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Carbohydrate Metabolism of Citrus Fruits

II. OXIDATION OF SUGARS BY AN AERODEHYDROGENASE FROM YOUNG ORANGE FRUITS*

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Enzymes that catalyze the oxidation of glucose and other sugars directly to the corresponding aldonic acids appear to be widely distributed in nature, since they have been found in bacteria (1–9), fungi (10, 11), algae (12), and animal tissue (13–15). In a preliminary communication (16), we have reported that the juice sacs of young oranges contain a system for the oxidation of sugars to aldonic acids. The present communication contains further information on the preparation and properties of this oxidase system. It has been shown to catalyze the oxidation of at least nine different sugars and thus appears to have a much wider range of specificity than enzymes previously investigated. Various tests suggest that the oxidation of most of these sugars may occur through the action of a single enzyme with a wide substrate specificity rather than by a family of enzymes, each with a high specificity for a single substrate.

EXPERIMENTAL PROCEDURE

Materials and Methods—Oranges (*Citrus sinensis*, var. Valencia) of various ages were obtained from Citrus Experiment Station groves and groves in Santa Ana, California. Studies to determine the distribution of the enzyme in various *Citrus* species were performed with fruit obtained from the groves at the Citrus Experiment Station. Samples were stored at 1° until used.

Unless otherwise specified, 0.25 M mannitol solution was used as solvent and suspending medium throughout the preparation and fractionation of the enzyme.

Standard manometric procedures were used to determine enzyme activity. The main chamber of the vessel contained a suitable aliquot of the enzyme preparation and 0.05 M phosphate buffer, pH 6.0, unless otherwise specified. The carbohydrate substrate was introduced from a side arm. Materials to be tested as inhibitors or activators were added from a second side arm. Alkali was routinely used in the center well. The enzyme preparations normally contained sufficient catalase or peroxidase to destroy hydrogen peroxide as it was formed.

The sugars were obtained from various commercial sources and their purity was checked by chromatography. The fructose was recrystallized from methanol before use.

The production of hydrogen peroxide by the oxidase reaction was established colorimetrically with commercial horse-radish peroxidase (Sigma Chemical Company) and *o*-dianisidine.

Nitrogen was determined by Kjeldahl digestion and direct nesslerization (17).

Chromatographic isolation and identification of reaction products was carried out with the use of the solvents butanol-acetic acid-water (52:13:35), ethyl acetate-pyridine-water (5:2:5, upper phase), and phenol-water (80:20) with Schleicher and Schuell No. 589, white ribbon filter paper. Sugars were detected on chromatograms with 1% *p*-anisidine hydrochloride in butanol (18) or a saturated solution of benzidine dihydrochloride in ethanol (19). Aldonic acids were visualized by dipping the papers in an acetone solution of potassium periodate after treatment with *p*-anisidine or benzidine hydrochloride (19). When C^{14}-labeled substrates were used, radioautograms of the chromatograms were prepared on Eastman Kodak "no-screen" x-ray film. The activity in the various chromatographic components was determined by cutting out the active areas and counting the paper sections under a Nuclear-Chicago D-47 gas flow counter equipped with a thin Mylar window. Active areas too large to fit on a single planchet were divided into smaller sections and total activity was taken as the sum of the activity of the sections.

Conditions for Extraction of Enzymes—Since the enzyme system was found localized in the juice sacs of very small fruits (see below), separation of the juice vesicles from the peels in quantity required special techniques. In small-scale studies, such as those concerning the effect of age on enzyme activity, the sacs were simply excised by hand, with a small, sharp, dental scraper. For larger preparations, where contamination by a certain amount of peel tissue would not be serious, a miniature reaming system was devised (Fig. 1). An aluminum reamer was attached to a stirring motor and the small fruit was reamed by holding it against the rapidly turning shaft. A continuous flow of extracting medium over the reamer was required to prevent clogging of the reamer vanes. For this purpose a circulating system was set up to allow the extracting solvent to be filtered, cooled, and played over the reamer as a relatively small volume was maintained.

Oranges under 10 g were cut in half transversely and the central pith removed with a small cork-borer. The sacs were reamed with 0.25 M mannitol as the suspending medium. After sufficient material was reamed to give a 10 to 20% suspension, the mixture was homogenized in a stainless steel blendor (Lourdes Instrument Corporation). For small scale preparations a glass tissue homogenizer was used. After filtering through several layers of cheesecloth, the residue was rehomogenized in a half volume and refiltered. The combined filtrates were centrifuged at 15,000 × *g*. Both the sediment and supernatant were retained since the activity was usually distributed about equally between the two fractions. The supernatant was stored at −18° or dialyzed against mannitol solution and then stored. The sediment was washed once with mannitol solution, resuspended in mannitol, and stored at −18°. At this point, the activity of the sediment

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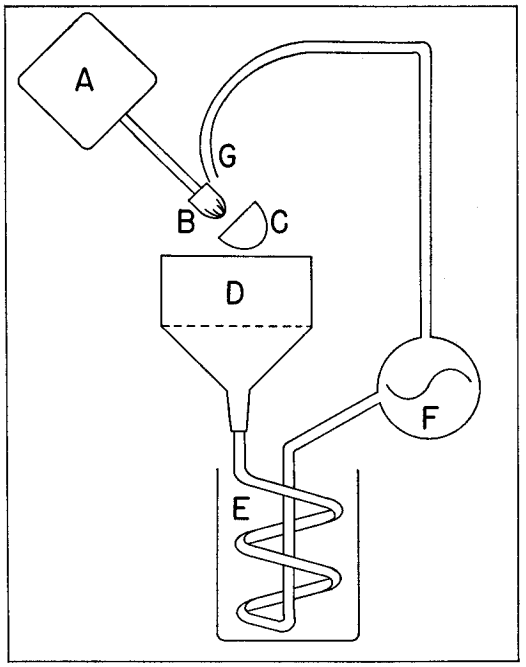

FIG. 1. Reamer for small oranges. *A*, stirring motor; *B*, aluminum reamer; *C*, orange half; *D*, buchner funnel with loose woven glass cloth filter; *E*, stainless steel cooling coils in ice bath; *F*, Rollflex circulating pump; *G*, delivery tube.

TABLE I

Fractionation of orange vesicle sugar aerodehydrogenase

Preparations obtained and assayed as in text.

Preparation	Volume	Total activity	Specific activity
	ml	*μl O₂/hr*	*μl O₂/hr/mg N*
Homogenate	50	1120	460
Clarified supernatant	45	480	410
Dialyzed supernatant	48	405	870
Methanol precipitate (0.75–1.25 vol.)	8	270	2120

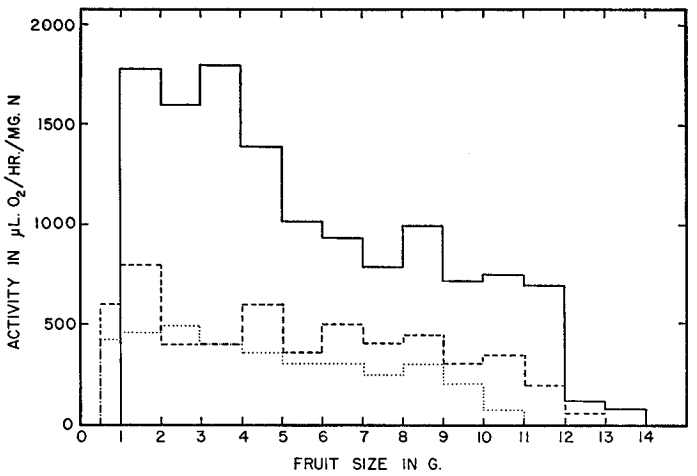

FIG. 2. Variation in activity of orange vesicle sugar aerodehydrogenase with size of fruit. Samples prepared and determined as indicated in the text. *Solid lines*, activity of dialyzed, soluble extract; *dotted lines*, activity of undialyzed, soluble extract; *dashed lines*, activity of particulate preparations.

fraction was quite stable and could be stored in the frozen state for long periods of time without significant loss in activity. The soluble material lost up to half of its activity during the period of dialysis or upon storage for a similar period. However, the remaining activity in this soluble fraction was quite stable to storage.

Effect of Variation of Extraction Procedures—Initial tests indicated that more activity was preserved by extraction in mannitol or sucrose than in water or buffer. Other agents were added to the basic extraction medium in attempts to solubilize more activity from the particulate fraction. Detergents (deoxycholeate, Alconox, Tween 80, Tween 20, Dreft, and Triton X-100 (Rohm and Haas Company)) were used at concentrations of 0.5% in the medium. With deoxycholeate or Alconox a greater proportion of activity was found in the soluble fraction. However, the presence of these detergents introduced difficulties in analysis and in fractionation, so that they were not generally used. Organic solvents, ethyl acetate, butanol, chloroform, and pyridine, had unpredictable effects without significantly improving extraction. Addition of β-thioethanol, cysteine or ethylenediaminetetraacetic acid did not affect extraction or stability during isolation.

Methanol Fractionation of Crude Extract—Twenty-five to 50 ml of the mannitol extract, buffered at pH 6.0 with 0.05 M phosphate buffer, was cooled at 0°. Over a period of 30 minutes 0.75 volumes of methanol, cooled by passing through a stainless steel tube in a dry ice bath, was added to the solution with constant stirring. The flow rate of the methanol was governed by a fine capillary tube drawn from polyethylene tubing. The precipitate formed was removed by centrifugation, another 0.5 volumes of methanol was then slowly added to the supernatant, and the suspension centrifuged. The sediment was taken up in 0.25 M mannitol solution, dialyzed for 2 hours against mannitol, and stored at −18°. This solution lost half of its activity during a storage period of 2 to 3 weeks. A summary of activity relations during fractionation is presented in Table I.

All further attempts to purify the enzyme have failed. Ammonium sulfate precipitation at various controlled acidities generally caused extensive inactivation. Negative results were also obtained in attempts to purify the enzyme by fractional elution after adsorption on DEAE-cellulose ion exchange agents (Bio-Rad Laboratories). The enzyme from oranges could not be recovered from the ion exchange agents, although, under similar conditions, the crude glucose oxidases from *Iridophycus flaccidum* or *Penicillium notatum* were stable and could be extensively purified.

Variation in Enzyme Content with Size of Fruit—Early experiments indicated that the enzyme was present only in small fruit. A study was, therefore, made to establish the actual change in enzyme during growth. Fruits ranged in size from less than a gram to greater than 20 g. Juice sacs were excised by hand into 8 ml of mannitol solution to a final volume of 10 ml before homogenizing in a glass homogenizer. After filtration through muslin and centrifugation, part of the supernatant was dialyzed and part was retained for analysis without dialysis. The sediment was washed once and resuspended in mannitol solution equal to the volume of the homogenate. Equal aliquots of each preparation were analyzed.

Fig. 2 shows that activity remains distributed between the sediment and the solution at all stages of growth. The particulate fraction and the undialyzed soluble fraction show relatively constant activity up to a weight of 10 to 11 g; as the weight in-

creases above 11 g, the activity rapidly decreases and disappears completely. Although there does not appear to be any activity in the undialyzed soluble fraction in the 12- to 14-g fruit, the dialyzed preparation shows some activity in this size range. Thus, it appears that there may be a dialyzable inhibitor in the extracts of the larger fruits.

Specificity of Enzyme—It is apparent, from the results presented in Table II, that the preparations oxidize a wide variety of sugars. The values in this table were obtained with the use of a methanol-fractionated preparation. The relative rates for most of these sugars were the same with the other preparations. Table III shows the comparison of rates for D-glucose, D-galactose, 2-deoxyglucose, and 2-glucosamine in three different fractions. The ratios of oxidative activity are constant for all these sugars in three preparations.

Additive experiments, in which two sugars were incubated simultaneously in a single vessel, showed that competition occurred between glucose and 2-deoxyglucose, glucose and mannose, or glucose and xylose. These results, presented in Table IV, suggest that a common enzyme is involved in the oxidation of all of these substrates. Additional support for this idea is found in the inhibiting action of a single agent, glucuronic acid, on the oxidation of glucose, galactose, and mannose.

Experiments with *alpha* and *beta* glucose were inconclusive because of the rather slow rate of oxidation in comparison to the rate of mutarotation of glucose.

Identification of Reaction Products—The product of enzymatic oxidation of C^{14}-glucose was identified as gluconic acid through chromatographic comparison with authentic C^{14}-gluconic acid formed by bromine oxidation of C^{14}-glucose (20). Each of the products showed a characteristic distribution of the activity into four major spots in two-dimensional chromatograms (Table V). For both the authentic and enzymatic samples the total activity was distributed in the following manner: spot A, 75 to 80%; spot B, 11 to 16%; spot C, 5 to 7%; and spot D, 2 to 4%.

For further confirmation, a portion of the C^{14}-gluconate obtained from the enzymatic oxidation of glucose was mixed with inactive gluconic acid and oxidized to arabinose with hydrogen peroxide (21). Chromatograms of the oxidation products showed perfect coincidence between the radioactive area and the colored spot obtained by reaction of the arabinose with *p*-anisidine hydrochloride.

Galactonic, lactobionic, and cellobionic acids were also prepared by bromine oxidation of the sugars. Chromatographic comparisons of the products of enzymatic oxidations of galactose, lactose, and cellobiose with the authentic samples verified that the enzymatic reaction formed the corresponding aldonic acids (Table V).

Effect of pH—Change of activity with variation of pH was determined for glucose with the use of phosphate-citrate and phosphate buffers with a dialyzed, soluble fraction. For each pH value, two flasks were used, one with and one without substrate. The actual pH of enzyme plus buffer was determined by direct measurement of a third preparation. Beginning at pH 6.5, an oxygen uptake was observed in the absence of substrate (Fig. 3). The rates observed in the vessels containing glucose are corrected

TABLE II

Relative rates of oxidation of various sugars by orange vesicle preparations

Vessels contained 0.5 ml of methanol-fractionated enzyme, 1 ml of 0.05 M phosphate buffer, pH 6.0, and 0.5 ml of sugar to give 0.11 M substrate.

Sugars*	Relative rates
D-Glucose	100
D-Galactose	92
Cellobiose	82
Lactose	74
Maltose	58
D-2-Deoxyglucose	56
D-Mannose	36
D-2-Glucosamine	18
D-Xylose	15
D-Arabinose	<1
N-Acetyl-D-2-glucosamine	<1

* Other sugars and sugar derivatives tested but showing no oxidation: L-glucose, L-arabinose, D-ribose, D-glucose 6-phosphate, D-glucose 1-phosphate, 2,3-dimethyl-D-glucose, L-fucose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, sucrose, melibiose, raffinose, dulcitol, mannitol, glycerol, and sorbitol.

TABLE III

Relative rates of oxidation of selected sugars by various fractions of orange vesicle preparations

Manometric vessels contained a suitable aliquot of enzyme preparations, 1 ml of phosphate buffer, pH 6.0, 0.5 ml of sugar to give substrate concentration of 0.11 M, and water to give total volume of 2.5 ml.

Preparation	Relative rates			
	D-glucose	D-galactose	2-Deoxy-glucose	2-Glucos-amine
Crude soluble, dialyzed	100	92	54	17
Particulate, washed	100	91	57	18
Methanol-fractionated	100	91	55	19

TABLE IV

Inhibition by sugars during oxidation by orange vesicle preparations

Vessels contained 1 ml of dialyzed, soluble enzyme, 1 ml of phosphate buffer, pH 6.0, 0.5 ml of each sugar, and water to give 3 ml of total volume. Glucose was at 0.022 M except where noted, other sugars at 0.11 M.

Sugars*	Relative rates
D-Glucose	100
D-2-Deoxyglucose	60
D-Glucose + D-2-deoxyglucose	83
D-Mannose	39
D-Glucose + D-mannose	86
D-Xylose	16
D-Glucose + D-xylose	68
D-Glucuronic acid	0
D-Glucose (0.11 M) + D-glucuronic acid	43
D-Galactose	99
D-Galactose + D-glucuronic acid	30
D-Mannose + D-glucuronic acid	10
D-Galacturonic acid	0
D-Glucose (0.11 M) + D-galacturonic acid	45

* Other sugars tested but showing no interaction: glucose 6-phosphate, fructose, and D-arabinose.

TABLE V

Chromatographic identification of aldonic acids formed by enzymatic oxidation of sugars

Authentic aldonic acids were formed by bromine oxidation of corresponding sugars. The values in the table are for R_G, the chromatographic movement relative to glucose. Solvent 1: butanol-acetic acid-water (32:13:35, volume for volume); solvent 2: phenol-water (80:20, weight per volume); solvent 3: pyridine-ethyl acetate-water (2:5:5, volume for volume, upper phase).

Material chromatographed		R_G		
		Solvent 1	Solvent 2	Solvent 3
C^{14}-gluconic acid*	A	0.95	0.18	0.19
	B	1.0	1.0	
	C	1.10	1.34	
	D	1.25	1.60	
C^{14}-glucose enzyme product*	A	0.97	0.18	0.19
	B	1.0	1.0	
	C	1.11	1.37	
	D	1.25	1.65	
Galactonic acid		0.85	1.08	0.18
Galactose enzyme product		0.85	1.08	0.17
Lactobionic acid		0.41	0.09	0.10
Lactose enzyme product		0.42	0.09	0.09
Cellobionic acid		0.48	0.11	0.10
Cellobiose enzyme product		0.49	0.11	0.10

* Values for solvent 1 and solvent 2 for C^{14}-gluconate and the C^{14}-glucose enzymatic reaction product were obtained from two-dimensional chromatograms (1st dimension with solvent 2). In this system, activity from labeled gluconate is always distributed between four major spots. All other data was taken from one-dimensional chromatograms.

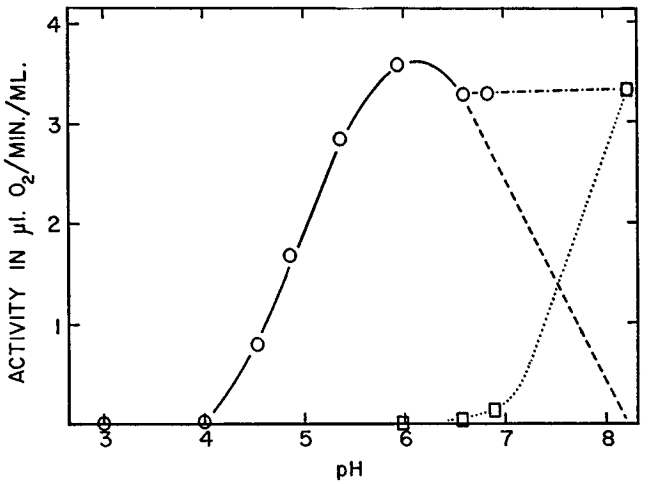

FIG. 3. Effect of pH on activity of orange vesicle sugar aerodehydrogenase. Activity determined manometrically as indicated in text using phosphate or citrate-phosphate buffers for control of pH. □, observed oxygen uptake in control flasks; ○, observed oxygen uptake in flasks containing glucose; *solid and dashed line*, curve caused by oxidation of glucose; *dotted line*, oxidation caused by endogenous substrate; *alternate dashes and dots*, observed oxidation caused by combined endogenous and glucose reactions.

for the endogenous blank. The reaction rates show an optimum around pH 6. Similar results were obtained with particulate preparations.

Activators and Stabilizers—No effect on activity or stability of normal preparations, or those partly inactivated by dialysis, was found upon incubation with flavin adenine dinucleotide, flavin mononucleotide, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, ethylenediaminetetraacetic acid, β-thioethanol, or cysteine. Extraction in the presence of the three latter compounds or dialysis or fractionation in their presence had no significant effect on the net amount of activity found at any stage of the enzyme preparation.

Inhibitors—Little or no effect on rate of oxidation of glucose was observed when sodium azide, *p*-chloromercuribenzoate, diethyldithiocarbamate, or acetate was incubated with the enzyme. Therefore, this enzyme differs from the oxidase from *Iridophycus* which was strongly inhibited by acetate and propionate (12). The reaction was inhibited by copper, mercury, silver, and barium ions.

The results shown in Table IV indicate that glucuronic and galacturonic acids are effective inhibitors. Further studies of the effect of glucuronic acid on the reaction with glucose, presented in Fig. 4, indicate that the inhibition is competitive. The reduction in over-all oxidation rate observed upon adding a sugar with a low rate of oxidation to one having a high rate (Table IV) indicates that competition also occurs between oxidizable sugars. Thus, mannose, 2-deoxyglucose, or xylose interfere with the oxidation of glucose.

Formation of Hydrogen Peroxide—If the enzyme is a flavoprotein aerodehydrogenase, similar to other glucose oxidases, formation of hydrogen peroxide during the reaction would be expected. No hydrogen peroxide accumulated during the reaction, presumably caused by the action of peroxidases and catalase, but it was possible to demonstrate the formation of hydrogen peroxide by indirect means. In the presence of hydrogen peroxide and peroxidase, *o*-dianisidine is oxidized by the peroxidase to a colored product which can then be measured spectrophotometrically (22). With glucose oxidase from fungi or from *Iridophycus* this reaction could be used as a quantitative measure of hydrogen peroxide produced. With orange vesicle preparations it was possible to

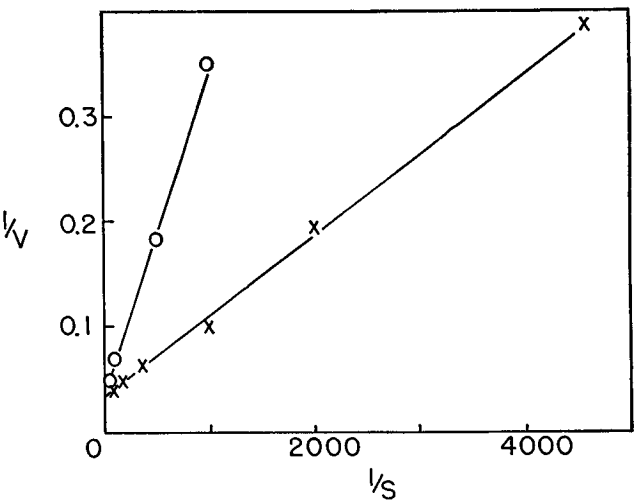

FIG. 4. Inhibition of orange vesicle sugar aerodehydrogenase by glucuronic acid. Reactions carried out with one ml of buffer, 0.1 M phosphate, one ml of dialyzed soluble enzyme, 0.5 ml of water, and 0.5 ml of glucose of a suitable dilution in the control flasks. 0.5 ml of glucuronic acid, adjusted to pH 6.0, replaced the water in the inhibited flasks. *S*, substrate concentration in moles per liter; *V*, rate of reaction in µmoles of oxygen absorbed per hour; *X*, glucose only; ○, glucose plus 0.10 M glucuronic acid.

demonstrate color formation that was dependent on the presence of glucose. However, competition between endogenous substrates and *o*-dianisidine for the peroxidase made quantitation impossible.

Reaction with Other Hydrogen Acceptors—No reduction of methylene blue, 2,6-dichlorophenolindophenol, 2,3,5-triphenyltetrazolium chloride, or cytochrome *c* could be observed either aerobically or anaerobically. Thus, it would appear that the system has a much higher specificity for the oxidant than it does for the reducing substrate. This specificity might be either for oxygen, directly, or for an endogenous acceptor which can react with oxygen but not with the dyes.

Distribution in Other Citrus Species—A number of other citrus fruits were examined to determine the distribution of this enzyme in related plants. A summary of this study is given in Table VI. Homogenates of the vesicles were prepared from each of the fruits and were tested. Endogenous respiration was determined before the addition of glucose. The figures given in the table are for the increase over the endogenous oxygen uptake. Oxygen uptake by homogenates of lemon vesicles (*C. limon*) was not enhanced significantly by addition of glucose but gluconic acid was identified as a minor product of the metabolism of C^{14}-glucose in excised, intact, lemon vesicles. Similarly, homogenates and extracts of orange albedo (white layer of the peel) were apparently inactive in manometric experiments but gluconic acid was found among other metabolic products after incubation of albedo slices with C^{14}-glucose. This would suggest that the enzyme may be active in some of the fruits or fruit parts which showed no activity in manometric experiments.

TABLE VI

Distribution of glucose aerodehydrogenase in Citrus species

Vesicles were excised from various fruits by hand, homogenized with equal amounts (weight per volume) of water, and samples taken for manometric analysis. Activity is given in μl O_2 per g fresh weight per hr.

Species tested	Fruit size range	Average yield of vesicles per fruit	Oxygen uptake
	g	*mg*	
Citrus			
sinensis (var. Valencia)	5	90	1120
(var. Navel)	8–10	350	660
macrophylla	2	100	1250
	18	1800	450
junios	5–8	130	1300
	10–15	290	410
vulgaris	5–9	150	390
macroptera	10–20	90	240
aurantifolia	5–8	125	210
aurantium	2	25	150
limetta			120
limon	9–10	200	Trace*
davoensis		170	Insignificant
grandis (Kao Panne)	40	50	Insignificant
hyptrix			Insignificant
unsheri		50	Insignificant
Poncirus trifolia	10–11	100	32

* No significant activity manometrically but gluconic acid appeared as a labeled product when intact, excised, vesicles were incubated with glucose-C^{14}.

DISCUSSION

Glucose oxidase from *Penicillium notatum* oxidizes glucose and 2-deoxyglucose at much greater rates than any other substrate, although it shows slight activity toward several other sugars (23). The enzyme from *Iridophycus flaccidum* is known to oxidize five sugars at comparable rates (12). The enzyme system from orange vesicles is capable of oxidizing at least nine sugars at significant rates and several factors suggest that this broad specificity may be contained in a single enzyme. The ratios of activity for several sugars are constant in crude or partially fractionated enzyme preparations. Glucuronic acid inhibits the action of the enzyme to a similar degree on glucose, galactose, or mannose. Mannose, 2-deoxyglucose, or xylose each acts as a substrate for the enzyme but also causes a reduction in the rate of oxidation of glucose indicating competition for sites on the same enzyme.

A single enzyme responsible for such multiple activities must have a rather loose configurational specificity. The high rates of oxidation of glucose, galactose, cellobiose, lactose, and maltose indicate that configuration or substitution on carbon 4 of the sugar has little effect on specificity. The relative rates of oxidation of glucose, 2-deoxyglucose, mannose, glucosamine, and acetylglucosamine suggest that the configuration about carbon 2 may have slight effect on activity but a steric interference may also be involved. The situation in reference to the role of carbon 6 appears confused since D-xylose may be oxidized whereas neither D- nor L-arabinose is oxidized at a significant rate. Glucuronic acid, having a carboxyl group in place of a primary alcohol group at carbon 6, cannot be oxidized but apparently retains a high degree of affinity for the enzyme as shown by its ability to inhibit the reaction with other sugars. No test of the configuration of carbon 3 has been made, although substitution of a methyl group in carbon 2 and 3 in dimethyl glucose has been shown to abolish activity. Sugars of the L-series (L-glucose, L-rhamnose, or L-fucose) did not appear to be reactive. It was not possible to determine conclusively whether β-glucose was oxidized in preference to α-glucose, since the rate of mutarotation of glucose was fast in comparison with the rate of oxidation in the preparation used.

It is also of note that the enzyme shows its maximal activity in the fruit during a period roughly corresponding to the period of maximal cell division in the vesicles (24) and disappears entirely shortly after cessation of cell division.

The function of this enzyme in citrus has not been determined. However, preliminary experiments with C^{14}-labeled glucose and gluconic acid in intact fruit and excised vesicles (25) have indicated that the orange may oxidize glucose to carbon dioxide through a pathway involving free gluconic acid.

SUMMARY

An enzyme prepared from the juice sacs of young oranges has been shown to catalyze the oxidation of at least nine sugars, D-glucose, D-galactose, D-mannose, D-2-deoxyglucose, D-2-glucosamine, D-xylose, cellobiose, lactose, and maltose, to the corresponding aldonic acids. Evidence has been presented which suggests that a single enzyme may be responsible for most of these oxidations. The enzyme appears to have the properties of a flavoprotein aerodehydrogenase, oxidizing sugars with simultaneous formation of hydrogen peroxide.

D-glucuronic acid and D-galacturonic acid act as competitive inhibitors for the oxidative reaction.

The enzyme appears to exist in the orange fruit only during, and for a short time after, the cell division phase of growth. Tests of young fruits of thirteen other *Citrus* species have shown a similar enzyme to be present in eight of them as well as in another relative, *Poncirus trifolia*.

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