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Further studies on the Natural Inhibitors Contained in Citrus Fruits Against Ascorbic Acid Oxidase

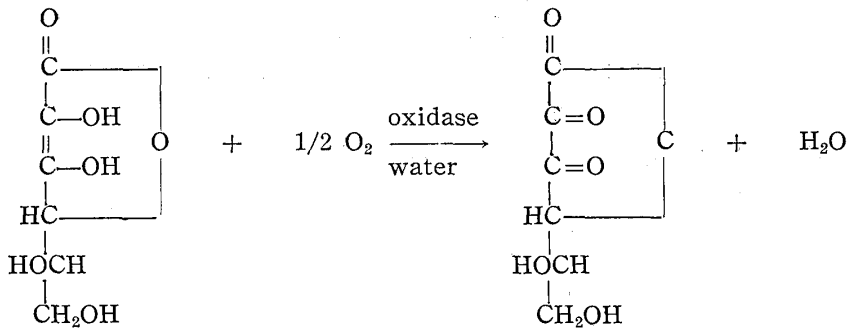
Chôten Inagaki and Atsuko Yuyama.
(稲 垣 長 典)　　(湯 山 厚 子)

Laboratory of Nutritoin Chemistry, Faculty of Home Economics,
Ochanomizu University, Tokyo, Japan

Introduction

From the time of Szent-Gyorgi,[1] numerous investigators working with a variety of plant extracts have pointed out that several plant juices possess the ability to catalyze the direct aerobic oxidation of Ascorbic acid.[2),3)] According to the investigation of Tauber,[2),4)] it was confirmed that this activity of juices is due to the presence of a specific enzyme in plant juices, namely, ascorbic acid oxidase.

Subsequent investigations on the character of the enzyme revealed that Ascorbic acid oxidase is a copper protein having non-dializable copper and that the enzyme catalyzes the direct aerobic oxidation of Ascorbic acid as the equation given below ;



l-Ascorbic acid　　Dehydroascorbic acid

The primary products of the oxidatian are Dehydroascorbic acid and H_2O. The oxidation of Ascorbic acid as catalyzed by the enzyme results in an oxygen consumption of one atom per mol of Ascorbic acid oxidized.

Ascorbic acid oxidase activity is greately inhibited by low concentrations of substances which from a stable complex with copper, such as cyanide, sodium diethyldithiocarbamate, sodium sulfide and potassium ethyl xanthate.[5] Substances of unknown composition that inhibit the activity of Ascorbic acid oxidase have been reported to be present in various plants and in human milk.[6),7)]

Inhibitors of Ascorbic acid oxidase contained in various fruits and vegetables have been studied by Inagaki, Fukuba and others,[8-10] and, a volatile organic compound in tomatoes, in organic anions, especially the chromate ion, in strawberries and anthocyanin pigment (nasunin) and globulin in egg-plants were reported to have an inhibitory activity upon Ascorbic acid oxidase. In citrus juices, two substance readily oxidized upon contact with air, the other being an inorganic compound present in ash of fruit juice were reported to have such an effect on Ascorbic acid oxidase by Inagaki and others.[11]

The present author tried to identify these two substaces, espeially an inorganic anion, however, no evidence of an inhibition by summer orange ash as well as by orange juice itself upon Ascorbic acid oxidase was obtained at least in this investigation.

Materials and Methods

1/30M l-Ascorbic acid solution——

0.5871 g of crystalline l-ascorbic acid kindly supplied by Takeda Phermarcy Co. was dissolved into a small amount of distilled water and the final volume was made up to 100 ml.

Buffer solution——

MacIlvaine's citrate-phosphate buffer series were used.——

Ascorbic acid oxidase preparation——

Ascorbic acid oxidase used in this investigation was prepared from pumpkin by the method by described by Fujita et al,[12] with some simplification. Pior to use, a small amount of this stored enzyme solution was diluted with distilled water and used as an enzyme solution.

Preparation of summer orange ash——

Peeled summer oranges were removed of their outer membrance and the segments were pulverized in an electric mixer, than squeezed and filtered through gauze. This orange juice was concentrated at a reduced pressure. The concentrate was placed in an evaporate dish to be carbonized and burned in a muffle at a temperature of about 560°C for approximately 30 hours. Yeild; 281 mg/600 ml of orange juice

Ash solution I——

500 mg of Summer orange ash was dissolved into 10 ml of distilled water and filtered through filter paper.

Ash solution II——

500 mg of summer orange ash was dissolved into 5% HCl solution and adjusted to pH 5.6 with 4% NaOH solution. The final volume was made up to 10 ml with distilled water, then fil-

tered.

Ash solution III——

500 mg of summer orange ash was dissolved into 0.1 M of citric acid and then 0.2 M of Na_2HPO_4 solution was added to adjust to pH 5.6. The final volume was made up to 20 ml with distilled water then filtered.

Orange juice——

Peeled summer oranges were removed of their outer membrance and the sements were squeezed and filtered through gauze. This was always newly prepared prior to use.

A pH 4.6 and a pH 5.6 orange juices.——

To the above mentioned orange juice a 3N KOH solation was added to adjust to pH 4.6 and to pH 5.6. Approximately 0.75 ml and 0.95 ml of 3N KOH solution were used to each of 10 ml of orange juices respectively.

The enzymatic activity was estimated in the rate of oxidation as measured by oxygen uptake with the aid of the conventional manometric method.[13] Warburg flasks equipped with two side arms were used.

All the experiments were carried at 30°C. After ten minutes of equilibration at this temperature, the materials in side arms were tipped into a main compartment of a flask and manometer readings were taken at three, four, or five minute intervals with shaking at the rate of 120/min. After the material in a side arm was tipped into a main compartment the side arm was rinsed with some of the fluid from the main compartment and this was tipped back into the main compartment.

Spectrophotometer was used to analyze the ash constituents.

Results

The result of a spectrophonometetric analysis of summer orange ash is shown in Table 1.

The inhibitory activity of summer orange ash upon ascorbic acid oxidase was examined following the procedure described in the previous paper,[11] in which Ash solution I was reported to have a complete inhibitory activity upon the enzyme. In the present study, however, a excessive amount of oxygen consumption was observed in the medium containing ash solution I. (Fig. 1, curve II) Moreover, when 1 ml of ash solution I in a side arm was tipped into the main compartment which contains 0.5 ml of Ascorbic acid solution and 1.5 ml of pH 5.6 buffer solution, a violent gas liberation was observed. (Fig. 1 curve IV) When 1.0 ml of this ash solution I

Table 1. Ash constituents of summer orange juice analysed by a spectrophotometer

sample		summer orange ash	supernatant of 10% ash soln. in N Hcl
Condition	Volts Amp. Sec.	Arc. 200 5 30	Arc. 200 5 30
Na		⫲·	+·
K		⫲·	⫩·
Ca		⫲	⫩
Mg		⫲	⫲
Fe		+	tr
P		⫩	tr·
Ti		—	—
Si		tr·	tr·
Cu		+	tr
Pb		—	—
Su		—	—
Mn		—	—
Hg		—	—
Zn		—	—
As		—	—
Sb		—	—
Al		tr	—
V		—	—
Cr		tr	—
Cd		—	—
Ni		—	—
Bi		—	—
Ag		—	—
Ba		—	—

— absence of the element
tr trace
+ presence of the element
⫩ presence of a considerable amount of the element
⫲ presence of a great amount of the element

tr<tr·<+
+<+·<⫩
⫩<⫩·<⫲

was tipped into 2 ml of a pH 5.6 buffer solution, the same gas liberation was observed. A pH measurement revealed that the pH values of ash pH values of ash solution I and of mixture of 1 ml of ash solution 1 plus 2 ml of a pH 5.6 buffer were 12 and 7.6, respectively.

In order to examine whether the gas liberated is CO_2 or not, 0.2 ml of 20% KOH solution was employed in a center well to absorb any CO_2. For it is well considered that some carbonate salts, which

liberate CO_2 when acidified are contained in the ash derived from orange juice. The result in Fig. 2 curves II and III show that the gas liberation is due to the CO_2 released on acidification. Furthermore, when 0.5 ml of Ascorbic acid solution was added to the enzyme-free medium which contained 1.0 ml of ash solution I and 1.5 ml of pH 5.6 buffer solution, the final pH of the medium became 8.6 at which Ascorbic acid was very unstable against auto-oxidation (Fig. 2 curve IV),[14] and an excessive amount of oxygen consumption was observed. (Fig. 2 curve II)

From these results we can conclude that the ash solution I is unsuitable to use for estimating its inhibitory activity on Ascorbic acid oxidase. Therefore, in subsequent trials ash solution II was used. Since the ash contained a great amount of carbonate, it was dissolved into a 5% HCl solution to liberate all the CO_2, then the solution was adjusted to pH 5.6 with 4% NaOH solution. This ash

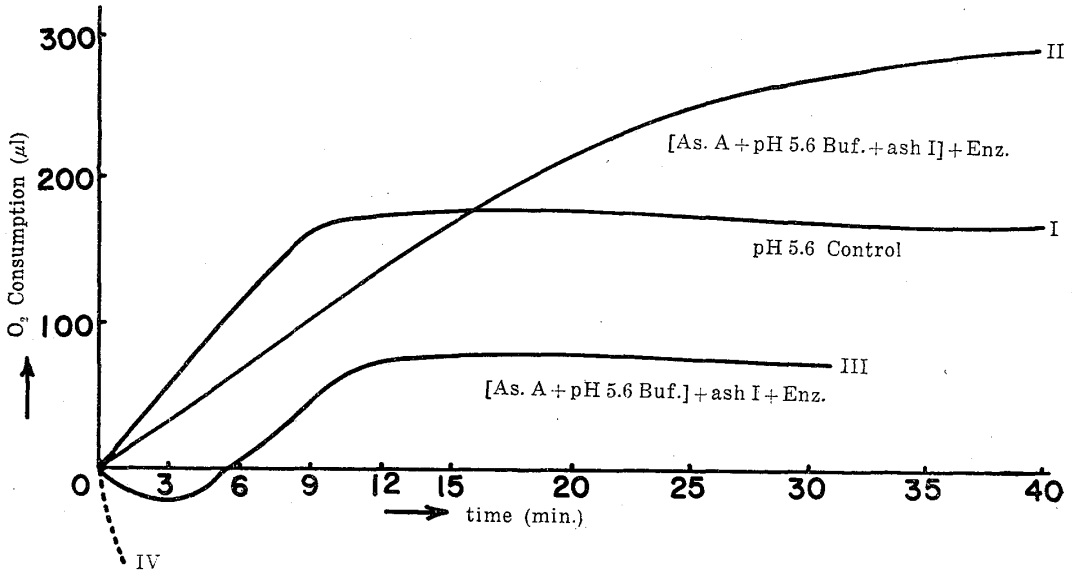


Fig. 1. Effect of ash solution I upon Ascorbic acid oxidase activity.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Ascorbic acid soln.	0.5	0.5	0.5	0.5
	pH 5.6 buffer soln.	1.0	1.0	1.0	1.5
	ash soln. I		1.0		
side arm	enzyme soln.	0.5	0.5	0.5	
	H_2O	1.0			
	ash soln. I			1.0	1.0

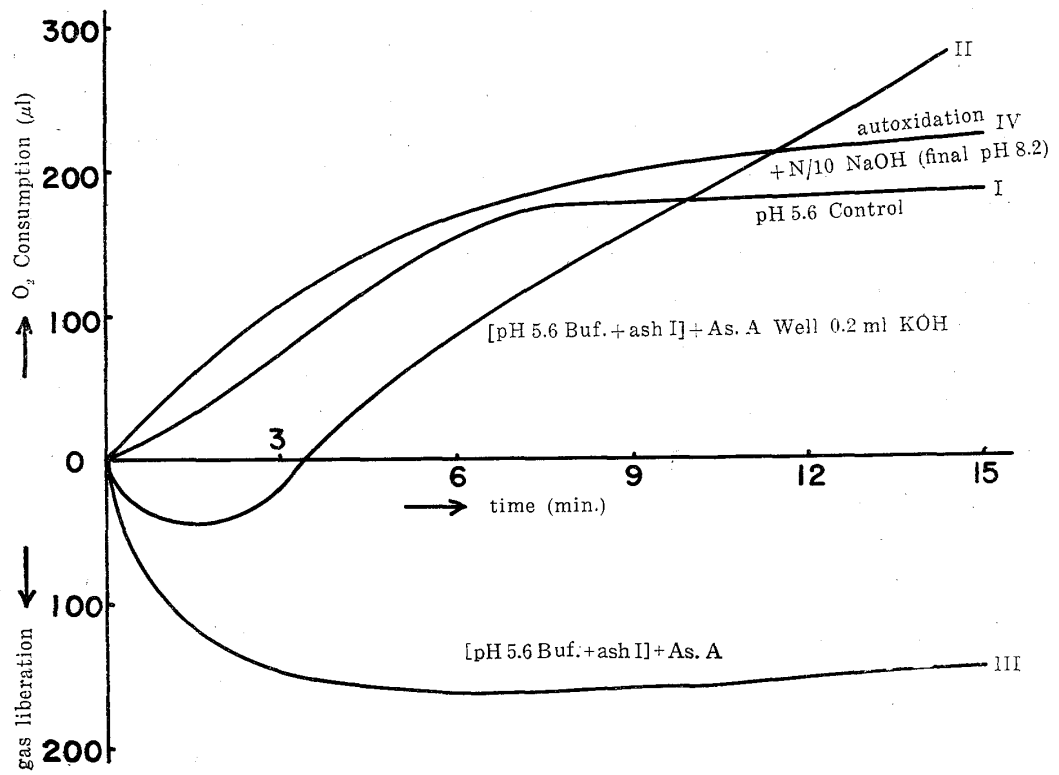


Fig. 2. Absorption of CO_2 liberated from the medium containing ash solution I by 20% KOH solution.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Ascorbic acid soln.	0.5			0.5
	pH 5.6 buffer soln.	1.0	1.5	1.5	
	ash soln. I		1.0	1.0	
	H_2O	1.0			2.0
side arm	enzyme soln.	0.5			
	Ascorbic acid soln.		0.5	0.5	
	N/10 NaOH soln.				0.5
well	20% OH soln.		0.2		
final pH		5.6	8.75	8.25	8.2

solution II was proved to have no effect upon the auto-oxidation of ascorbic acid. (Fig. 3, curve IV)

Fig. 3 curve II indicates that the initial velocity of the enzyme activity decreased by some 30% when ash solution II was added. In this case, however, after a precise determinetion of the final pH of the medium with ash solution II was always approximately 5.15, inspite of the addition of a pH 5.6 ash solution to a pH 5.6 medium. When 1 ml of a pH 5.6 asd solution was added to 1 ml of a pH 5.6

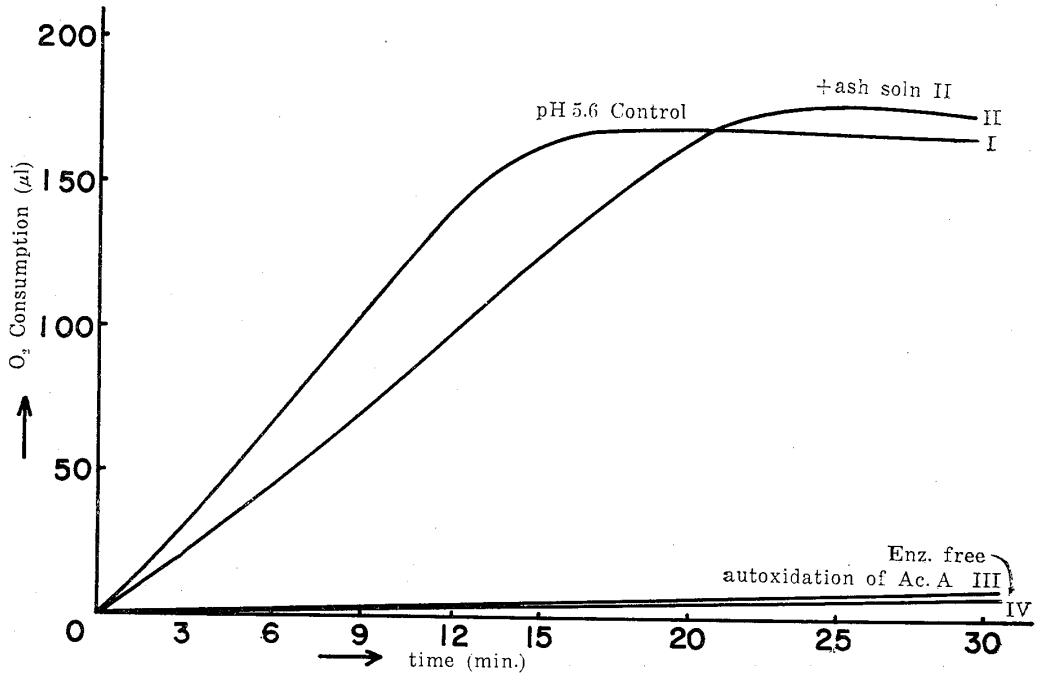


Fig. 3. Effect of ash solution II upon Ascorbic acid oxidase activity.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Ascorbic acid soln.	0.5	0.5		0.5
	pH 5.6 buffer soln.	1.0	1.0	1.0	1.0
	H_2O			1.5	0.5
side arm	enzyme soln.	0.5	0.5		1.0
	ash soln. II (pH 5.6)		1.0		
	H_2O	1.0			
	Ascorbic acid soln.			0.5	
final pH measured		5.7	5.15	5.65	5.15

buffer solution (citrate-phosphate buffer), the same pH drop was observed.

To make it clear whether or not the 30% inhibition of Ascorbic acid oxidase activity by ash solution II might be attributed to the inactivation of the enzyme due to this pH drop, a pH 5.15 buffer solution was prepared and the enzyme activity in the medium with ash solution II was compared with that in a control medium at pH 5.15. Fig. 4 curve II and III show that no clear effect of summer orange ash on the activity of Ascorbic acid oxidase takes place.

When a pH 6.0 buffer solution was used instead of a pH 5.6 buffer in the medium with ash solution II the final pH became ap-

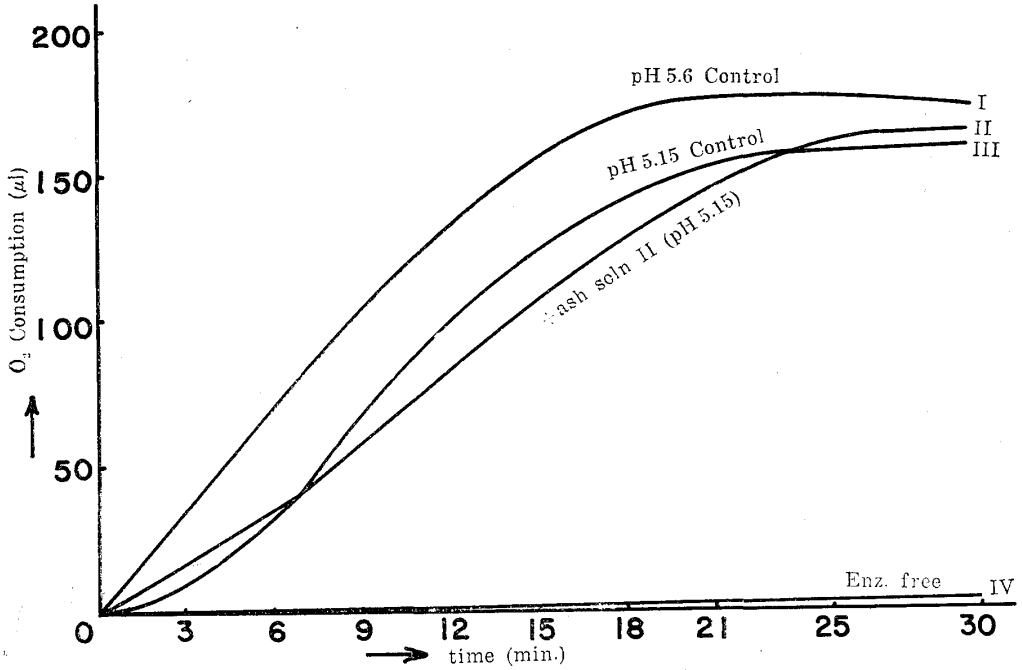


Fig. 4 Effect of ash solution II on Ascorbic acid oxidase activity at pH 5.15.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Ascorbic acid soln.	0.5	0.5	0.5	0.5
	pH 5.6 buffer soln.	1.0	1.0		1.0
	pH 5.0 buffer soln.			1.0	
side arm	enzyme soln.	0.5	0.5	0.5	
	ash soln. II (pH 5.6)	1.0	1.0		1.0
	H_2O			1.0	0.5
final pH measured		5.7	5.15	5.15	5.1

proximately 5.7. This time the activity of the enzyme in this medium was comparable to that in a control activity of the enzyme in this medium was comparable to that in a control medium at pH 5.6. In this case also, the influence of the addition of summer orange ash was not obvious. (Fig. 5 curve I and II) Fig. 5 curve III shows that ash solution III has no inhibitory effect upon Ascorbic acid oxidase activity.

Influence of pH on the activity of Ascorbic acid oxidase was studied and the result is shown in Fig. 6. It is clear that the enzyme is markedly inactivated according to the reduction of its pH value from the optium pH 5.6. Between pH 5.6 and 6.0 there appears to be no significant difference in the rate of Ascorbic acid oxidase activity. Due to the fact that aqueous solutions of Ascorbic

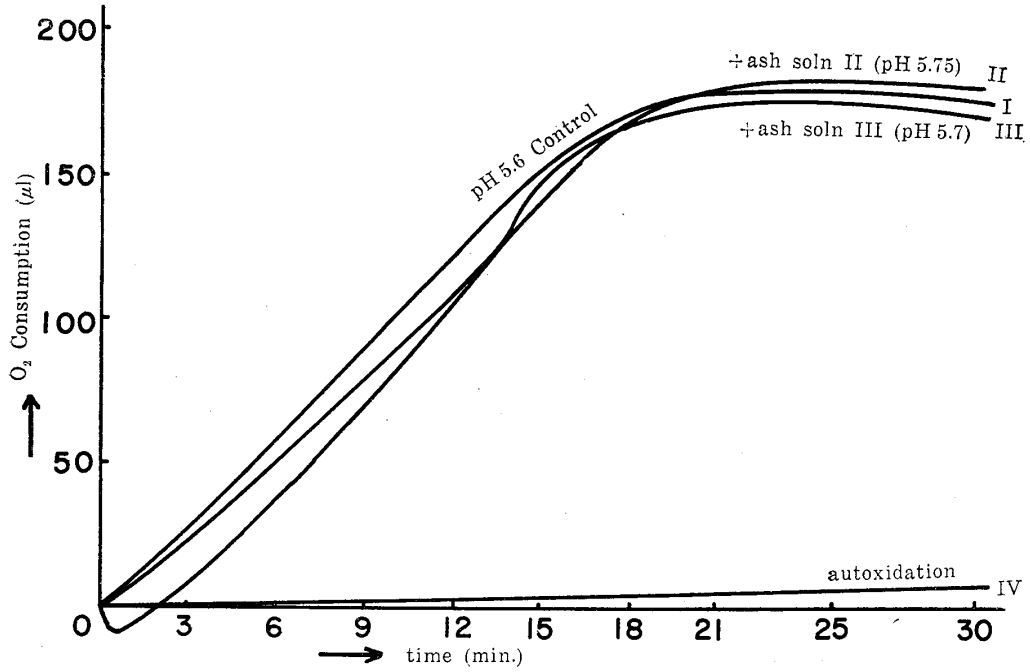


Fig. 5. Effect of ash solution II and III on Ascorbic acid oxidase activity at pH 5.6.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Acsorbic acid	0.5	0.5	0.5	0.5
	pH 5.6 buffer soln.	1.0		1.0	1.0
	H_2O				1.5
	pH 6.0 buffer soln.		1.0		
side arm	enzyme soln.	0.5	0.5	0.5	
	ash soln. II (pH 5.6)		1.0		
	ash soln. III (pH 5.6)			1.0	
	H_2O	1.0			
final pH measured		5.72	5.75	5.7	5.7

acid buffered above pH 6.6 are subjected to extensive autoxidation the result on the alkali side may not represent the right enzyme activity.

Because of the failure to find the inhibition of the activity to Ascorbic acid oxidase by summer orange ash, the inhibitory activity by orange juice itself was reinvestigated.

The oxygen uptake of the medium contained 1 ml of summer orange juice in 30 min. was zero (Fig. 7 curve III) however, the final pH of the medium was 3.2 at which Ascorbic acid oxidase is completely inactive as is inferred from Fig. 6 while the pH of

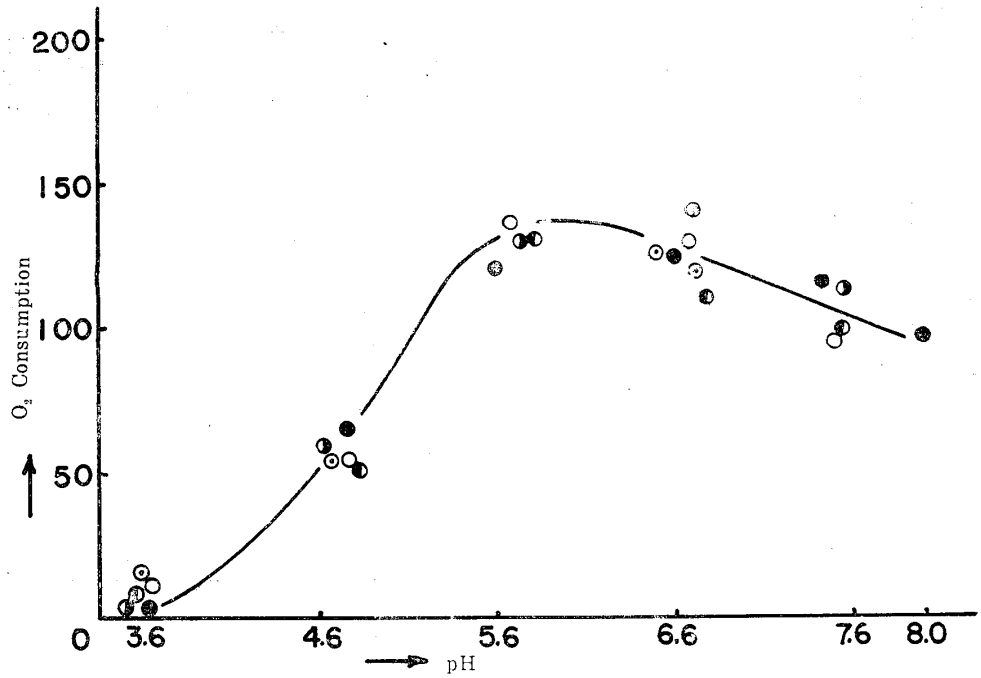


Fig. 6. Influence of pH upon the activity of Ascorbic acid oxidase from pumpkin. Each dot indicates the amount of oxygen consumption of Ascorbic acid during the first 15 min. by Ascorbic acid oxydase at varying pH. (Concentration of Ascorbic acid solution; $5/3\times10^{-5}$ M)

the orange juice was 2.6.

Since the acidity of citrus juices is due primarily to citric and maleic acid[15] it is quite natural that the pH of the buffered with citrate-phosphate buffer system drops upon the addition of even a small amount of citrus juice. Therefore a pH adjustment of the orange juice was necessary.

pH values of 4.6 and 5.6 were obtained by adding 0.75 ml and 0.9 ml of 3N KOH solution to 10 ml of orange juice respectively and the inhibitory activity was examined at these pH values. As is shown in Fig. 7 curves II and III, and Fig. 8 curves I and II, no significant difference of the enzyme activity between the medium with orange juice and that without it can be detected at both pH 4.6 and pH 5.6.

Although the presence of copper in summer orange ash was revealed by spectrophotometric analysis, (Table 1) and was also reported detected in citrus juice,[15] the oxygen uptake of Ascorbic acid which observed in 30 min. at pH 4.6 in the enzyme-free medium with 1 ml of orange juice having the same pH value (Fig. 7 curve V) was approximately equal to that obtained by the autoxidation of Ascorbic acid at the same pH value in the enzyme-free medium (Fig. 7 curve

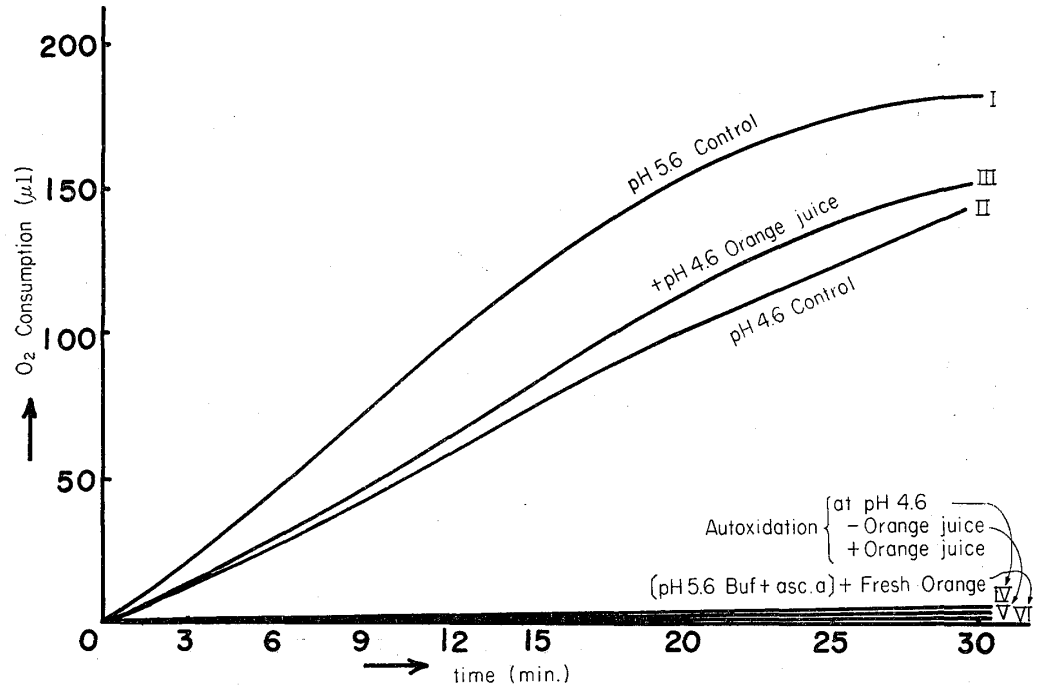


Fig. 7. Effect of a fresh and a pH 4.6 orange juice on Ascorbic acid oxidase activity.

Composition of reaction media

	No.	I	II	III	IV	V	VI
main compartment	Ascrbic acid	0.5	0.5	0.5	0.5	0.5	0.5
	pH 5.6 buffer soln.	1.0					1.0
	pH 4.6 buffer soln.		1.0	1.0	1.0	1.0	
	H_2O				1.5	0.5	
side arm	enz. soln.	0.5	0.5	0.5			0.5
	pH 4.6 orange juice			1.0		1.0	
	H_2O	1.0	1.0				
	fresh orange juice						1.0
final pH measured		5.7	4.65	4.61	4.65	4.61	3.2

IV). This was also demonstrated at pH 5.6 (Fig. 8 curves III and IV).

From these results, it seems probable that orange juice does not prosses an Ascorbic acid oxidase activity, nor does the copper therein catalyze the oxidation of Ascorbic acid.

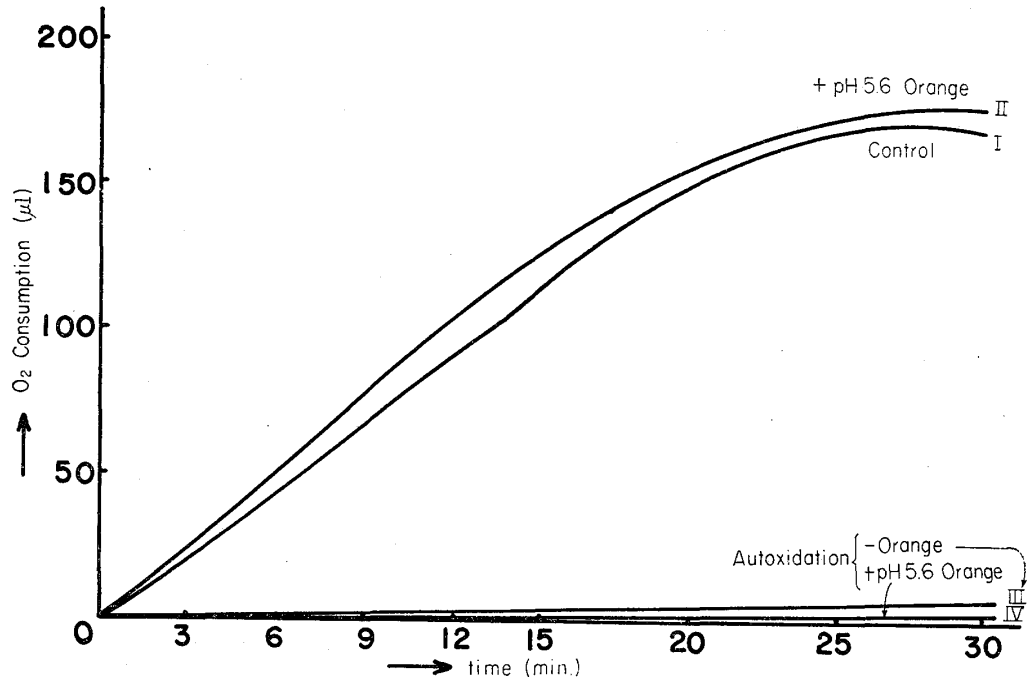


Fig. 8. Effect of a pH 5.6 orange juice on Ascorbic acid oxidase activity.

Composition of reaction media

	No.	I	II	III	IV
main compartment	Ascorbic acid soln.	0.5	0.5	0.5	0.5
	pH 5.6 buffer soln.	1.0	1.0	1.0	1.0
	H_2O			1.5	0.5
side arm	enzyme soln.	0.5	0.5		
	pH 5.6 orange juice		1.0		1.0
	H_2O	1.0			
final pH		5.7	5.55	5.6	5.55

Discussion

From the foregoing results, it seems impossible to demonstrate the existence of a specific substance or substances in orange juice as well as in the ash solutions that would have an inhibitory effect upon Ascorbic acid oxidase. Orange juice itself has no definite effect upon Ascorbic acid oxidase activity and it seems likely that the apparent inhibition by orange juice may be attributed to the low pH of the juice. As in the study by the previous report pH measurement was neglected, it seems that they did not offer a definite proof of an inhibitory activity upon the ascorbic acid oxidase.

It was reported that the ash varies in its composition with the

pressure use to extract the juice from the fruit and with the amount of pulp left in the juice.[15] In the present study, orange juice, from which the ash was derived, was obtained by pulverizing in an electric mixer, than sqeezed and filtered through gauze, whereas in the previous study by the previous report, orange segments were directly sqeezed and filtered through gauze without the aid of a mixer. The former reported that ash solution I inhibited Ascorbic acid oxidase completely, whereas in the present study an excessive amount of oxygen uptake was observed in the presence of ash solution I. One of the reasons for this discrepancy may be ascribed to the difference in the pH of the aqeous solution of ash caused by the difference in the composition of ash.

From this point of view, it is probable that the complete inhibition reported by the former may be due to the cancelling-out effect of the oxygen uptake and CO_2 liberation.

The inhibitory activity reported lost its activity when the ash solution is passed through an anion exchange resin column as well as a cation exchange resin column. When ash solution I is passed through an anion exchange resin column, carbonate ions will be absorbed so that it is reasonable that the ash solution passed through the column loose the apparent inhibitory activity upon Ascorbic acid oxidase by liberation of CO_2.

When ash solution I is passed through a cation exchange resin column, the acidity of the column will result in the liberation of CO_2, so that it will be also expected that the ash solution passed through the column again loose effect on the Ascorbic acid oxidase activity.

The pH drop observed by the addition of ash solution II to a pH 5.6 buffer may be due to the presence of phosphates in the ash, and resulting decrease of pH of the citrate phosphate buffer system owing to varying phosphate ion charges.

In any case, however, it is too early to discuss the inhibitory activity of summer orange ash on Ascorbic acid oxidase, as long as no significant evidence is obtained of the inhibitory activity of orange juice itself on Ascorbic acid oxidase.

Summary

1. Summer orange ash solutions were examined in their inhibitory activities upon Ascorbic acid oxidase activity. Inhibitory effects of a fresh orange juice and orange juices adjusted to a pH 4.6 and a pH 5.6 were studied.

2. The evidence to demonstrate the existence of a specific sub-

stance that have an inhibitory effect upon Ascorbic acid oxidase was not obtained in ash solutions. As in the study by the previous report pH measurement was neglected, it seems that they did not offer a definite proof of an inhibitory activity upon Ascorbic acid oxidase.

3. Influence of pH upon activity of Asscorbic acid oxidase was studied.

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