues to involve alcohols, aldehydes, ketones, and acids.

The data in Table II also show that the reduced form of NADP predominates. Since many biosynthetic pathways require the reducing power of NADPH, these data suggest that the redox state in citrus is favorable for reductive biosynthesis.

Change in Coenzyme Redox Ratios with Fruit Maturation. The NAD and NADP redox ratios from immature and mature orange and grapefruit are shown in Table III. These data show that the NADH/NAD ratio for both fruit increased as the acidity declined and the Brix/acid ratio increased with maturity. This change in coenzyme redox ratio may reflect the decline in respiration during maturation reported by Bain (1958). As respiration declines, the fruit has less capacity to reoxidize NADH with O_2, but the demand for NAD is probably not lessened. As a consequence, the coenzyme ratio shifts toward the reduced form. This shifting toward higher NADH/NAD ratio has been associated with maturation and the transition toward anaerobiosis in plant tissue (Yamamoto, 1963).

In animal tissue, increase in coenzyme redox ratio affects a diversion from citrate to glucose formation (Williamson *et al.*, 1967a). If a similar response obtains in citrus, it should be accompanied by an increase in the Brix/acid ratio of the juice. This suggests the rise in Brix/acid as fruit matures could be attributed to the increase in the NADH/NAD ratio.

In contrast to this shift in NADH/NAD, maturation had little effect on NADPH/NADP or on ratio of NADP + NADPH to NAD + NADH. This stable ratio of NADPH/NADP indicates that the coenzyme capacity for reductive biosynthesis in citrus does not change during maturation. The redox ratio in apple pulp declined during the respiration climacteric that accompanied ripening (Rhodes and Wooltorton, 1968). Yamamoto (1963) found that senescence in a variety of plants was accompanied by a decline in NADP redox ratio in all of the plant organs.

Concentration of Coenzymes in Frozen and Thawed Grapefruit. Slowly freezing and thawing grapefruit produced large changes in dinucleotide concentration and redox ratios in juice vesicles. The data in Table IV show that very little NADPH remained 24 hours after thawing. On the other hand, the concentration of NADH was little changed 144 hours after thawing. Slow freezing and thawing alters permeability of biological membranes (Heber, 1967); therefore, in frozen and thawed grapefruit, citric acid would probably pass more freely from the acid vacuole into the cytoplasm. Both NADPH and NADH were shown to be labile to citrus juice acids. Therefore, this loss of NADPH and retention of NADH suggests NADH is probably protected by its association with mitochondrial protein while NADPH is susceptible because of its free state in the cytoplasm. Such a distribution of NADH and NADPH has been reported for animal tissue (McLean *et al.*, 1967).

After 72 hours the concentration of the oxidized forms had decreased to less than half of the zero time values. The concentration of NADH diminished less than the oxidized form so that the ratio of reduced to oxidized NAD was almost doubled after 72 hours.

The increase in NADH/NAD of frozen and thawed grapefruit was not accompanied by a decrease in titratable acidity as was observed with maturing fruit. Acidity was constant throughout the 144-hour experiment. Nevertheless, freezing and thawing has been reported to decrease acidity of both oranges and grapefruit. Rouse *et al.*, (1958) found that fruit harvested severa' weeks after being freeze-damaged on the tree have a lower acidity than undamaged fruit from the same trees.

CONCLUSION AND APPLICATION

Demonstration that juice vesicles contain the coenzymes for oxidoreductases is evidence that many of these oxidation-reduction reactions take place in the vesicle. Some of the reactions, no doubt, are involved in the formation of specific flavor compounds. More significant to ready application, however, is the demonstration that NADH/NAD increased when acidity decreased with maturation. This accompanying change in coenzyme redox ratio suggests that the coenzyme may function as a major regulator of citrate formation, as proposed by Williamson *et al.*, (1967b). They showed that the shift of NAD toward the reduced form depressed the synthesis of oxalacetate which, in turn controls the rate of citrate formation. This relationship between acidity and coenzyme redox ratio suggests that it may be possible to control acidity of citrus fruit by controlling the coenzyme redox ratio.

Table IV. Dinucleotide Changes in Juice after Freezing and Thawing Whole Grapefruit[a]

Post-Thaw Time at 20°C., Hrs.	Nanomoles per 10-Gram Vesicles					
	NAD	NADH	NADH/NAD	NADP	NADPH	NADPH/NADP
0	53 ± 5	8.3 ± 1.6	0.16	8.7 ± 2.3	12 ± 2.2	1.4
24	55 ± 3	9.2 ± 2.1	0.17	8.3 ± 2.4	1.1 ± 0.5	0.13
72	22 ± 3	6.8 ± 2.1	0.31	3.0 ± 1.6	0.9 ± 0.5	0.30
144	18 ± 2	6.3 ± 1.5	0.35	0	0	0

[a] Thermal history is described under methods. Each value ± standard error of the mean represents the mean of four samples.

ACKNOWLEDGMENT

Technical assistance of Sidney Hughes is gratefully acknowledged. Special acknowledgment is made of the help of R. E. Berry in preparing this manuscript.

LITERATURE CITED

Attaway, J. A., Metcalf, J. F., *Proc. Florida State Hort. Soc.* **79,** 307 (1966).
Attaway, J. A., Pieringer, A. P., Barabas, L. J., *Phytochem.* **6,** 25 (1967).
Bain, J. M., *Aust. J. Bot.*, **6,** 1 (1958).
Dixon, M., Webb, E. C., "Enzymes," pp. 672–700, Academic Press, New York, N. Y., 1964.
Greenbaum, A., Clark, J., McLean, P., *Biochem. J.* **95,** 161 (1965).
Heber, U., *Plant Physiol.* **42,** 1343 (1967).
Hulme, A. C., Jones, J. D., Wooltorton, L. S. C., *Phytochem.* **3,** 173 (1964).
Hulme, A. C., Rhodes, M. J. C., Wooltorton, L. S. C., *Phytochem.* **6,** 1343 (1967).
McLean, P., Grunbaum, A. L., Brown, J., Greenslade, K. R., *Biochem. J.* **105,** 1013 (1967).
Potty, V. H., *J. Food Sci.*, **34,** 231 (1969).
Rhodes, M. J. C., Wooltorton, L. S. C., *Phytochem.* **7,** 337 (1968).
Richards, J. H., Hendrickson, J. B., "Biosynthesis of Steroids Terpenes and Acetogenins," pp. 122, 173, W. A. Benjamin, Inc. New York, N. Y., 1964.
Rouse, A. H., Atkins, C. D., Moore, E. L., *Proc. Florida State Hort. Soc.* **71,** 216 (1958).
Smith, M. H., *Nature* **213,** 627 (1967).
Stanley, W. L., Citrus Flavors in "Flavor Research and Food Acceptance," edited by L. B. Sjostrom, Ed., Reinhold Publ. Co., New York, N. Y., 1958.
Ville, C. A., *Biochem. J.* **83,** 191 (1962).
Williamson, D. H., Lund, P., Krebs, H. A., *Biochem. J.* **103,** 514 (1967a).
Williamson, J. R., Olson, M. S., Herezea, B. E., Coles, H. S., *Biochem. Biophys. Res. Commun.* **27,** 595 (1967b).
Wolford, R. W., Attaway, J. A., J. AGR. FOOD CHEM. **15,** 369 (1967).
Wolford, R. W., Attaway, J. A., Alberding, G. E., Atkins, C. D., *J. Food Sci.* **28,** 320 (1963).
Yamamoto, Y., *Plant Physiol.* **38,** 45 (1963).

Received for review May 2, 1969. Accepted July 19, 1969. U. S. Fruit and Vegetable Products Laboratory, Winter Haven, Fla. is one of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture. References to specific commercial products do not constitute endorsement.