Natural Inhibitors Against Ascorbic Acid Oxidase Contained in Egg Apples.*

(Studies on the Natural Inhibitors Against Ascorbic Acid Oxidase. Part III)

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Introduction

In the previous papers, we have reported that the natural inhibitors against the cucumber ascorbic acid oxidase were contained in the fresh tomato juice and also in the strawberry juice, and we have also made a statement of these two inhibitors: the inhibiting action by the former juice was due to the volatile organic compounds contained in this, while this action by the latter was mainly due to the presence of the inorganic anion, especially chromate ion, contained in this juice.[1][2]

Pursuing further the investigations of the same inhibitors, we could find the presence of the inhibitors for the same enzyme in the egg apple juice.

In this paper, we would report the nature of this inhibitor and the mechanism of its inhibiting reaction.

Experimental

Reagents—

1. *l*-Ascorbic acid solution. 1.760 mg of crystalline *l*-ascorbic acid are dissolved with 70 ml. of McIlvaine's phosphate-citrate buffer solution (pH. 5.6) and *N*/2 NaOH solution is added in this solution so as to adjust the pH of the solution to 5.6 and then the volume of this solution is made up to 100 ml. with the same buffer solution. The concentration of this solution with *l*-ascorbic acid is *N*/10.

2. Buffer solution. McIlvaine's phosphate-citrate buffer (pH. 5.6) is used.

3. Cucumber juice and egg apple juice. These materials are not specially selected and the fruits, as fresh as possible, are used. The pressed juice of the grated cucumber or egg apple is here to be designated as the cucumber juice or egg-apple juice.

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4. Nasunin. The anthocyanine pigment contained in the pericarp of the egg-apple, 'nasunin', is extracted according to the Kuroda and Wada's methed[3] from the grated pericarp with methyl alcohol containing 3% of HCl. The methyl alcoholic solution of lead acetate is added to this extract, then the precipitate formed by the treatment is centrifuged off and discarded. The precipitate formed by the further addition of acetic acid to this extract is also separated out, and this precipitate (lead complex of the pigments) is again dissolved in methyl alcohol containing 2% of HCl. After the separating of the non-dissolved matters, mainly lead chloride, ethyl ether is added to alcoholic solution, and by this the pigment chloride is sedimented. The purification of this pigment is performed by the use of picrate. The molecular formula is $C_{36}H_{37}O_{19}Cl \cdot 10H_2O$.

Procedures—

All the determinations were carried out by Warburg's manometer at 30°C.

Results

Fig. 1. was obtained after the determination of the absorption of oxygen in 30 minutes by the reaction media having the compositions cited as in Table I, and from this result we can assume the presence of the natural inhibitor against the cucumber ascorbic acid oxidase in the egg-apple juice.

Table I. Compositions of the reaction media
(Temperature for reaction: 30°C)

		1	2	3	4	5	6	7
Flask	*N*/10 *l*-Ascorbic acid (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Egg-apple juice (ml.)	—	0.1	0.2	0.3	0.4	0.5	1.0
	Buffer solution (ml.)	1.5	1.4	1.3	1.2	1.1	1.0	0.5
Side arm	Cucumber juice (ml.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5

It was necessary to know whether the inhibitors were contained in the pericarp or in the sarcocarp, because the nature of those two greatly differ from each other. The next experiment was carried out separately with the pressed juice of the sarcocarp and that of the pericarp. The results, shown in Fig. 2, showed us that the inhibitors were distributed uniformly in both parts of the fruit.

It was important to know then whether the inhibitors contained in these two parts were the same one or the different ones. To clarify this point, we have done two different treatments on these juices: one

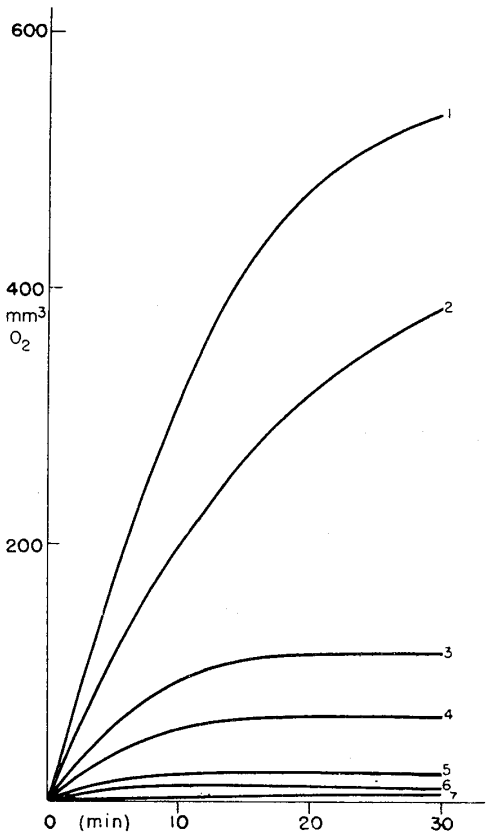

Fig. 1. Inhibition of the cucumber ascorbic acid oxidase's action by the addition of the egg-apple juice.
(Conditions for reaction were cited in Table I.)

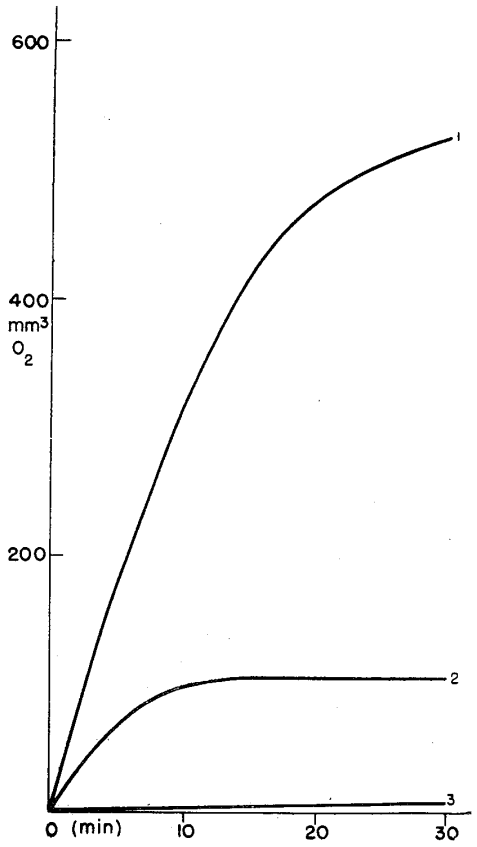

Fig. 2. Comparison of the inhibiting activities of the juice obtained from the pericarp of egg-apple with that obtained from the sarcocarp.
Compositions of the reaction media:

N/10 Ascorbic acid	0.5 ml.
Egg-apple juice	0.5 ml.
Cucumber juice	0.5 ml.
Buffer solution	1.0 ml.

1. Without egg-apple juice
(0.5 ml. of buffer solution was further added so as to make the total volume of the reaction medium 2.5 ml.)
2. Egg-apple juice obtained from the pericarp
3. Egg-apple juice obtained from the sarcocarp.

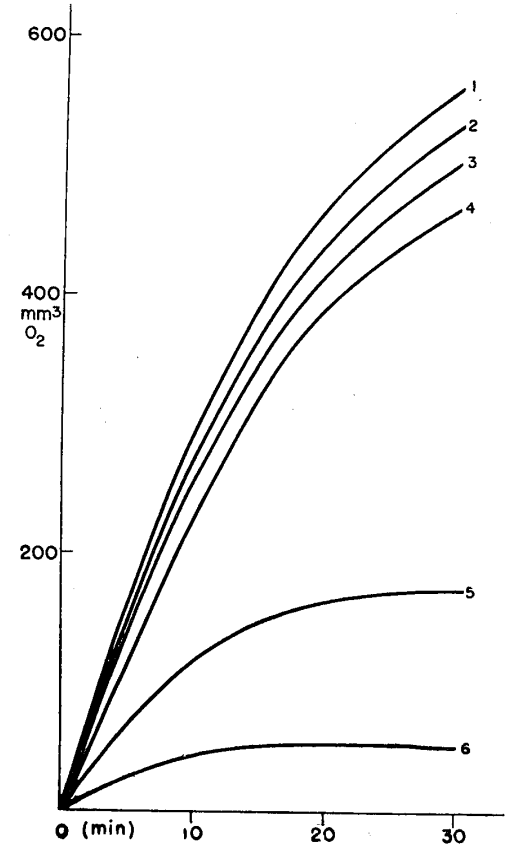

Fig. 3. The changes of the inhibiting activities by the various treatments.

Methanol treatment

The pericarp juice and the sarcocarp juice were separately added with the same volume of absolute methanol, and the precipitate formed by this was centrifuged off and the remains were then concentrated *in vacuo*.

Boiling treatment

These juices were boiled for 10 minutes, and then the precipitates formed were centrifuged off.

(Compositions of the reaction media were the same as that shown in Fig. 2.)

1. Without egg-apple juice
2. Methanol-treated sarcocarp juice
3. Buffer suspension of the precipitate formed from the sarcocarp juice by the methanol treatment.
4. Boiled sarcocarp juice
5. Boiled pericarp juice
6. Methanol-treated pericarp juice.

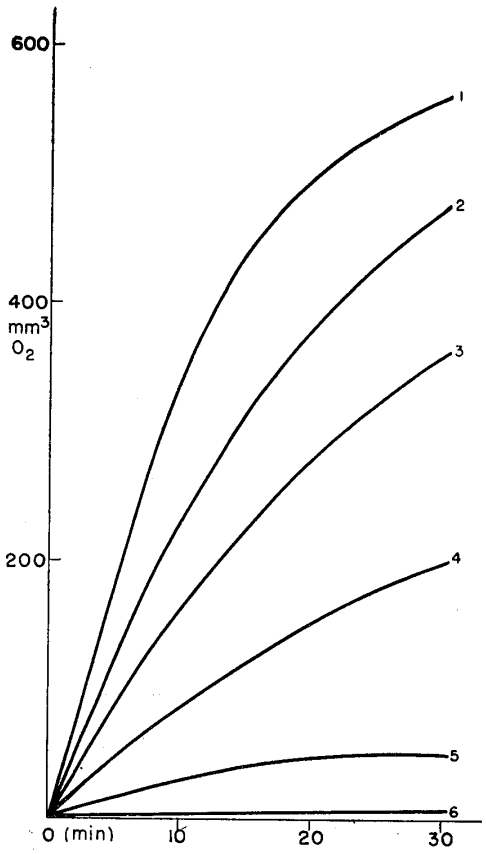

Fig. 4. Inhibition of the cucumber ascorbic acid oxidase's action by the addition of egg-apple pigment.

Concentration of the pigment:
5% aqueous solution of the crude preparation of egg-apple anthocyanin pigment.

Compositions of the reaction media:
the same as that given in Fig. 2. (The sum of the pigment solution added and the buffer solution was adjusted to 1.5 ml.)

1. Without pigment solution
2. 0.1 ml. of pigment solution added
3. 0.3 ml. 〃 〃
4. 0.5 ml. 〃 〃
5. 0.7 ml. 〃 〃
6. 1.0 ml. 〃 〃

was the methanol treatment and the other was the boiling treatment, the behaviors of two kinds of juices for these were determined under the conditions cited in Fig. 3.

From these results, it was assumed that these two juices had the different kinds of inhibitors, the one contained in the pericarp was thermostable, while the other obtained from the sarcocarp was thermolabile. The nature of the inhibitor contained in the pericarp was further investigated. From the thermostability and the solubility on 50% methanol solution, some relationship between this inhibitor and the pigment in the pericarp seemed to be present.

The inhibiting activity of the 5% aqueous solution of the crude pigment preparation, which was not recrystallized as picrate in the course of the extraction of nasunin cited in the experimental part, was therefore determined on the reaction media having the composition shown in Fig. 4.

The pigment characteristic of the egg-apple is 'nasunin', anthocyanin having purplish red color, and so the correlation between this anthocyanin and the inhibiting activity was a question to be solved. This point was made clear with the media composed of 2% aqueous solution of crystalline nasunin, *N*/10 ascorbic acid, cucumber juice, and buffer solution. The results obtained were as those shown in Fig. 5.

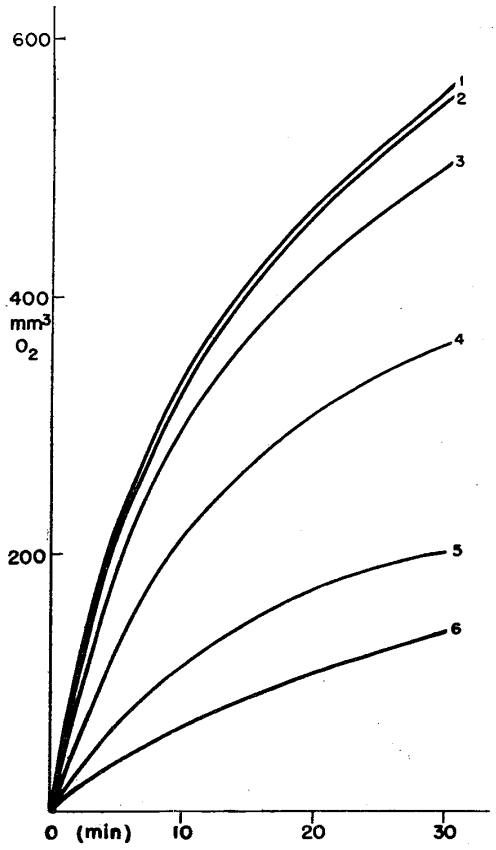

Fig. 5. Inhibition of the cucumber ascorbic acid oxidase's action by the addition of crystalline nasunin solution to the reaction media.

Concentration of nasunin solution: 2%

1. Without nasunin solution
2. Nasunin solution 0.1 ml. added
3. 〃 〃 0.3 ml.
4. 〃 〃 0.5 ml.
5. 〃 〃 0.7 ml.
6. 〃 〃 1.0 ml.

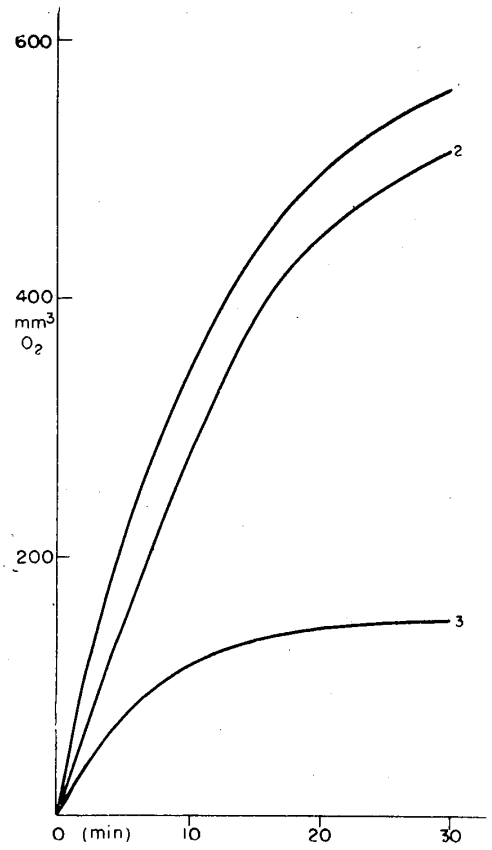

Fig. 6. Inhibition of the cucumber ascorbic acid oxidase' action by the addition of the salted and nondialysable fraction of the egg-apple sarcocarp juice.

1. Without juice.
2. Supernatant of nondialysable fraction was added
3. Buffer suspension of the precipitate separated from the nondialysable fraction was added

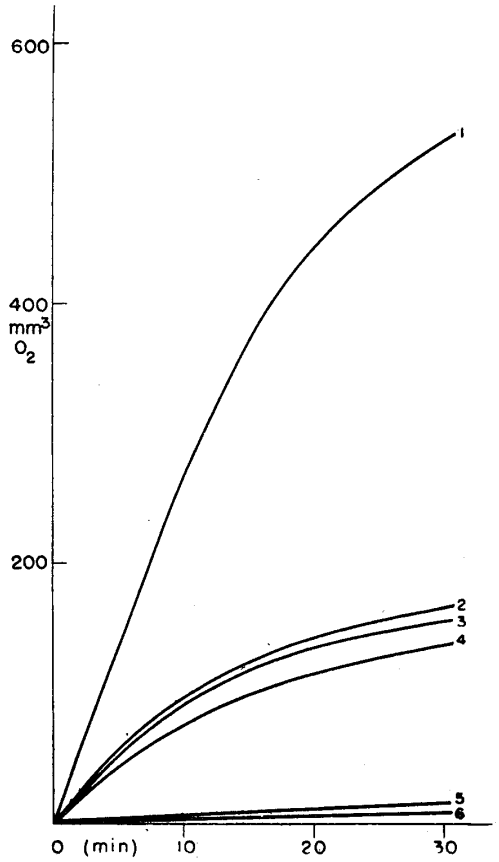

Fig. 7. Fractionation of the natural inhibitor contained in the sarcocarp of the egg-apple by the various concentrations of ammonium sulfate.

1. Without egg-apple juice
2. Buffer suspension of the precipitate formed during the 10/10–7/10 saturation against ammonium sulfate.
3. With that of 7/10–5/10
4. 〃 〃 5/10–3/10
5. 〃 〃 3/10–1/10
6. 〃 〃 1/10–0

From these results, we can assume that the inhibiting action on the ascorbic acid oxidase by the juice obtained from the pericarp of the egg-apple is mainly due to the presence of nasunin in the juice.

We have done the experiments on the nature of the inhibitor contained in the sarcocarp juice. This is, as mentioned above, thermolabile and loses its activity by the addition of methanol to the juice, so we could presume its nature as belonging to a protein-like compound. The protein fraction contained in the sarcocarp juice was salted out by the addition of ammonium sulfate (10/10 saturation), and this fraction was separated out from the mother liquor by filteration in a refrigerator. We could observe the inhibiting action with this precipitate and not with the filtrate. Then this precipitate was again dissolved with the buffer solution, and this solution was dialysed in the refrigerator against water for 24 hours, and after the dialysis, the solution contained in the celophane sack was centrifuged, and then the supernatant and the precipitate were separated out. The precipitate was again suspended in the buffer solution. The inhibiting activities of this supernatant and the buffer suspension of this precipitate were shown in Fig. 6.

The concentration of ammonium sulfate at which the most active fraction of this inhibiting protein was salted out was determined by the precipitates gathered at 0–1/10, 1/10–3/10, 3/10–5/10, 5/10–7/10 and 7/10–10/10 saturation against ammonium sulfate. The result was il-

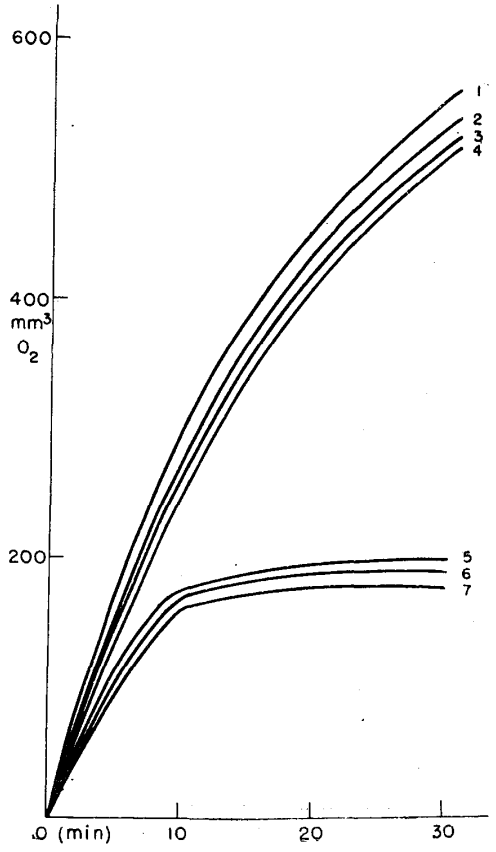

Fig. 8. The behavior of the precipitate formed during the dialysis against the various concentrations of sodium chloride.

1. Without egg-apple juice
2. Solution of 15% NaCl soluble part of this precipitate
3. Solution of 10% NaCl soluble part
4. Solution of 5% NaCl soluble part
5. Buffer suspension of 15% NaCl insoluble part
6. Buffer suspension of 10% NaCl insoluble part
7. Buffer suspension of 5% NaCl insoluble part

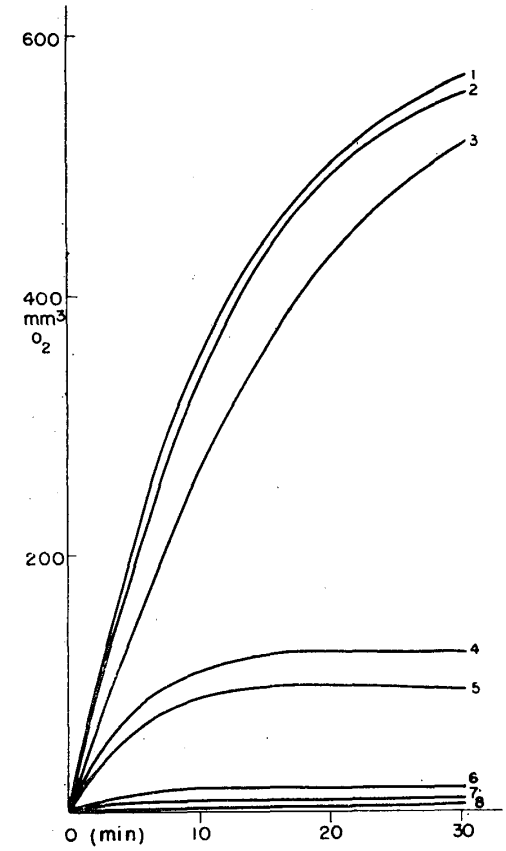

Fig. 9. Inhibiting action of egg-apple juice for the oxidation of ascorbic acid by $CuSO_4$. Compositions of the reaction media:

N/10 Ascorbic acid	0.5 ml.
M/100 $CuSO_4$	0.5 ml.
Buffer solution	1.0—1.4 ml.
Sarcocarp juice	0–1.0 ml.

1. 0.5 ml. of cucumber juice were added instead of $CuSO_4$ solution
2. Without sarcocarp juice
3. 0.1 ml. of juice was added
4. 0.3 ml. 〃 〃
5. 0.5 ml. of juice were added instead of $CuSO_4$
6. 0.5 ml. of juice were added
7. 0.7 ml. 〃 〃
8. 1.0 ml. 〃 〃

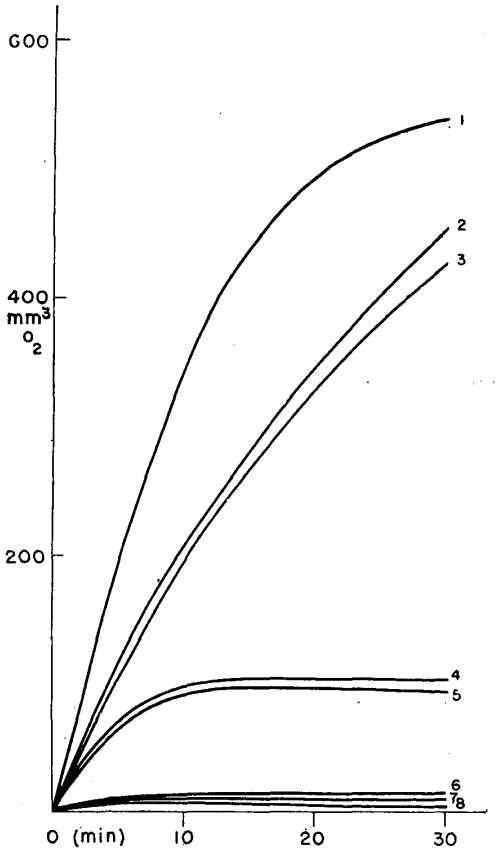

Fig. 10. Inhibiting action of egg-apple juice for the oxidation of ascorbic acid by M/1.000 $CuSO_4$ solution.
(The compositions of the reaction media and the reaction numbers were the same as those given in Fig. 9.)

lustrated in Fig. 7, and from this, it is assumed that this inhibiting protein is easily separated out with the low concentration of salt solution, and that this protein has a globulin-like property.

If this inhibiting protein is globulin, it must be dissolved with 10 % NaCl solution. To confirm this point, the precipitate obtained after centrifugation of the non-dialysate of the salted protein was treated with 5%, 10%, and 15% NaCl solution. As shown in Fig. 8, the inhibitor was not removed to the NaCl solution.

In order to ascertain the mechanism of the inhibition by this globulin-like protein for the cucumber ascorbic acid oxidase, the action of this protein for the oxidation of ascorbic acid by $M/100$ and also $M/1.000$ concentrations of $CuSO_4$ solutions were ascertained, and Figs. 9 and 10 were obtained as the results,

Discussion

As shown in the experimental part, we could find two kinds of inhibitors for ascorbic acid oxidase in egg-apple juice, one is nasunin and the other is a globulin-like protein.

Of the anthocyanin pigments experimented upon, the inhibiting activity was only recognized with nasunin and not with the other anthocyanin pigments extracted from dahlia flowers, leaves of the beefsteak plant, and also from seeds of red beans, and so, the relationship between the differences in the molecular structure of their anthocyanin and their inhibiting activity was assumed to be the interesting point to throw light on the investigation of the mechanism of this inhibiting reaction.

The inhibitor contained in the sarcocarp juice was thermolabile and salted out with only the addition of the low concentration of ammonium sulfate, and so this one is assumed to be a protein having a large molecular weight, namely, a globulun-like protein. If this protein is globulin, it must be dissoluable with 10% NaCl solution, while as shown in Fig. 8, when the salted and dialysed protein fraction was used as the purified inhibitor, the inhibitor did not pass into the solution. Nevertheless, this fact was not sufficient to prove that it was a mistake to assume this protein as globulin, but it was also assumed that the decrease in solubility against NaCl solution was caused during the salting out and the dialysis owing to the denaturation of the protein. On this point, we must make further investigation to confirm whether the facts are correct.

It is worth noting here that, as illustrated in Figs. 9 and 10, curve 5, egg-apple juice itself had some activity as ascorbic acid oxidase, and in the following reaction conditions with the reaction medium composed of $N/10$ ascorbic acid 0.5 ml., buffer solution 1.5 ml., and egg-apple

juice 0.5 ml., the reaction temperature, 30°C. and the reaction time, 30 minutes, this system could absorb 100 $\mu l.$ of oxygen, and under the same conditions, when the egg-apple juice was replaced by the cucumber juice, this value increased to 560 $\mu l.$, so the egg-apple juice had about one fifth of the ascorbic acid oxidase activity of the cucumber juice. The reason why these two kinds of oxidase react to interfere with each other was the most important point, and we shall explain this in the following paper.

The sarcocarp juice also had the inhibiting activity on the ascorbic acid oxidation with $CuSO_4$. This was probably due to the combination of this protein with Cu, and so the inhibition of this sarcocarp juice on the ascorbic acid oxidate's action was perhaps performed by the combination of the globulin and the prothetic group of the enzyme.

Summary

Our investigations have shown that there exist natural inhibitors in the egg-apple juice which act upon the ascorbic acid oxidase.

1. The egg-apple juice has two different kinds of inhibitors, and the one is thermostable, while the other is thermolabile.
2. The thermostable inhibitor is mainly contained in the pericarp, and the inhibiting action was performed by nasunin, that is the anthocyanin pigment contained in this, while anthocyanin pigments extracted from the other materials have no inhibiting reaction.
3. The thermolabile inhibitor is mainly contained in the sarcocarp, and this inhibitor is assumed to be globulin owing to its behaviors with regard to $(NH_4)_2SO_4$ and also the dialysis.
4. The sarcocarp juice showed an inhibiting activity against the ascorbic acid oxidation with $CuSO_4$, and from this fact it is assumed that this inhibitor make its activity by the combination with the prothetic group of the oxidase.

Literature

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3. C. Kuroda and M. Wada, *Bull. Chem. Soc. Japan*, **11**, 283 (1936)

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