CCCIX. THE REVERSIBLE INHIBITION OF β -MALT-AMYLASE BY ASCORBIC ACID AND RELATED COMPOUNDS.

By CHARLES SAMUEL HANES.¹

From the Ontario Research Foundation, Toronto, Canada.

(Received September 30th, 1935.)

THE activities of several enzymes appear to be markedly influenced by reduced ascorbic acid. Papain is reported to be inhibited by ascorbic acid alone but activated by ascorbic acid *plus* ferrous sulphate [Maschmann and Helmert, 1934]. Cathepsin is activated by ascorbic acid and the activation is increased by the presence of iron [Euler *et al.*, 1934]. An activation is also reported for arginase [Edlbacher and Leuthardt, 1933]. Inhibitions have been reported in the cases of urease [Edlbacher and Leuthardt, 1933] and β -malt-amylase [Purr, 1934].

In the present investigation the observation of Purr that β -malt-amylase is strongly inhibited by reduced ascorbic acid has been confirmed. Moreover, evidence has been found which suggests that the effect can be attributed to the presence of the dienol grouping in the vitamin and that it is not due merely to the reducing properties of this grouping, since (1) two other dienol compounds, dihydroxymaleic acid and reductone, exert the same type of inhibition, and (2) the inhibition by a dienol compound is reversed not only by oxidative destruction of the dienol grouping but also by the addition of a variety of other reducing substances including hydrogen cyanide, sodium hydrosulphite and several sulphydryl compounds. This latter effect, the reversal of dienol inhibition by other reagents of a reducing character, even though the actual mechanism of this reactivation remains obscure, would seem to exclude the possibility that the effect of the vitamin and the other dienol compounds is due merely to their reducing properties.

The results to be described thus suggest that the inhibitory action on β -malt-amylase is of the nature of a specific effect and they accordingly lend plausibility to the view that the vitamin function of ascorbic acid may be related to its ability to function as a regulator of enzyme activity within the cell.

Preparation of β -malt-amylase.

The enzyme was prepared from ungerminated barley by a method based on that of van Klinkenberg [1931]. The seed coat, aleurone layer and most of the embryonic tissue were removed from a specimen of high-grade malting barley by grinding in an abrasive mill. The product, which resembled commercial "Pearl Barley", was ground to a fine flour.

200 g. of the flour were extracted with 700 ml. 50 % ethyl alcohol for 2 hours. After centrifuging, the residue was again extracted with 250 ml. 50 % alcohol and again centrifuged down. The supernatant liquors were combined and the enzyme precipitated by the addition of alcohol to a final concentration of 80 %. The precipitate was removed on the centrifuge and washed once by re-suspension in 80 % alcohol. The washed residue was thoroughly drained on a suction filter

¹ Now at the Low Temperature Research Station, Cambridge.

and was then suspended in 200 ml. of water. An insoluble fraction was removed by filtration after 5 days.

Three such concentrated stock enzyme solutions (prepared from two different specimens of barley) were used in the present investigation, all of which gave similar results. The stock enzymes were stored under toluene at 0° in which conditions only slight changes in activity (less than 10 %) occurred in an 8-week period.

Portions of stock enzyme sufficient for 1 or 2 days' experiments were removed as required and after suitable dilution (6 to 10-fold in different cases) were stored in the reaction thermostat at 25°.

Reagents.

Conductivity water (from an all-pyrex still) was used in the preparation of enzyme, substrate and all reagents used in the pretreatment of the enzyme. Solutions used in pretreatment were adjusted to the natural acidity of the enzyme (p_H 6.4) by appropriate additions of sodium hydroxide.

The following diénol compounds were used:

1. Ascorbic acid as supplied by British Drug Houses.

2. Glucoreductone ($\text{CHO} \cdot \text{COH} \cdot \text{CHOH}$) was prepared from glucose by the method of Euler and Martius [1933]. The product after recrystallisation from acetone and ethyl acetate had properties in general agreement with the description of these authors although no claim can be made for its absolute purity, as will be clear from the following observations: mol. wt. from the rise of boiling point in acetone solution (Pregl method), 90, 99, 93, average 94; acid equivalent, 87.5; iodine equivalent, 92.

3. Dihydroxymaleic acid ($\text{HOOC} \cdot \text{COH} \cdot \text{COH} \cdot \text{COOH}$) was prepared from tartaric acid by the procedure of Fenton [1905], solid carbon dioxide and acetone being used as a refrigerant during the oxidation with hydrogen peroxide. The anhydrous substance gave theoretical values for titratable acidity and iodine consumption. In some experiments the disodium salt of this acid (prepared by neutralising an alcoholic solution of the acid with alcoholic sodium hydroxide) was used.

Solutions of the diénol compounds were freshly prepared a short time (rarely more than 20 min.) before mixing with the enzyme. Sodium hydrosulphite was used within 2 min. of dissolving. Other reagents, hydrogen cyanide, sulphhydryl compounds *etc.*, were prepared daily.

The various reducing compounds in the low final concentrations in which they occurred in the starch digests were found to have no appreciable influence on the determination of maltose production in the digests of starch.

Procedure for pretreatment of enzyme and activity determinations.

Throughout the investigation a standard volume of 2 ml. of enzyme (containing from 0.8 to 1.4 mg. dry matter) has been used. Pretreatment of the enzyme with the various reagents studied has usually been carried out individually on these standard 2 ml. portions contained in small vials supported in the bath at 25°, although in some experiments it was more convenient to pretreat a larger volume from which a number of samples (equivalent in volume to 2 ml. of the original) were taken for activity determination.

Activity values are based on determinations of the initial velocity of maltose production in digests of soluble starch at 25° and p_H 4.7 (the optimum value). The standard volume of enzyme, diluted to 5 ml. by the addition of water or reagents used in pretreatment, is added from the vial to a digest flask, the lip of

the vial being first lightly smeared with vaseline. The digest flask contains 25 ml. 0.66 % soluble starch (saturated with toluene) and 3 ml. *N*/5 acetate buffer of p_H 4.7.¹ The vial is rapidly rinsed with the reaction mixture and thereafter 5 ml. samples are removed at intervals for maltose determination by the methods described earlier [Hanes, 1929; 1932]. The first sample is taken at 0.7 min. and subsequent samples usually at intervals of 1.2 min. In most experiments four samples were taken from each digest, the observations being thus restricted to the first 5–6 min. of the reaction.

It has been shown earlier [Hanes, 1932] that under these conditions the progress curves of maltose production normally remain almost linear, there being only a small decrease in the reaction velocity during the short period under observation. This has been found to hold true in the present study for digests with untreated enzyme, or enzyme reactivated after diénol inhibition. With inhibited enzyme, however (particularly after pretreatments with ascorbic acid and reductone), the rate of maltose production frequently falls off with somewhat variable rapidity which indicates a further development of the inhibition after the enzyme (with diénol compound) is added to the buffered substrate. At least part of this increased inhibition results from the presence of the more acid buffer of the digest, but the presence of heavy metal impurities with the starch may also contribute (*v. p.* 2599). This feature has the effect of increasing the experimental error in deriving initial slope values with inhibited enzyme, but the initial slope method clearly remains the most desirable basis of assessing activity as it provides as nearly as possible a measure of the activity of the enzyme at the termination of its controlled pretreatment. Examples of progress curve data will be seen in Table V, and in Fig. 5.

EXPERIMENTAL.

The time course of the diénol inhibition.

(a) *Development.* In a number of experiments in which the rate of development of inhibition was followed it became clear that the effect was not “instantaneous” but (except with very low concentrations of inhibitor) required a period of several minutes for maximum development. This will be sufficiently illustrated by the graphs of Fig. 1 in which the observed inhibitions are plotted against duration of pretreatment with what may be termed high, medium and low amounts of reduced ascorbic acid—2.5, 0.1 and 0.03 mg. respectively per standard volume (2 ml.) of enzyme. In these cases the ascorbic acid in 1 ml. of solution was added to 2 ml. of enzyme for the pretreatment, 2 ml. of water being added immediately before starting the digest.

Data from three different experiments with 0.1 mg. ascorbic acid have been plotted to illustrate the degree of reproducibility of the results. They would indicate a maximum inhibition of about 80 % developing in 10–15 min. With 2.5 mg. ascorbic acid the full inhibition of 98 % had developed within 7 min.² Only with the lowest amount of ascorbic acid (0.03 mg.) does the earliest activity determination (after 1 min. pretreatment) show maximum inhibition. In this case the subsequent determinations show that the inhibition becomes progressively smaller.

¹ The final volume of the standard digest is thus 33 ml. and the starch concentration 0.50 %. For substrate we find most suitable potato starch “solubilised” according to the procedure of Small [1919]. The moisture content is determined by drying to constant weight at 110° in a high vacuum in the presence of P_2O_5 .

² With intermediate doses 0.2–0.7 mg. a slight progressive increase in inhibition has been observed for periods up to 5 hours. An example is shown in Fig. 2.

(b) "*Spontaneous*" regeneration after dienol inhibition. The phenomenon of regeneration of activity, which is noticeable in the inhibition-time curve with low ascorbic acid in Fig. 1, has been investigated in some detail and has been found to be associated with the destruction of the inhibiting dienol group by aerobic oxidation. With increasing amounts of inhibitor, regeneration is slower in appearing and smaller in extent. We give in Table I observations extending over longer periods which illustrate the magnitude of the effect with different concentrations of ascorbic acid.

Fig. 1. The time course of inhibition by three concentrations of ascorbic acid, 0.03, 0.1 and 2.5 mg. respectively, in 1 ml. added to 2 ml. enzyme. Observations from three experiments with the intermediate concentration are shown.

Table I. *Regeneration of activity during prolonged ascorbic acid treatment.*

2 ml. enzyme pretreated with 1 ml. ascorbic acid solution.			
mg. ascorbic acid in 1 ml.	Relative activity after pretreatment for 1 hour	20 hours	45 hours
0.1	21	44	—
0.2	12	36	51
0.7	4	16	22

The elucidation of this feature has brought to light an interesting difference in the behaviour of the three dienol compounds, in that the regeneration of activity during treatment with ascorbic acid is much slower than with dihydroxymaleic acid or reductone.

Experiments were carried out in which the activity of the enzyme and the degree of oxidation of the dienol compound mixed with it were followed simultaneously. For the latter determination 5 ml. samples of the enzyme-inhibitor solution were acidified with 2 ml. 5% acetic acid; an excess of iodine was added by pipette and back-titrated with sodium thiosulphate in the presence of 4 drops of 1% soluble starch. Under these conditions the enzyme alone showed no iodine consumption.

In Fig. 2 are given the results of such an experiment in which three portions of the same enzyme solution were treated with ascorbic acid, dihydroxymaleic acid and reductone of the same concentration. The pretreatment was carried out in each case by the addition of 40 ml. of $M/800$ solution of the dienol compound

to 80 ml. of enzyme contained in a 500 ml. flask, the solutions being kept aerated by frequent whirling of the contents about the walls. Samples of 5 ml. for determination of iodine consumption and 3 ml. for activity determination were taken at intervals. In order to insure the same acidity in the three solutions, the dienol compounds after first neutralising to p_H 6.4 with NaOH were made up to volume with the addition of phosphate buffer of this p_H to give a concentration of $M/200$. Accordingly the pretreatment of the enzyme was carried out in the presence of $M/600$ phosphate of p_H 6.4 and the final concentration of inhibitor was $M/2400$ in each case.¹

Fig. 2. The time course of inhibition (upper) and iodine-reducing capacity (lower) during treatments of the enzyme with $M/2400$ ascorbic acid (*A*), reductone (*R*) and dihydroxymaleic acid (*M*).

In the upper part of Fig. 2 it will be seen that the enzyme treated with ascorbic acid showed a rapid fall in activity to approximately 3% of the original value, rising in the course of 5 hours to 7%. A subsequent determination at 24 hours (not shown) indicated a further regeneration to 32% of the original. In contrast to this effect it will be seen that the inhibitions by the two other dienol compounds were of a transient nature. The inhibition by reductone, initially of the same order as that by ascorbic acid, decreased very rapidly so that in 30 min. the activity had risen to 85% of the control value; after the rapid regeneration to this level the activity rose only very slowly, reaching 91.5% of the control value after 24 hours (not shown). A subsequent determination (32 hours) showed no further recovery. The dihydroxymaleic acid inhibition, considerably smaller initially than the others, also decreased rapidly and after 3 hours the activity was identical with that of the untreated control.

In the lower part of Fig. 2 are shown the iodine consumption data for the three enzyme-inhibitor mixtures expressed in ml. 0.004 *N* iodine per 5 ml. In the ascorbic acid-enzyme mixture a relatively slow decrease in iodine consumption occurred. The value fell from 0.84 to 0.67 in 5 hours, and (not shown) to 0.415 after 23 hours. In the cases of the other dienol compounds, however, rapid

¹ For convenience in comparing with other treatments this corresponds to the addition of 1 ml. containing 0.22 mg. ascorbic acid, 0.185 mg. dihydroxymaleic acid and 0.11 mg. reductone, respectively, to the standard 2 ml. volume of enzyme.

oxidation of the dienol group occurs so that the iodine consumption values fall to zero in 1 hour with dihydroxymaleic acid and in 15 min. with reductone. It is noteworthy that the complete disappearance of the reducing group occurred in each case before the activity of the enzyme had regenerated to its maximum extent.

It is clear from the results of this experiment that the regeneration of the enzyme is closely correlated with the destruction of the inhibiting dienol group (probably by aerobic oxidation) and that the differences in duration of the inhibitions produced by the three substances are due to differences in the stability of the dienol group as it occurs in them.

Two further points in this experiment are worthy of note—namely that the iodine consumption values immediately after mixing enzyme and dienol solutions are appreciably lower than the theoretical values for the dienol alone, and that in the cases of reductone and dihydroxymaleic acid the complete oxidation of the dienol group occurs before the regeneration of the enzyme activity is complete. These points, which suggest that a small amount of the dienol compounds combines with some constituent of the enzyme preparation, are being further investigated.

Fig. 3. The time course of inhibition (upper) and iodine-reducing capacity (lower) during treatments with *M*/800 ascorbic acid and reductone.

We give in Fig. 3 the results of a similar experiment in which portions of enzyme were pretreated with *M*/750 (final concentration) ascorbic acid and reductone (slightly more than three times the strength used in the preceding experiment). This corresponds to the pretreatment of 2 ml. enzyme with 1 ml. containing 0.704 mg. ascorbic acid and 0.352 mg. reductone. As before the dienol compounds after neutralisation to p_H 6.4 were dissolved in *M*/200 phosphate buffer of this p_H . The iodine consumption determinations were made on 3 ml. samples.

To facilitate comparison we have included in Fig. 3 the activity-progress curve for *M*/2400 reductone re-drawn from Fig. 2. It will be seen that the increased concentration of reductone resulted in a slightly greater initial inhibition and that the subsequent regeneration is considerably slower and less

complete. As before the determinations of iodine consumption indicate a rapid oxidation of reductone and a very slow oxidation of ascorbic acid. In contrast to the results of Fig. 2, however, the iodine consumption values did not fall to zero but after 100 min. treatment there remained a small residual reducing power which decreased extremely slowly.

*Effect of the enzyme preparation on the oxidation of the
three dienol compounds.*

Experiments of the type just described led to a study of rates of oxidation of the three dienol compounds in the presence and absence of small amounts of the enzyme preparation. Mawson [1935] and others have reported that extracts of animal tissues possess the property of protecting ascorbic acid against aerobic oxidation. We have found that the β -amylase preparation, an extract of ungerminated barley, has a similar action in decreasing considerably the rate of oxidation of ascorbic acid. In contrast, however, to its stabilising action toward ascorbic acid the presence of the enzyme preparation markedly accelerates the oxidation of the other two dienol compounds.

The following experiment will serve to illustrate this difference in the action of the enzyme extract toward the three compounds.

$M/500$ solutions of ascorbic acid, reductone and dihydroxymaleic acid, neutralised to p_H 6.4 with NaOH and made up in 0.005 M phosphate of the same p_H were used. Within 1.5 min. of dissolving the dry substances in the previously measured NaOH and buffer (already diluted to volume) two 50 ml. portions of each dienol solution were treated as follows: to one were added 3 ml. of water, to the other 3 ml. of undiluted stock enzyme solution. Samples of 10 ml. were taken at intervals for determinations of iodine consumption in the manner described earlier (p. 2591). Throughout the experiment the solutions were aerated every 3 or 4 min. by whirling around the walls of the 500 ml. flasks in which they were contained. The final concentration of the dienol compounds was 0.0185 M throughout (which is considerably stronger than in either of the two preceding inhibition experiments) and in the solutions to which enzyme extract was added the dry matter from the extract amounted to 0.28 mg. per ml. of the final solution (which is smaller than under the normal pretreatment conditions).

From the iodine consumption values the progress of oxidation of the dienol group in the six solutions was calculated; the results appear in Table II.

Table II. *Aerobic oxidation of dienol compounds in presence and
absence of added enzyme preparation.*

Dienol compounds 0.0185 M throughout; p_H 6.4; temp. 21.5–22.5°.

Time min.	Percentage oxidation of dienol group					
	I		III		V	
	Ascorbic acid		Reductone		Dihydroxymaleic acid	
	Alone	+ Extract	Alone	+ Extract	Alone	+ Extract
15	13.9	4.8	18.3	38.8	4.9	12.2
30	23.2	5.7	25.5	57.2	7.0	18.4
45	32.2	6.7	32.2	66.5	—	—
60	39.8	7.5	37.8	79.8	10.5	27.3
75	47.0	8.1	43.6	87.5	11.8	31.0
95	—	—	—	—	13.5	36.7

By comparing columns I, III and V it will be seen that in the absence of the enzyme extract dihydroxymaleic acid is oxidised least rapidly, ascorbic

acid and reductone being oxidised at considerably greater and roughly similar rates. (The initial rate for reductone is slightly higher but falls off appreciably.) With added extract, however, there is a marked change in the rates; ascorbic acid is oxidised much less rapidly than before, and the rate of oxidation of the other two compounds is considerably increased. Thus the addition of the extract has decreased the oxidation of ascorbic acid for the 75-min. period from 47 to 8.1%—whereas the oxidation of reductone is increased from 43.6 to 87.5% and that of dihydroxymaleic from 11.8 to 31.0%.

From results of this type it is concluded that the enzyme extract contains a factor (or factors) of unknown nature which protects the dienol group of ascorbic acid against aerobic oxidation, but that under the same conditions the oxidation of reductone and dihydroxymaleic acid is greatly accelerated. Further investigation is required before the nature of this effect can be discussed.

This peculiar difference in the behaviour of the three dienol compounds in the presence of the enzyme extract offers a clear explanation of the differences in the duration of their inhibitory action on β -amylase. Ascorbic acid which is stabilised against oxidation by the extract produces the most permanent inhibition, whereas reductone whose oxidation is most rapid in the presence of the extract produces the most transient inhibition, dihydroxymaleic acid being intermediate in both respects.¹

The effect of oxidisers dienol compounds and the addition of oxidants with the reduced compounds.

As would be expected from the foregoing experiments the oxidation of the dienol compounds by aeration before mixing with the enzyme destroys their inhibitory power. The effects of 10-min. pretreatments with reduced and aerobically oxidised ascorbic acid and reductone are given in Table III.

Table III.

	Relative activity
2 ml. enzyme. Untreated control (initial velocity 0.320)	100
2 ml. enzyme + 1 ml. $\equiv$ 0.2 mg. reduced ascorbic acid—10 min.	12
2 ml. enzyme + 1 ml. $\equiv$ 0.2 mg. oxidised ascorbic acid*—10 min.	97.5
2 ml. enzyme + 1 ml. $\equiv$ 0.1 mg. reduced reductone—10 min.	15
2 ml. enzyme + 1 ml. $\equiv$ 0.1 mg. oxidised reductone†—10 min.	98

* Solution previously aerated 48 hours. Iodine consumption 3% of original.

† Solution previously aerated 24 hours. Iodine consumption 1.5% of original.

Owing to the extreme sensitivity of β -malt-amylase to inactivation in the presence of slight traces of most oxidising reagents the choice of reagents other than molecular oxygen for the oxidation of the dienol group is limited. Pretreatment of the enzyme with ascorbic acid previously oxidised with iodine produces considerable inhibition although not as much as reduced ascorbic acid. For example, 5-min. treatments of 2 ml. enzyme with 0.35 mg. of reduced and

¹ The selective stabilisation of ascorbic acid by barley extracts seems of interest not only in connection with the present subject but also in suggesting a possible explanation of the specificity of ascorbic acid as an antiscorbutic factor. It seems possible that the failure of reductone and dihydroxymaleic acid to protect against scurvy (as reported by Euler and Martius [1933] and Dahlmer [1934] respectively) may be due to the accelerated oxidative destruction of these dienol compounds in the presence of tissues in contrast to ascorbic acid which is known to be protected against oxidation. This suggestion is being investigated with a wider range of dienol compounds than has hitherto been available and with a variety of plant and animal extracts.

iodine-oxidised ascorbic acid produced inhibitions of 82.5 and 53.5 % respectively. We are inclined to believe however that the inhibition in the latter case was due to traces of free iodine which in a concentration of $3.3 \times 10^{-6} N$ would account for this effect.¹

In an investigation of the effects of a variety of oxidation systems on β -malt-amylase (which will be described in a subsequent paper) it was found that potassium ferricyanide and methylene blue produce only very slow inactivation. We have accordingly tried the effect of adding these substances with reduced ascorbic acid during pretreatments of the enzyme. Typical results are given in Table IV.

Table IV. *Pretreatment with ascorbic acid in the presence of methylene blue and potassium ferricyanide.*

Enzyme, 2 ml.; ascorbic acid, 0.1 mg. in 1 ml.; methylene blue and potassium ferricyanide, 1 ml. of 0.001 M solution.	
	Relative activity
Enzyme. Untreated (initial velocity 0.405)	100
Enzyme + ascorbic acid. Pretreated 15 min.	22
Enzyme + ascorbic acid + methylene blue. Pretreated 15 min.	57
Enzyme + ferricyanide. Pretreated 15 min.	97
Enzyme + ascorbic acid + ferricyanide. Pretreated 15 min.	86.5
Enzyme + ascorbic acid + ferricyanide. Pretreated 25 min.	95

It will be seen that methylene blue (which oxidises ascorbic acid only slowly) decreases the inhibition appreciably and ferricyanide (which oxidises ascorbic acid moderately rapidly) almost entirely removes the inhibition.

It can be concluded from the results of the foregoing experiments that the strong inhibition of β -amylase is due to the reduced forms of the dienol compounds used. The regeneration of the activity of the enzyme observed is clearly associated with the aerobic oxidation of the inhibiting compound and it has been shown that treatment with the previously oxidised compounds produces no inhibition. Moreover, the presence of methylene blue and ferricyanide to a great extent prevents the inhibition.

Fig. 4. Relation between degree of inhibition and concentration of ascorbic acid. Pretreatment period 10 min. The indicated amounts of ascorbic acid dissolved in 1 ml. were added to 2 ml. enzyme.

¹ Such inhibition by iodine-oxidised ascorbic acid, unlike that by the reduced substance, is not reversed by treatment with cyanide or hydrosulphite. This is also true of inactivation by iodine.

Relation between inhibition and concentration of ascorbic acid.

In many experiments, particularly those of an exploratory nature, a pre-treatment period of 10 min. was used. Consequently, although no single experiment was designed to investigate the relationship between amount of ascorbic acid and inhibition, there are available a number of observations of the inhibitions produced by 10-min. treatments with different amounts of ascorbic acid. These observations, which were made at different times on three different enzyme preparations, serve to illustrate the general form of the relation (Fig. 4). (In all these cases 2 ml. of enzyme were pretreated with 1 ml. of solution containing the indicated amounts of ascorbic acid.)

The degree of inhibition rises rapidly with increasing amounts of ascorbic acid up to about 0.2 mg. (per 3 ml. enzyme-inhibitor mixture) but above this range of concentration the degree of inhibition rises only very slightly with increasing amounts of ascorbic acid.

Table V. *Progress of maltose production in digests with untreated enzyme, inhibited enzyme and reactivated enzyme.*

Enzyme throughout, 2 ml.; ascorbic acid (asc.), 1 ml. 0.001 *M* solution ($\equiv$ 0.176 mg.); dihydroxymaleic acid (D.H.M.), 1 ml. 0.001 *M* solution ($\equiv$ 0.148 mg.); reductone, 1 ml. 0.002 *M* solution ($\equiv$ 0.176 mg.); cysteine, 1 ml. 0.5 mg. calculated as hydrochloride.

<i>t</i> min.	mg. maltose per 5 ml.	Slope mg./min.	Initial velocity*	<i>t</i> min.	mg. maltose per 5 ml.	Slope mg./min.	Initial velocity*
Controls.							
(1) Enzyme untreated.				(2) Enzyme + cysteine pretreated 5 min.			
0.7	0.426	0.348	0.350	0.7	0.495	0.328	0.335
1.9	0.844	0.330	(100 rel.)	1.9	0.888	0.318	(95.5 rel.)
3.1	1.239	0.3065		3.1	1.270	0.327	
4.3	1.607	0.311		4.3	1.662	0.345	
5.5	1.980			5.5	2.076		
Ascorbic acid—treated.							
(3) Enzyme + ascorbic acid—pretreated 10 min.				(4) Enzyme + ascorbic acid—pretreated 10 min., then cysteine 5 min.			
0.7	0.294	0.072	0.080	0.7	0.479	0.306	0.310
1.9	0.380	0.066	(23 rel.)	1.9	0.846	0.310	(88.5 rel.)
3.1	0.459	0.020		3.1	1.207	0.298	
4.3	0.483	0.014		4.3	1.565	0.300	
5.5	0.500			5.5	1.924		
Reductone—treated.							
(5) Enzyme + reductone—pretreated 10 min.				(6) Enzyme + reductone—pretreated 10 min., then cysteine 5 min.			
0.7	0.326	0.050	0.060	0.7	0.640	0.300	0.310
1.9	0.386	0.019	(17 rel.)	1.9	1.000	0.277	(88.5 rel.)
3.1	0.409	0.004		3.1	1.332	0.270	
4.3	0.414			4.3	1.656		
Dihydroxymaleic acid—treated.							
(7) Enzyme + D.H.M.—pretreated 10 min.				(8) Enzyme + D.H.M.—pretreated 10 min., then cysteine 5 min.			
0.7	0.404	0.214	0.215	0.7	0.529	0.353	0.360
1.9	0.660	0.216	(61.5 rel.)	1.9	0.952	0.334	(103 rel.)
3.1	0.919	0.206		3.1	1.352	0.331	
4.3	1.166	0.202		4.3	1.749	0.323	
5.5	1.408			5.5	2.137		

* Extrapolated values.

Reversal of dienol inhibition by hydrogen cyanide, sulphydryl compounds and sodium hydrosulphite.

In discussing the progress curves of maltose production (on which activity values are based) attention has already been drawn to the data of Table VI in which is shown the re-activation of the enzyme by cysteine after inhibition by the three dienol compounds.

It will be seen that the control enzyme is not appreciably influenced by treatment with cysteine (Digests 1 and 2). After treatment with ascorbic acid, reductone and dihydroxymaleic acid the activity was reduced to 23, 17 and 61.5 % respectively of the control value (Digests 3, 5 and 7) and after the 5-min. cysteine treatment these values were increased to 88.5, 88.5 and 103 % of the control (Digests 4, 6 and 8). There is thus a nearly complete removal of the inhibition by cysteine in all cases.

Other reagents effective in reversing the inhibition are hydrogen cyanide, sodium hydrosulphite, thiosalicylic and thiolacetic acids. With the β -amylase preparations used in this investigation¹ none of these substances affected the activity of the non-inhibited enzyme to an appreciable extent except after prolonged storage at 0° when small activations by cysteine were observed, the greatest activation of this type being a 16 % increase in activity after pretreatment of 2 ml. enzyme for 20 min. with 4 mg. cysteine. In general pretreatment of the control enzyme with these reducing compounds resulted in barely significant inhibitions (of the order of 1 to 5 %). It may therefore be stated with assurance that the great increases in activity observed after treating the dienol-inhibited enzyme with these compounds are due to a reversal of the dienol inhibition.

In Table VI are given the results of three typical experiments illustrating the reversal of various dienol inhibitions by the group of activators mentioned above.

Table VI.

Exp.	Inhibition by 1 ml. containing	Reactivation by	I.V.	Relative activity
A	Control	—	0.322	100
	"	2 ml. 0.1N HCN—5 min.	0.318	99
	0.4 mg. maleic acid—10 min.	—	0.319	99
	0.154 mg. D.H.M.*—10 min.	—	0.136	42
	"	2 ml. 0.1N HCN—5 min.	0.285	88.5
	"	2 ml. $\equiv$ 1 mg. cysteine—8 min.	0.320	99.5
	"	2 ml. $\equiv$ 1 mg. Na hydrosulphite—5 min.	0.317	98.5
B	Control	—	0.410	100
	"	1 ml. $\equiv$ 2 mg. cysteine—5 min.	0.435	106
	0.75 mg. ascorbic acid—10 min.	—	0.018	4.5
	"	2 ml. 0.1N HCN—2 min.	0.320	78
	"	2 ml. 0.1N HCN—7 min.	0.380	92.5
	"	1 ml. $\equiv$ 2 mg. cysteine—5 min.	0.330	80.5
	"	1 ml. $\equiv$ 2 mg. thiosalicylic acid—7 min.	0.375	91.5
C	Control	—	0.315	77
	"	2 ml. $\equiv$ 2 mg. hydrosulphite—6 min.	0.342	100
	0.1 mg. reductone—10 min.	—	0.330	96.5
	"	2 ml. 0.1N HCN—6 min.	0.110	32
	"	2 ml. $\equiv$ 4 mg. cysteine—6 min.	0.330	96.5
	"	2 ml. $\equiv$ 2 mg. hydrosulphite—6 min.	0.315	92
	"	—	0.280	82

* D.H.M. = dihydroxymaleic acid.

¹ In an investigation briefly reported earlier [Hanes and Barker, 1931] cyanide activations of certain β -amylase preparations from barley were observed.

Exp. A shows complete reversal of a 58% inhibition with dihydroxymaleic acid by cysteine and hydrosulphite treatments and a considerable reactivation by cyanide. The activity of the non-inhibited control was not influenced by cyanide pretreatment. We have included also with this experiment an observation which shows that maleic acid (neutralised to p_H 6.4 as usual with reagents used in pretreatment) does not influence the activity of the enzyme.

In Exp. B are shown the effects of a variety of reactivation treatments (using cyanide and three different sulphydryl compounds) after an almost complete inhibition by ascorbic acid, and in Exp. C are shown reactivations after reductone inhibition. The control enzymes in these cases showed respectively a slight activation with cysteine and a slight inhibition with sodium hydrosulphite.

It is felt that the examples given in Tables V and VI are sufficient to establish the fact that the inhibition of β -malt-amylase by the three dienol compounds can be reversed completely or nearly completely by treatment with cyanide, hydrosulphite and various sulphydryl compounds. On the basis of numerous additional data now at hand it may be said that the concentrations of the different activators used in the above experiments are sufficiently great to induce maximum rates of reactivation, although in some of the experiments the duration of treatment was not long enough to allow the maximum reactivation to occur. A fuller discussion of the time relations of the reactivation and the relationship between the activity of the enzyme and the concentrations of dienol and activator is, however, beyond the scope of the data now available.

The effects of other factors on the inhibition by ascorbic acid.

(a) *The effects of added iron and copper.* Papain although inhibited by ascorbic acid alone is activated by treatment with ascorbic acid *plus* ferrous sulphate according to the observations of Maschmann and Helmert [1934]. In view of this effect, β -malt-amylase was subjected to similar treatment. It was found that the addition of ferrous sulphate with ascorbic acid resulted in slightly increased inhibition. Pretreatment of β -amylase with ferrous sulphate alone causes considerable inactivation; thus a 3-min. pretreatment with $0.9 \times 10^{-5} M$ ferrous sulphate produced an 8% inhibition. With the higher concentrations such as were applied to papain by Maschmann and Helmert considerably greater inhibition results. We give an example in which the concentrations of iron and ascorbic acid are the same as those reported to activate papain. 2 ml. portions of enzyme were pretreated 10.5 min. with (a) 1 ml. ferrous sulphate ($\equiv 3.15$ mg. $FeSO_4 \cdot 7H_2O$), (b) 1 ml. ascorbic acid ($\equiv 0.2$ mg.), (c) ferrous sulphate *plus* ascorbic acid. The observed inhibitions were 63, 86 and 89% respectively. It can be concluded that added ferrous iron does not have the effect of converting the inhibitory effect of ascorbic acid into an activation in the case of β -amylase.

The addition of traces of copper sulphate in concentrations so low as to produce only slight inhibition when used alone were found greatly to increase the inhibition by ascorbic acid and reductone. It was found moreover that such inhibition in the presence of copper was only slightly reversible by subsequent treatment with hydrogen cyanide. Examples of this effect are shown in Table VII.

We are here reminded of the observations of Maschmann and Helmert on papain and Euler *et al.* [1934] on liver cathepsin that the addition of traces of cupric salts with ascorbic acid causes inhibition.

Table VII.

A. Effect of addition of traces of copper sulphate with ascorbic acid.

	I.V.	Relative activity
(1) Control	0.376	100
(2) Control + 20 γ CuSO ₄ —5 min.	0.340	90.5
(3) Enzyme + 0.1 mg. ascorbic acid—5 min.	0.095	25
(4) As (3) followed by 2 ml. 0.1 <i>N</i> HCN—8 min.	0.352	93.5
(5) Enzyme + 0.1 mg. ascorbic acid + 20 γ CuSO ₄ —5 min.	0.005	1.3
(6) As (5) followed by 2 ml. 0.1 <i>N</i> HCN—8 min.	0.155	41

B. Effect of addition of traces of copper sulphate with reductone.

(1) Control	0.310	100
(2) Enzyme + 0.1 mg. reductone—10 min.	0.044	14
(3) As (2) followed by 2 ml. 0.1 <i>N</i> HCN—6 min.	0.295	95
(4) Enzyme + 0.1 mg. reductone + 2 γ CuSO ₄ —10 min.	0	0
(5) As (4) followed by 2 ml. 0.1 <i>N</i> HCN—6 min.	0.130	42

(b) *The presence of acetate buffer.* The addition of the acetate buffer, which was used in maintaining the acidity of the starch digests, to the enzyme during pretreatment with ascorbic acid was found to increase the inhibition. For example, the addition of 0.01 *M* acetate of p_H 4.7 during a 10-min. pretreatment with 0.05 mg. ascorbic acid increased the inhibition from 53 to 86 %. Whether this effect is attributable to the increased acidity or to the presence of heavy metal impurities in the buffer has not been determined. It is mentioned merely as offering an explanation, at least in part, of the falling reaction rates observed in digests with inhibited enzyme (p. 2590 above).

(c) *Exclusion of oxygen during pretreatment.* We mention finally an experiment in which the enzyme was pretreated with ascorbic acid in the absence of oxygen. Use was made of a small glass vessel with two limbs in which were placed separately 2 ml. enzyme and 1 ml. of ascorbic acid containing 0.2 mg. The vessel was evacuated by means of an oil pump so as to induce gentle boiling of the solutions at room temperature. This was continued for 5 min., the vessel

Fig. 5. Progress curves of maltose production with untreated enzyme and enzyme pretreated 15 min. with 0.2 mg. ascorbic acid (a) in air, (b) in absence of oxygen.

being shaken continuously. The two solutions were then mixed, tipping from one limb to the other several times, and were left 15 min. in the evacuated vessel. The vessel was then opened and the inhibitor-enzyme mixture was added

to the digest, the vessel being rinsed out with the digest fluid. A second portion of enzyme was then pretreated in the same vessel without evacuation. The progress curves for these enzymes, together with that of the untreated control, are shown in Fig. 5.

It will be seen that the exclusion of oxygen during pretreatment had no significant effect upon the development of the inhibition, the initial velocities of maltose production being the same and indicating an inhibition of approximately 80 % of the untreated control. (The more rapid fall in the reaction rate in the progress curve with enzyme pretreated anaerobically is possibly not significant since as we have mentioned earlier considerable variability in this effect is encountered.)

DISCUSSION.

The experimental results demonstrate that β -malt-amyase is inhibited by the reduced forms of *l*-ascorbic acid, reductone and dihydroxymaleic acid. These compounds, whose formulae are set out below, have in common the possession of the strongly reducing dienol grouping but are in other respects widely dissimilar.

That the inhibition of the enzyme by the three compounds is of the same nature is strongly indicated by certain peculiarities of the effect in each case. The unusual features of this particular type of inhibition are that it is reversed on the one hand by oxidative treatments which destroy the dienol grouping, and on the other hand by the addition of a variety of reducing substances (of widely different reducing power). We are thus led to the conclusion that the inhibition is due to the presence of the dienol grouping, and moreover that the inhibitory action of this grouping is not due merely to its reducing properties but is more specific in nature since the effect is reversed by the addition of other reducing substances which have no appreciable effect on the activity of the non-inhibited enzyme.

It is worthy of mention that dihydroxymaleic acid produces a considerably smaller inhibition than ascorbic acid or reductone in the same molar concentration, the latter two compounds inhibiting to approximately the same extent (*cf.* p. 2592). This fact suggests that the particularly reactive "endiol" configuration which occurs in ascorbic acid and reductone possesses a greater inhibitory power than the dienol grouping as it occurs in dihydroxymaleic acid. Further investigation to test this suggestion is desirable.

The most unusual feature of this inhibition by dienol compounds is its reversibility either by oxidative treatments or by the addition of a variety of reducing substances. Thus we have presented data illustrating reactivations on the one hand by molecular oxygen, methylene blue and ferricyanide, and on the other by sodium hydrosulphite, sulphydryl compounds and cyanide.

The mechanism of the reversal of inhibition by oxidative treatments would appear to be relatively simple and would seem to be the result of the destruction of the inhibiting group by oxidation. (It seems probable from the results with ferricyanide that the oxidation of ascorbic acid to the reversibly oxidised state destroys the inhibitory action although this cannot be held to be definitely established, *cf.* p. 2596.)

The mechanism underlying the reversal of the dienol inhibition by cyanide, hydrosulphite and sulphydryl compounds remains obscure and our present data are inadequate for its elucidation. The different cases in which activations of other hydrolytic enzymes have been reported by members of this same group of substances would appear to involve effects of two distinct types: (1) the removal of heavy metal inhibitors (in which case the activators are effective by virtue of their ability to form complexes with the inhibiting metal ion); (2) the reversal of inactivations which are the result of reversible oxidation of some essential part of the enzyme complex [*e.g.* Hellerman and Perkins, 1934]. It is evident that serious difficulties are encountered in attempting explanations of the inhibition of β -amylase by dienols and the reactivation by the group of activators mentioned, along either of these recognised lines. It is hoped that additional data bearing on this problem will be available shortly.

Although the mechanism remains obscure, the effect appears of considerable interest as representing a type of reaction which may be of importance in the physiology of ascorbic acid. We refer in particular to the opposing actions of sulphydryl compounds on the one hand and of ascorbic acid on the other in their effects on this enzyme. In this connection the observation of Birch and Dann [1933] that ascorbic acid and glutathione occur commonly together in the tissues of the animal body is suggestive. While these authors regard the parallelism in distribution as evidence that the two substances are components of a single oxidation system, our present effect suggests the possibility that they may function as the opposing elements of an enzyme regulatory mechanism.

It may be stated finally that there remains the possibility that effects of a similar nature to those described for β -malt-amylase may occur with other enzyme systems. In particular, attention is called to the cases of papain and urease which are inhibited by ascorbic acid according to the reports of Maschmann and Helmert [1934] and Edlbacher and Leuthardt [1933] respectively. (It is to be noted that papain, unlike β -amylase, is activated by ascorbic acid *plus* iron.) Both papain and urease are known to be activated by cyanide, hydrosulphite and sulphydryl compounds after certain "heavy metal" and "oxidative" inactivations [Hellerman and Perkins, 1934; Hellerman *et al.*, 1933] and it would be of great interest to know whether the same group of substances is effective in reversing the reported inhibitions of these enzymes by ascorbic acid, as is the case with β -malt-amylase.

SUMMARY.

1. Treatment of β -malt-amylase with reduced ascorbic acid results in inhibition. The inhibition is not "instantaneous" but requires a measurable time for maximum development. During prolonged pretreatment of the enzyme with ascorbic acid a slow regeneration of activity occurs.

2. Reductone and dihydroxymaleic acid likewise inhibit the enzyme. The inhibitions by equivalent concentrations of ascorbic acid and reductone are of the same order, which is considerably greater than that by dihydroxymaleic acid.

3. During pretreatment of the enzyme with reductone and dihydroxymaleic acid a relatively rapid regeneration of activity occurs. This reversal of the inhibition is shown to be related to the destruction of the dienol grouping by oxidation.

4. It is shown that the presence of the enzyme extract protects ascorbic acid against oxidation whereas it accelerates the destruction of reductone and dihydroxymaleic acid.

5. Preliminary aerobic oxidation of ascorbic acid and reductone destroys their inhibitory power.

6. The addition of methylene blue and ferricyanide (which exert only very slight inhibitions on the enzyme) reverses the inhibition by ascorbic acid.

7. The addition of hydrogen cyanide, sodium hydrosulphite, cysteine, thio-salicyclic acid and thiolacetic acid reverses the inhibition by the three dienol compounds.

8. The addition of ferrous sulphate has little effect upon the degree of inhibition by ascorbic acid but traces of copper cause increased inhibition.

9. Exclusion of oxygen does not significantly affect the development of the inhibition.

I wish to express my indebtedness to Mr James Alexander for invaluable technical assistance.

REFERENCES.

- Birch and Dann (1933). *Nature*, **131**, 469.
Dahlmer (1934). *Deutsch. med. Woch.* **60**, 1200.
Edlbacher and Leuthardt (1933). *Klin. Woch.* **12**, 1843.
Euler, Karrer and Zeheuder (1934). *Helv. Chim. Acta*, **17**, 157.
— and Martius (1933). *Liebig's Ann.* **505**, 73.
Fenton (1905). *J. Chem. Soc.* **87**, 804.
Hanes (1929). *Biochem. J.* **23**, 99.
— (1932). *Biochem. J.* **26**, 1406.
— and Barker (1931). *Proc. Roy. Soc. Lond.* B **108**, 95.
Hellerman and Perkins (1934). *J. Biol. Chem.* **107**, 241.
— Perkins and Clark (1933). *Proc. Nat. Acad. Sci.* **19**, 855.
van Klinkenberg (1931). *Proc. Acad. Sci. Amsterdam*, **34**, 893.
Maschmann and Helmert (1934). *Z. physiol. Chem.* **224**, 56.
Mawson (1935). *Biochem. J.* **29**, 569.
Purr (1934). *Biochem. J.* **28**, 1141.
Small (1919). *J. Amer. Chem. Soc.* **41**, 113.