was brought to *p*H 8 with cyclohexylamine before chromatography.

By the above analysis, hydrolysates of crystalline phosphoinositides from beef heart, wheat germ (supplied by Dr. M. Faure) and a crude phosphoinositide from liver (isolated from Viobin liver fat by solvent fractionation), all showed the same two inositol monophosphates in the same proportions. The two compounds are tentatively identified as *myo*-inositol 1-phosphate (major component) and *myo*-inositol 2-phosphate (minor component).

Acknowledgments.—This work was supported in part by grants from the Nutrition Foundation and the U. S. Public Health Service (Grant A884).

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The Inhibition of β-Amylase by Ascorbic Acid[1]

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RECEIVED AUGUST 25, 1958

The effect of ascorbic acid on β-amylase has been shown to be the result of the formation of an inactive cuprous enzyme. The extent of this inactivation at a fixed ascorbic acid concentration has been shown to be proportional to the copper concentration. The same type of inactivation has been encountered with bisulfite–copper ion solutions demonstrating that this effect is general rather than specific for ascorbic acid. Ascorbic acid actually decreases the extent of inhibition caused by mercuric ions. In this case the potent inactivator, mercuric ion, is reduced to the less active mercurous ion. This effect again demonstrates that the ascorbic acid acts through the reduction of cupric ion to cuprous followed by the formation of the inactive cuprous enzyme compound.

Introduction

The inhibition of β-amylase by ascorbic acid was reported some years ago by Purr[3] and Hanes.[4] The problem was later studied by Seshagirirao and Giri[5] who divided the action in two parts. They reported a reversible inhibition in the presence of ascorbic acid alone and an irreversible inactivation in the presence of ascorbic acid plus cupric ion. Ito and Abe[6] further divided the inactivation into a reversible and an irreversible phase.

Hanes suggested a reaction between the dienol of the ascorbic acid and the sulfhydryl of the enzyme as the cause of inhibition. Seshagirirao and Giri showed that any substance which prevented the oxidation of ascorbic acid also prevented the inhibition. Most of these compounds presumably complexed with the cupric ion. However, no explanation for the inhibition phase was offered. They again suggested the same possible reaction put forth by Hanes.

More recently Rowe and Weill[7] investigated the nature of the inhibition. They found that the inhibition was non-competitive with respect to ascorbic acid. This was contrasted with typical sulfhydryl reagents such as *p*-chloromercuribenzoate which show a competitive type of inhibition. These results suggest that the inhibition is not the result of any direct reaction between the dienol of the vitamin and the sulfhydryl of the enzyme.

The results of the current study demonstrate that the difference between the inhibition and the so-called irreversible inactivation is merely one of degree. All of the data suggest that the function of the ascorbic acid is to reduce cupric ion to cuprous. This then reacts with the enzyme to inactivate it. In this respect the results are similar to those reported by Mapson[8] for urease in that inactivation is due to the formation of a cuprous mercaptide complex.

Results and Discussion

In previous work[6] it has been shown that ascorbic acid resulted in a reversibly non-competitive inhibition with the enzyme in the absence of added cupric ions. The inhibition is dependent upon the oxidation of ascorbic acid. Raising the *p*H will increase the rate of oxidation of ascorbic acid and subsequently the amount of inhibition. Figure 1 shows the increased inhibition by ascorbic acid as the *p*H is increased.

Any substance which decreases the rate of oxidation of ascorbic acid should also reduce the extent of the inhibition. Mapson[9] has shown that KCl will decrease the rate of oxidation of ascorbic acid, presumably through the formation of a $CuCl_2^-$ ion with cuprous ion.

The effect of KCl in retarding the oxidation of ascorbic acid at *p*H 4.5 has been verified. This same concentration of KCl also removes a considerable amount of the inhibition of enzyme activity caused by the vitamin. If an antioxidant does not retard the oxidation of ascorbic acid, it does not remove the inhibiting effect of the ascorbic acid. Dihydrocaffeic acid is an example of this type.

No cupric ions have been added to the inhibition studies previously reported; however, the decrease in inhibition caused by KCl suggested that cupric ions might be present in very small quantities. Large volumes of the reaction mixture were concentrated, and it was demonstrated that there was about 1 part of copper per 20 million parts of solution. While this amount of copper is not sufficient to cause an "irreversible" inactivation, it is sufficient to induce a reversible inactivation or inhibition.

(1) This paper has been presented in part at the North Jersey Meeting in Miniature of the A.C.S. in January, 1958.

(2) Submitted in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

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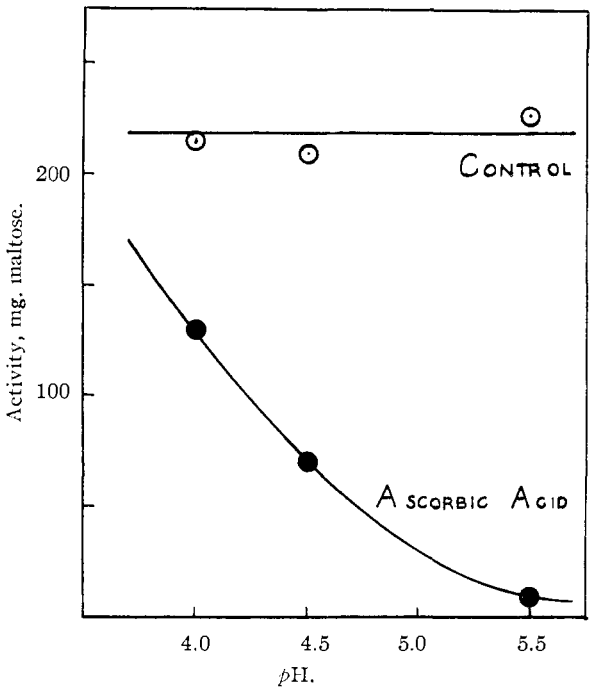

Fig. 1.—Effect of 10^{-3} M ascorbic acid on the inhibition of β-amylase.

Ascorbic acid alone and cupric ions alone appear to have little effect in inactivating β-amylase. Ascorbic acid and cupric ions together in sufficient concentration will completely inactivate β-amylase. This is shown in Table I and confirms the similar results obtained by other investigators.[5,6]

TABLE I

INFLUENCE OF COPPER ON THE ASCORBIC ACID INACTIVATION OF β-AMYLASE

Sample[a]	Cu^{++} concn., M	Ascorbic concn., M	Activity[b]	% Recovery
1	.....	..	3.49	..
2	4×10^{-5}	..	3.07	89
3	4×10^{-5}	10^{-2}	0.16	5
4	.....	10^{-2}	3.10	92

[a] Contains 100 mg. enzyme. [b] Activity in mg. maltose formed at 60 minutes using 2 mg. of enzyme after reactivation by cysteine.

It has been shown that a metal–ascorbic acid system can hydroxylate some aromatic compounds.[10] Since alteration of the tyrosine grouping in β-amylase has been shown to lead to inactivation,[11] it seemed plausible that the cupric–ascorbic acid system might be inducing oxidation of the tyrosine moiety of the protein. The most active of the hydroxylating systems is ascorbic acid–ferrous ion. Table II shows that this system exerts no inactivating effect on the enzyme. Attempts to analyze for oxidized tyrosine in the enzyme by the method of Arnow[12] have proven unsuccessful. There was also no discernible alteration in the infrared spectrum of the dialyzed, lyophilized enzyme. This suggests that the inactivation of β-amylase by a metal–ascorbic acid system is not involved with tyrosine oxidation. It has already been demonstrated that there is no stoichiometric relationship between ascorbic acid and the –SH of the enzyme.[7]

TABLE II

EFFECT OF ASCORBIC ACID–FERROUS ION ON β-AMYLASE

Sample[a]	Fe^{++} concn., M	Ascorbic concn., M	Activity[b]	% Recovery
1	.....	..	3.49	..
2	4×10^{-5}	..	3.18	92
3	4×10^{-5}	10^{-2}	3.15	91

[a] Contains 100 mg. enzyme. [b] Activity in mg. maltose at 60 minutes using 2 mg. enzyme after reactivation by cysteine.

Since the copper–ascorbic acid induced inactivation may not be effected by means of an oxidation mechanism, there remained the possibility of a metal–enzyme complex, preferentially with the essential sulfhydryl moiety. The least concentration of copper needed to cause inactivation in the presence of ascorbic acid was determined. The per cent. activity of β-amylase corresponding to a range of copper concentrations in the presence of 0.01 M ascorbic acid are shown in Fig. 2. It is evident that the least concentration of copper needed to cause appreciable inactivation is between 8 and 10 times 10^{-6} M copper.

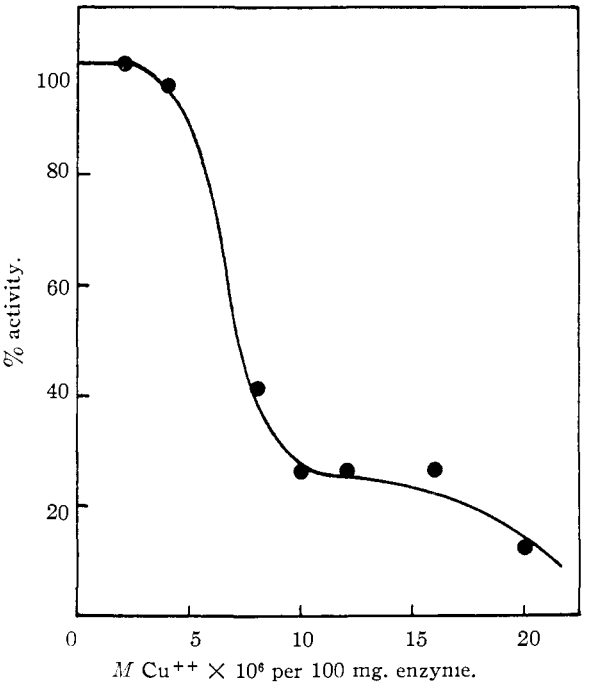

Fig. 2.—Least amount of copper necessary to cause appreciable inactivation with 10^{-2} M ascorbic acid.

The results of Fig. 2 show the minimum amounts of copper necessary to inactivate the enzyme in the presence of ascorbic acid. If the copper is bound by the enzyme, then it should be possible to add fresh enzyme to the lower levels of copper concentration without any loss in activity of the freshly added enzyme. The data in Table III show that no significant amount of the freshly added enzyme was inactivated. All of the copper had evidently been bound by the first portion of the enzyme used

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TABLE III

ACTIVITY OF ENZYME ADDED TO Cu^{++}–ASCORBIC ACID INACTIVATED ENZYME

Cu^{++} concn.[a]	Fresh enzyme, mg./ml.	Total activity	Activity of recovered enzyme	% Recovery of added enzyme
0	..	1.57	..	..
8	..	0.37		
	1	1.91	1.54	98
10	..	0.22		
	1	1.61	1.39	89

[a] Concentration of $CuCl_2$ times 10^6 per 100 mg. enzyme in the presence of 10^{-2} *M* ascorbic acid. Aliquots containing 1 mg. per ml. were used for activity assay expressed as mg. maltose.

and consequently was not available to inactivate the additional untreated enzyme.

Since we have apparently established that the copper is bound to the enzyme, it is desirable to see whether the copper is forming a complex with the essential sulfhydryl groups of the enzyme and in turn causing inactivation. A typical mercaptide-reagent, *p*-chloromercuribenzoate (PCMB), was used to block the essential sulfhydryl groups of the enzyme. The enzyme, totally inactivated with PCMB, could be reversibly reactivated with an excess of cysteine. The PCMB-inactivated enzyme was mixed with copper and ascorbic acid in concentrations that would ordinarily inactivate the enzyme. After the appointed time cysteine was added and some activity of the enzyme reappeared. This is shown in Table IV and indicated that a sulfhydryl–copper complex (in the presence of ascorbic acid) is involved in the inactivation of β-amylase.

TABLE IV

PROTECTION OF β-AMYLASE AGAINST COPPER–ASCORBIC ACID INHIBITION

PCMB concn.[a]	Cu^{++} concn., *M*	Ascorbic concn., *M*	Cysteine reactivation	Activity[b]	% Recovery
4	..	..	No	0.00	..
4	..	..	Yes	2.17	64
4	10^{-5}	10^{-2}	No	0.02	..
4	10^{-5}	10^{-2}	Yes	1.34	40

[a] Concentration of PCMB × 10^4 used to inactivate 100 mg. enzyme. [b] Activity in mg. maltose at 60 minutes using 2 mg. enzyme.

Although the activities were not completely recovered, presumably due to an excess of PCMB present, the copper (in the presence of ascorbic acid) was not able to exert its full inactivation effect so long as the essential sulfhydryl groups of the enzyme were tied up with PCMB.

It has been previously described[5,6] that a permanent irreversible inactivation occurs when cupric ions and ascorbic acid are present. This has been found to be true only when inactivation reversal by excess cysteine takes place for a short duration (0.5 hr.). At the lowest concentration of copper required to inactivate β-amylase in the presence of 10^{-2} *M* ascorbic acid, excess cysteine will not cause reactivation to occur within a 30-minute interval. Upon prolonged contact (24 hours) with cysteine, complete reversal of the copper–ascorbic acid inactivation can be afforded. The amount of reactivity that occurs is decreased as the concentration of copper is increased. The reversal of a previously mentioned irreversible inactivation caused by copper and ascorbic acid is shown in Table V.

TABLE V

CYSTEINE REACTIVATION OF THE INACTIVATED ENZYME

Cu^{++} concn.,[a] *M*	Ascorbic concn., *M*	Time in cysteine, hr.	Activity[b]	% Reactivity
0	10^{-2}	0.5	3.36	..
0	10^{-2}	24	3.41	..
10^{-5}	10^{-2}	0.5	0.89	26
10^{-5}	10^{-2}	24	3.12	92
4×10^{-5}	10^{-2}	0.5	0.00	0
4×10^{-5}	10^{-2}	24	.56	16

[a] Contains 100 mg. enzyme. [b] Activity as mg. maltose formed at 60 minutes using 2 mg. enzyme after reactivation by cysteine.

If the inactivation is the result of the formation of the cuprous ion, then similar results should be obtained with any reducing agent which can produce cuprous ion *in situ* as long as it is not removed in a complex. The data in Table VI show the effect of bisulfite. While the bisulfite itself does not have too much effect, the bisulfite plus cupric ions produce an inactivation similar to the ascorbic acid plus cupric ion.

TABLE VI

ENZYME INACTIVATION INVOLVING Cu^{++}–BISULFITE

Sample[a]	$NaHSO_3$ concn., *M*	Cu^{++} concn., *M*	Activity[b]	% Reactivity
1	..	..	3.70	..
2	10^{-2}	..	2.63	71
3	10^{-2}	10^{-5}	0.82	22
4	..	10^{-5}	3.69	99

[a] Contains 100 mg. enzyme. [b] Activity as mg. maltose formed at 60 minutes using 2 mg. enzyme after reactivation by cysteine.

The cuprous–mercaptide complex resulting in enzyme inactivation is relatively stable as seen by its inability to reverse in cysteine except on prolonged contact. When a sample of copper–ascorbic acid inactivated enzyme is exhaustively dialyzed against water for four days, 33% of its activity is recovered. The concentration of copper remaining after dialysis is 6.4×10^{-6} *M*. This amount of copper, determined after oxidation and concentration of the dialyzed enzyme, corresponds to that concentration found on the almost vertical portion of the curve in Fig. 2.

Various ion-exchange resins were used with the object of adsorbing that copper which was not bound to the enzyme and remained in solution. Washing the enzyme solution through columns proved unsuccessful since the majority of the enzyme was adsorbed on the resins. By agitating the enzyme mixture in the presence of the ion-exchange resins during inactivation, it was possible to retain most of the enzyme activity in the control and yet have total pickup of free copper in the solution of the inactivated enzyme. Using Amberlite MB-3, the results were similar to those obtained in the dialysis experiments.

While cuprous is a more effective inactivator than cupric, the situation is reversed with mercury ions. Mercuric ions should be more effective than mercurous and thus mercuric alone should produce more inactivation than mercuric ion plus ascorbic acid. The data in Table VII confirm this.

TABLE VII

COMPARISON OF THE INACTIVATION BY MERCURY

Sample[a]	Hg^{++} concn., M	Hg^{+} concn., M	Ascorbic concn., M	% Activity
1	10^{-4}	..	..	6.5
2	10^{-4}	..	10^{-2}	39.0
3	..	10^{-4}	..	28.0

[a] Contains 100 mg. enzyme.

Conclusion

Ascorbic acid inactivates β-amylase by reducing cupric to cuprous ion with subsequent formation of a cuprous-mercaptide complex. The reversibility of the reaction depends upon the amounts of reagents used and the length of exposure to the reactivating agent, cysteine, in this case.

While the reversible inhibition of activity has previously been shown to depend upon ascorbic acid concentration, the extent of the so-called "irreversible" inactivation depends upon copper concentration as well. Many of the samples thus inactivated could be restored to activity depending upon the length of time exposed to the reactivating agent. The fact that reactivation was only partial at high cupric ion concentrations does not alter the postulation. At these higher concentrations the proteins precipitated and seemed to be altered vastly.

It may be postulated that there is no essential difference between the inhibition and so-called irreversible inactivation. It is merely a difference of degree. The apparent inhibitory action of ascorbic acid, then, is its ability to reduce the cupric to cuprous which in turn complexes with the essential sulfhydryl of the enzyme. Mercury, on the other hand, has an opposite effect inasmuch as the mercuric is a more effective complexing agent. The inhibition in the presence of mercury ions is diminished by ascorbic acid.

Experimental

Materials.—The β-amylase was purchased from the Wallerstein Co., Inc. (New York, N. Y.). The enzyme, prepared from barley, was free of α-amylase activity and contained only inorganic salts as impurities. The ascorbic acid was Lot 9636, obtained from Hoffmann-LaRoche, Inc. (Roche Park, Nutley, N. J.). The enzyme substrate was Takamine soluble starch (Takamine Laboratory, Inc., Clifton, N. J.). The reagents used were Fisher Reagent Grade and recrystallized where necessary. CuCl was prepared in this Laboratory and stored under anhydrous ether. Ferrous sulfate and mercuric chloride were used as sources of ferrous and mercuric ions, respectively. Mercurous nitrate, used as a source of mercurous ions, was solubilized in a minimum of nitric acid. Blanks were run in all enzyme activities.

Oxidation Measurements.—The rate of oxidation of ascorbic acid alone and in the presence of various reagents was determined with the Beckman Model D.U. Spectrophotometer at 265 mμ in the presence of 0.005 *M* acetate buffer at *p*H 4.5. Ascorbic acid has been found to obey Beer's law in concentrations from 1 to 5×10^{-5} *M*. Low actinic glassware was used in all oxidation rate studies.

Inhibition Procedure.—Two mg. of enzyme were added to a 1% starch solution in the presence of 0.05 *M* acetate buffer at *p*H 4.5 or any other desired *p*H. Ascorbic acid, KCl, dihydrocaffeic acid and other reagents in desired concentration were added to the starch solution immediately prior to addition of the enzyme. Activities were determined as mg. of maltose produced and determined as outlined in the following inactivation procedure.

Inactivation Procedure.—One hundred mg. of the enzyme preparation was reacted with the designated amounts of cupric chloride, ferrous chloride, mercuric chloride and ascorbic acid. The reaction mixture contained 0.05 *M* acetate buffer at *p*H 4.5 and was incubated at 25° for 1 hr. in a 50-ml. volumetric flask. After incubation, 10 ml. of the reaction mixture was added to 10 ml. of 0.01 *M* cysteine and allowed to stand for 30 minutes. Two ml. of the solution containing 1 mg. enzyme per ml. was then added to a 1% starch solution and incubated for 30 or 60 minutes. The activity of the enzyme in each case was calculated as mg. of maltose produced. The maltose was determined by an iodine thiosulfate method.[13] A 5-ml. aliquot of the reaction mixture was added to 1 ml. of 1.0 *M* sodium carbonate solution to stop the enzyme action and diluted to 10 ml. Five ml. of 0.02 *N* iodine was added to a measured portion (2 ml.) of this solution and allowed to stand for 30 minutes at 20°. After acidification, the excess iodine was back titrated with 0.005 *N* thiosulfate.

When fresh enzyme was added to the copper-ascorbic inactivated enzyme to show that the enzyme picked up the copper, two samples of enzyme were used. After treatment with copper plus ascorbic acid and incubation for 1 hr., aliquots were removed and added to cysteine. Fresh enzyme was added to a second sample of each, allowed to stand for an hour, and then added to cysteine after which activities were determined.

In experiments involving inactivation prevention by PCMB followed by copper-ascorbic acid treatment, activities were determined at four stages. (a) Two hundred mg. of enzyme were treated for 0.5 hr. with 4×10^{-4} *M* PCMB. An aliquot containing 2 mg. of enzyme was removed for activity assay. Two samples of this PCMB treated enzyme were set up at this point. (b) One sample was added to cysteine for 0.5 hr. and then the amount of reactivation determined. (c) The second sample containing 100 mg. of the PCMB-treated enzyme was added to the copper and ascorbic acid and diluted to 50 ml. volume. The copper concentration was 10^{-5} *M* and ascorbic acid 10^{-2} *M*. An aliquot containing 2 mg. of enzyme was then removed for activity assay. (d) A second portion was added to an equal volume of cysteine for 0.5 hr. after which activity was determined on it.

When longer contact with the cysteine was desired, *i.e.*, 24 hr., the cysteine-reaction mixture stood in the refrigerator to prevent bacterial contamination.

Dialysis of the treated enzyme samples consisted of putting the entire sample in a cellophane tubing and dialyzing against demineralized water at refrigerator temperature, 3°. At the completion of dialysis, an aliquot containing 2 mg. of enzyme was used for activity assay while the rest was boiled down in the presence of 1 ml. of concentrated HNO_3 for copper analysis. All copper analyses were determined by using the diethyldithiocarbamate test.

Acknowledgments.—Funds for this research were made available by the Rutgers University Research Council and U. S. Public Health Service Grant A 1771 Bio.

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