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The tissue functions of vitamin C from a chemical point of view are still essentially unknown, although many empirical physiological and histological relationships have been clearly established (1, 2). Its sensitivity to aerobic oxidation when copper is present as a catalyst (3, 4) is perhaps its most striking characteristic *in vitro*.

Subsequent to the description of a "hexoxidase" in cabbage leaves by Szent-Györgyi (5), several investigators have reported the existence of an "ascorbic acid oxidase" in other plants and fruits. Such an "enzyme" was reported to be present in apple juice by Zilva (6), in the pods of the drumstick-tree by Srinivasan (7), and in cauliflower juice by Hopkins and Morgan (8). Kertesz, Dearborn, and Mack (9) have studied the destruction by heat of an oxidizing agent in a number of vegetable extracts. Tauber, Kleiner, and Mishkind (10) have reported the concentration of a similar factor from Hubbard squash. Although the latter workers suggested that their "enzyme" was of a different character than the original "hexoxidase" of Szent-Györgyi, the evidence was not conclusive, as shown by the statement of Hopkins and Morgan (8) that there were "insufficient grounds" for distinguishing these enzymes.

Barron, Barron, and Klemperer (11) have questioned the enzymic nature of the catalyst. The more consistent inhibition produced by cyanide compared to 8-hydroxyquinoline on a series

of vegetable and fruit juices led them to suggest that, "It is . . . quite probable that hemochromogens are the main catalysts for the oxidation of ascorbic acid in these biological fluids possessing no inhibitory mechanisms." McFarlane (12) reported that sodium diethyldithiocarbamate served as a copper inhibitor against the aerobic oxidation of vitamin C in aqueous solution.

An observation in this laboratory that diethyldithiocarbamate completely inhibited the aerobic oxidation of ascorbic acid in cucumber juice, which is very active catalytically, led to a study based on the hypothesis that copper was chiefly responsible for the catalytic effect of "oxidase" preparations.[1] The problem was approached from two points of view: (a) the effect of a series of copper inhibitors upon typical "enzymes," and (b) a comparison of the reported "enzymes" with copper plus proteins.

EXPERIMENTAL

The rate of oxidation of ascorbic acid was measured manometrically in air-filled Warburg vessels. The "enzymes" and copper were invariably placed in the main vessel and ascorbic acid solution in the side arm, with a total volume of 3.3 ml. including 0.3 ml. of 20 per cent KOH in the alkali cup. Unless otherwise indicated, 0.01 mM of ascorbic acid was used, equivalent theoretically to 112 c.mm. of oxygen under standard conditions. To avoid contamination by copper, Kahlbaum's phosphates were employed, and triple distilled water from all-glass Pyrex stills was used for making up solutions and rinsing apparatus. The crystalline ascorbic acid was oxidized very slowly in this kind of water (approximately 5 c.mm. of O_2 per hour). Experiments were performed at 37°. The rate of reaction was expressed as c.mm. of O_2 per hour, extrapolated when necessary from the linear rate obtained for the major part of the reaction.

The rate of catalysis established by copper-abumin or copper-gelatin mixtures was approximately proportional to the concentration of copper and inversely proportional to the concentration of protein (cf. Fig. 1).

The experimental protein solutions contained sufficient copper

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[1] The authors wish to thank Dr. M. O. Schultze for the copper analyses reported in this paper, and Professor E. B. Hart for the samples of copper amide biuret and copper hematoporphyrin.

to give an oxidation rate comparable to that of the "enzymes." As shown in Table I, the concentration of copper in the enzyme preparation was about the same as that in the copper-gelatin solutions. In the albumin solutions the copper concentration was higher. The method of Fischer and Leopoldi (13) was used in making copper analyses.

Although there was sufficient copper in some of the buffers to cause a slow oxidation of ascorbic acid, this effect was negligible in the presence of protein. No difference in the rate of oxidation
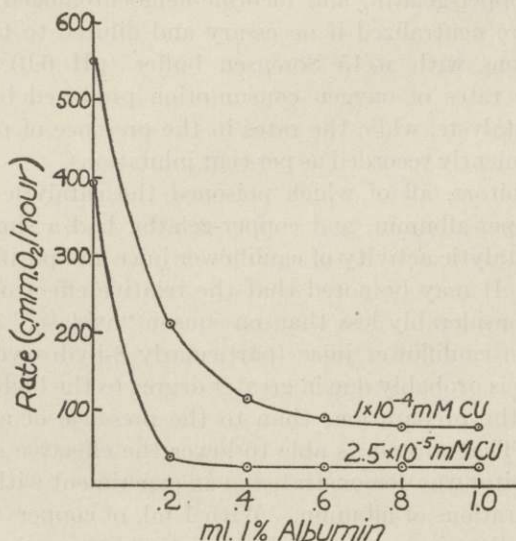

Fig. 1. Effect of ovalbumin on copper catalysis of the oxidation of ascorbic acid. Ascorbic acid, 0.01 mM, pH = 5.8, M/15 PO_4 buffer, $T = 37°$.

by the "enzymes" or copper-albumin could be detected when small or large quantities of buffer were used.

Preparation of Enzymes—Although only a few experiments dealt with cabbage and cucumber press-juice, "purified squash oxidase" and cauliflower press-juice were studied in more detail.

The final dried acetone precipitate of squash "oxidase," prepared as described by Tauber, Kleiner, and Mishkind (10), was only slightly soluble in water. Although the soluble portion was active, boiling for 5 minutes did not destroy the activity. Furthermore, the greater part of the activity was associated with material that was coarsely suspended as altered protein. For this reason

it was preferred to suspend the final acetone precipitate in water, aerate until free of acetone, and utilize this preparation as squash "oxidase." By determining the units of activity in the alcohol extract and the final product it was found that the preparations were of equal strength to those of the original investigators.

Effect of Inhibitors—Seven inhibitors that have been used for qualitative and quantitative tests for copper were examined for their inhibitory power on the press-juice of cabbage and cauliflower, the squash "oxidase" described above, copper, copper-albumin, copper-gelatin, and nicotine-hemochromogen. The inhibitors were neutralized if necessary and diluted to the desired concentrations with M/15 Sørensen buffer (pH 6.0). Table I records the rates of oxygen consumption produced by the unpoisoned catalysts, while the rates in the presence of poisons are more conveniently recorded as per cent inhibitions.

The inhibitors, all of which poisoned the catalytic action of copper, copper-albumin, and copper-gelatin, had a similar effect upon the catalytic activity of cauliflower juice and purified squash "oxidase." It may be noted that the relative effect on cabbage juice was considerably less than on squash "oxidase," and somewhat less on cauliflower juice (particularly 8-hydroxyquinoline). This finding is probably due in greater degree to the higher protein content of the preparations than to the presence of non-copper catalysts. That protein is able to lower the effective concentration of inhibitor was demonstrated in an experiment with high and low concentrations of albumin. With 1 ml. of copper + 1 ml. of 1 per cent albumin as the catalyst, diethyldithiocarbamate and 8-hydroxyquinoline produced inhibitions of 92 and 94 per cent respectively, but with 1 ml. of copper + 1 ml. of 6 per cent albumin as catalyst, there was only 40 and 45 per cent inhibition. The latter protein concentration corresponded approximately to the per cent of solids present in the cauliflower press-juice, whereas squash "oxidase" contained considerably less solids. Therefore the amount of inhibitor used was increased to the quantities reported in Table I, although in nearly all cases much less inhibitor produced comparable effects. The effect of these higher concentrations of inhibitors could hardly be due to a non-specific blocking of an enzyme surface, since (a) the seven compounds selected had copper-combining capacity as their only common

characteristic, and (b) much higher concentrations of non-specific inhibitors such as urethane and sodium fluoride had little or no effect.

TABLE I

Effect of Copper Inhibitors

Ascorbic acid, 0.01 mm; $T = 37°$; pH = 6.0 ± 0.1.

Inhibitor	Catalysts						
	Cu, 3×10^{-5} mm	Cu-albumin, 2×10^{-4} mm Cu	Cu-gelatin, 6×10^{-5} mm Cu	Nicotine-hemo-chromogen, 3×10^{-4} mm hemin	Cauli-flower press-juice, 5×10^{-5} mm Cu	Squash "oxidase," 3×10^{-5} mm Cu	Cabbage juice
	Rate of O_2 consumption, c.mm. O_2 per hr.						
None	420	260	240	320	220	290	90
	Per cent inhibition						
Diethyldithiocarbamate, 3×10^{-3} mm*	100	96	98	0	76	100	68
8-Hydroxyquinoline, 7×10^{-3} mm	99	92	96	0	42	94	62
Pyridine, 1 mm, KCNS, 1 mm	92	90	98	−37†	84	85	50
Sodium cyanide, 0.04 mm	98	95	97	48	88	95	56
Potassium ferrocyanide, 0.025 mm	93	87	90	0	58	89	32
Potassium ethyl xanthate, 0.06 mm	95	78	88	0	83	97	68
Sodium sulfide, 0.025 mm	98	86	85	8	91	96	35

* The quantities of copper, ascorbic acid, and inhibitors recorded in this table represent the total amounts in the 3 cc. volume of reactants. The total amount of albumin and gelatin in 3 cc. was 10 mg.

† 37 per cent increase in O_2 consumption rate.

The results with inhibitors are somewhat different from the results reported by Barron *et al.* (11) in which cabbage and squash juice were not inhibited by 8-hydroxyquinoline, although markedly affected by cyanide. The apparent discrepancy may be due

to different inhibitor to protein ratios in the preparations used. Cyanide is an active inhibitor against both copper and hemochromogen. The results recorded in Table I show that nicotine-hemochromogen was not affected by the series of inhibitors used. The high concentration of pyridine in pyridine-KCNS apparently produced a more effective concentration of pyridine hemochromogen.

Effect of Heat—Although the catalytic power of copper salts is not inhibited by boiling, the "enzymes" are heat-sensitive. Copper-albumin showed an 84 per cent decrease in its catalytic power after 5 minutes heating at 100°, parallel with a 75 to 85 per cent destruction of the cauliflower, squash, and cucumber "enzymes." That this phenomenon is coincident with coagulation of protein is suggested by (a) the heat stability of the non-coagulable copper-gelatin, (b) the stability of cucumber "enzyme" at 55° (no coagulation) and its destruction at 100° (coagulation), and (c) the fact that 88 per cent of the original copper was found by analysis in the heat-precipitated cauliflower protein, and 48 per cent in the case of squash. Since this precipitated copper is practically inactive, the heat coagulation of protein must involve a binding of the copper in a non-catalytic form. It is also of interest that albumin, when heat-coagulated in the absence of copper, has a greater inhibitory effect on copper catalysis than the original protein. We have not studied the possible relationship of the above phenomena to sulfhydryl groups.

Optimum pH—In their original paper Barron, DeMeio, and Klemperer (4) showed that the catalytic power of inorganic copper was greatly dependent upon the pH, but that in the non-autoxidizable range of ascorbic acid (below pH 7.6) there was no distinct optimum. The use of citrate-phosphate buffer in the presence of copper did not alter the above relationship.

This relation is greatly changed, however, in the presence of albumin or gelatin, in which case a distinct optimum pH range was found, comparable to that of the "enzymes." Fig. 2 illustrates the similarity between the postulated enzymes and the copper-protein complexes.

Kinetics—Szent-Györgyi (5) found, with cabbage "oxidase," an increase of velocity with increasing substrate concentration, and postulated a complex reaction involving an intermediate sub-

stance X. Since Tauber *et al.* (10) found, with their squash "oxidase," that no increase in velocity occurred with increasing substrate concentration, they concluded that their "enzyme" was of different character than that of Szent-Györgyi. Since both findings are in harmony with the Michaelis-Menten theory, their difference may depend only on the substrate to "enzyme" ratio.
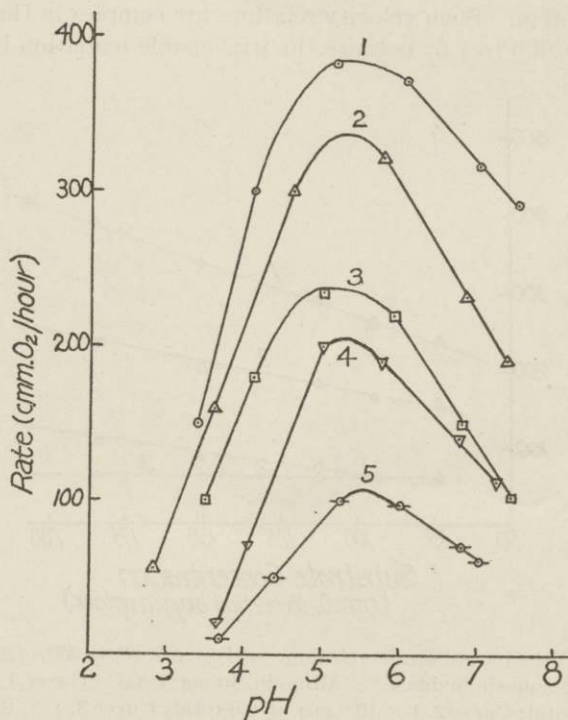

FIG. 2. Optimum pH of preparations. Ascorbic acid, 0.01 mM, $T = 37°$, PO_4-citrate buffer. Cu in Cu-albumin, 3×10^{-4} mM (total); in Cu-gelatin, 6×10^{-5} mM. Albumin and gelatin, 10 mg. total. Curve 1, copper-albumin; Curve 2, squash "oxidase;" Curve 3, copper-gelatin; Curve 4, cauliflower juice; Curve 5, cabbage juice.

The results given in Fig. 3 show that it is possible to duplicate Szent-Györgyi's results with squash "oxidase." The effect of changing the substrate to copper-albumin ratio is also shown in Fig. 3. It will be noted that both types of curve may be obtained in accordance with the variation in this ratio.

It has been noted before that the rate of oxidation of ascorbic acid follows a linear course until the vitamin has almost completely disappeared. Hopkins and Morgan (8) have suggested that, "it is perhaps necessary to assume that it [the enzyme] activates both the reduced and oxidized molecules of ascorbic acid." The same phenomenon is noted, however, in the presence of copper-protein. Such velocity relations are complex in the higher pH ranges (6.0 to 7.6) because the irreversible oxidation becomes
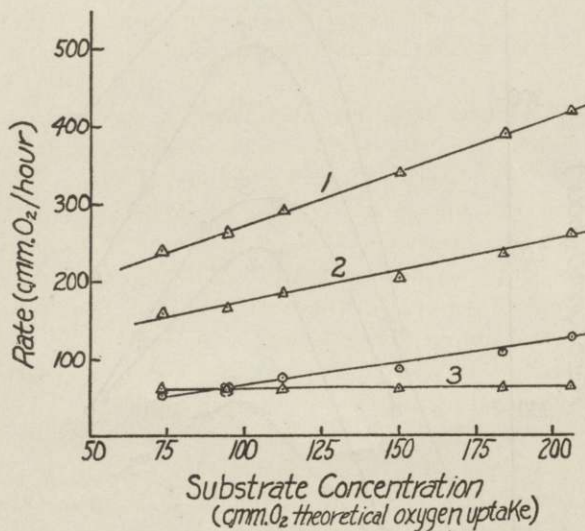

FIG. 3. Velocity-substrate relation. pH = 6.0, $T = 37°$. △ copper-albumin, ⊙ squash "oxidase." Albumin, 10 mg. total. Curve 1, 4×10^{-4} mM of Cu total; Curve 2, 1×10^{-4} mM of Cu total; Curve 3, 1×10^{-5} mM of Cu total.

increasingly prominent. In fact, at pH 7.4 the oxygen consumption of a copper-catalyzed vitamin solution was nearly twice that indicated by simultaneous indophenol titration of the same solution. This continued and irreversible oxidation superimposed on the reversible oxidation (equivalent to $-2H$) makes the velocity of the latter difficult to measure and interpret.

Inactivation by Acid—Tauber, Kleiner, and Mishkind (10) found that purified squash "oxidase" was inactivated at pH 2.0. Squash "oxidase," cauliflower juice, and copper-albumin were

adjusted to pH 2.0 and allowed to stand for 2 hours. They were then tested for their catalytic activity at pH 6.0 and compared with the untreated preparations at the same dilution. The inactivations amounted to 75, 68, and 64 per cent for squash, cauliflower, and copper-albumin respectively. Cauliflower juice was partially inactivated at pH 3.0. Acid inactivation of the preparations appears comparable to heat coagulation, a process in which copper is bound in a non-catalytic form by the denatured proteins.

Tryptic Digestion—Tauber *et al.* (10) also observed a progressive decrease in the activity of their preparation due to the action of trypsin.

Triplicate 90 hour digestions at 37° were carried out on squash "oxidase," cauliflower juice, and copper-albumin with 0.5 per cent trypsin (Fairchild Brothers and Foster) at pH 6.8 to 7.2. Samples of the tryptic digests were tested for their catalytic power at four different time intervals. The results were not so consistent as those indicated by the above workers. The maximum inhibitions obtained were 40 per cent in the case of squash, 18 per cent with cauliflower, and practically none with copper-albumin. If copper were liberated from the protein by digestion of the latter, one might expect either no decrease in catalytic activity or an increase. A significant result was obtained, however, upon testing the effect of small amounts of the tryptic digests on the catalytic power of copper. The results recorded in Table II indicate that the products of proteolysis of the squash and cauliflower proteins inhibit copper catalysis to a greater extent than the original material. Digested albumin showed little change in its inhibitory effect on copper.

Although copper-albumin showed no decrease in activity upon digestion, neither did its products of proteolysis inhibit added copper more than the original material. The latter observation has also been made with albumin containing no copper during digestion.

Combined Forms of Copper—Evidently the linkage between copper and protein is one in which copper is free to undergo alternate oxidation and reduction and to combine with copper inhibitors. In this connection the findings of Ettisch, Sachsse, and Beck (14) and of Borsook and Thimann (15) are of interest in

demonstrating the formation of un-ionized complexes with proteins and amino acids. The following points in our work are of interest in relation to the catalytic action of combined copper: (a) Cauliflower press-juice assumed a brown color when diethyldithiocarbamate was added, indicating the formation of a complex similar to that formed with inorganic copper salts. The juice lost practically none of its activity, however, when dialyzed for as long as 24 hours at pH 4.0 to 7.5. (b) Albumin and gelatin in equal weights possessed considerably different inhibitory powers on the copper catalysis of ascorbic acid oxidation. The lesser inhibitory power of gelatin is of interest in connection with its low sulfur content and non-coagulability. (c) Copper amide biuret (16, 17), a combined form of copper, was found to catalyze

TABLE II

Inhibition of Copper Catalysis by Original and Digested Preparations
Ascorbic acid, 0.01 mM; $T = 37°$; pH = 6.1.

Digested material	Rate of O₂ consumption, c.mm. O₂ per hr.			
	Original	Digested	Original + Cu (1 × 10^{-4} mM)	Digested + Cu (1 × 10^{-4} mM)
Squash "oxidase"............	88	55	142	63
Cauliflower juice............	61	51	102	66
Copper-albumin.............	44	48	88	80

the oxidation of ascorbic acid at the same rate as an equimolar amount of inorganic copper in the pH range of 4.0 to 7.5. It was inhibited quantitatively by diethyldithiocarbamate (2 moles of inhibitor per mole of Cu), with the formation of a typical brown copper complex. (d) Copper hematoporphyrin (16), unlike hemin, was able to function as a catalyst for the oxidation of ascorbic acid. The catalytic action of this "bound" copper was also completely inhibited by diethyldithiocarbamate. (e) The usual activity of glutathione in blocking the catalytic activity of copper is decreased rapidly as the concentration of protein increases.

DISCUSSION

It appears from our investigations that copper may be combined with proteins in such a manner that it retains its catalytic rôle in

the oxidation of ascorbic acid.   It is not assumed that the copper-albumin mixture that we have studied is the exact counterpart of the copper-protein present in vegetable preparations, but rather, that the properties of such mixtures and complexes provide an experimental basis for understanding the catalytic behavior of minute quantities of copper in natural products.

Although more copper was required to produce a given catalytic effect in the presence of ovalbumin than was found in the vegetable press-juices, copper + gelatin and copper + edestin proved to be nearly like the vegetable juices in this respect.   Furthermore, a large fraction of the copper in the copper-albumin mixture was dialyzable, whereas that in the "enzymes" was not.   On the other hand, the proteins present in the "enzyme" preparations possessed an efficiency comparable with albumin in the manner in which they protected ascorbic acid from oxidation by added copper.

The concept of a specific "oxidase" for the oxidation of ascorbic acid has been a factor in the belief that this vitamin is an important catalyst in biological oxidation.   The view that copper may serve as this "enzyme" does not, *per se*, argue for or against such a rôle for the vitamin.

The evidence given concerning the copper nature of the catalyst in vegetable juices has an immediate significance in relation to the preservation of the vitamin in foods and in vitamin analysis, but further study will be necessary to find whether there is a relationship between the vitamin and copper in living tissues.

SUMMARY

1. The catalytic activity of squash and cauliflower juices on the oxidation of ascorbic acid, previously ascribed to a specific "oxidase," is attributed to the copper present in combination with protein material.

2. Seven copper inhibitors produced nearly complete poisoning of the "enzymes," as well as of inorganic copper and copper-protein mixtures.   These inhibitors did not affect the catalytic function of nicotine-hemochromogen in a manner which would suggest that this type of substance is responsible for the oxidation of ascorbic acid by cauliflower juice or "purified squash oxidase."

3. The catalytic properties of copper are changed greatly by the

presence of proteins. A mixture of copper and albumin assumes the characteristic properties of the "enzymes." It displays an optimum pH similar to that of the "enzymes," is inactivated by heat and acid, and shows similar velocity relations to the quantity of substrate.

4. The type of union which may exist between copper and protein has been discussed and the catalytic ability of "bound" copper illustrated by copper amide biuret and copper hematophorphyrin.

5. Although the above conclusions apply only to cauliflower juice, "purified squash oxidase," cucumber juice, and cabbage juice, it is suggested that the other "ascorbic acid oxidases" described in the literature are probably not essentially different from these. A study of several other vegetable and fruit juices is in progress in this laboratory.

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