ACTIVATION AND OTHER PROPERTIES OF ASCORBIC ACID OXIDASE*

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INTRODUCTION

The reaction inactivation of several copper oxidases, particularly ascorbic acid oxidase, (AAO) has been the subject of previous investigations summarized by DAWSON AND TARPLEY[1] and SINGER AND KEARNEY[2]. In the absence of certain protective agents, these enzymes appear to be rapidly inactivated during catalysis of the aerobic oxidation of the appropriate substrate. POWERS AND DAWSON[3] classified the inactivation of the copper enzyme, AAO, into two categories: (a) inactivation due to environmental conditions during the reaction reduced by inert proteins such as gelatin, egg albumin, denatured catalase or peroxidase, and (b) inactivation due to some factor inherent in the ascorbic acid–AAO reaction, sensitive only to heme-containing proteins such as catalase and peroxidase.

Interest in the activation and protection of AAO was renewed by the paper of GEMMILL[4] on the activating effect of thyroxine on the AAO system. FRIEDEN[5] confirmed GEMMILL's report and observed that the activating and protecting action was not specific for thyroxine or proteins, but was shown by any effective cupric ion complexer such as EDTA, cyanide, cysteine, and other amino acids. It was observed that the copper complexers appear to increase the initial rate of AAO action as determined by the Warburg technique and also to protect the enzyme against reaction inactivation. These findings were of particular interest because of the subsequent observation that AAO is very sensitive to cupric ion[6] and that only when the enzyme is aerobically active is there an exchange of enzyme copper for radio-active cupric ion[7].

Additional evidence was presented pertaining to the possible sulfhydryl dependence of AAO. FRIEDEN AND NAILE[8] reported the inhibition of AAO by the organic mercurials such as phenylmercuric chloride. However, this inhibition could be reversed by cysteine, glutathione, thyroxine, ribonucleic acid, some nucleotides, and some

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amino acids. Thus while this spectrum of activations, inhibitions and reversals was suggestive, the essentiality of sensitive chemical groups other than or in addition to sulfhydryl groups of the enzyme was not precluded[9].

Of possible import to the behavior of AAO are the recent reports of the existence of a highly reactive DPNH-oxidizing intermediate in the AAO system by KERN AND RACKER[10] and WOISLOIT, NASON AND TERRELL[11, 12]. It was shown that in both the alcohol dehydrogenase and quinone reductase system, rapid oxidation of reduced DPN formed *in situ* was observed when both ascorbate and AAO was added. Neither component alone nor cupric ion and ascorbate was able to effect a similar oxidation. Earlier, MATHEWS[13] had postulated a "monodehydroascorbate" as an intermediate in the conversion of ascorbate to dehydroascorbate. POWERS AND DAWSON[3] had also previously suggested that the inactivation of AAO might be due to some factor inherent in the AAO reaction.

This report summarizes our recent studies on the behavior of different preparations of AAO treated with a variety of conditions and chemical reagents.

EXPERIMENTAL

Methods

AAO activity at 30.0° C was determined by two independent methods essentially as described by POWERS, LEWIS AND DAWSON[14] and RACKER[15]. The Warburg technique involves the addition of enzyme to the main compartment of the Warburg vessel, immediately prior to equilibration in a final volume of 3.00 ml. Also in the main compartment, except where noted, were sufficient sodium phosphate buffer, pH 7.2, for a final concentration of 0.0100 *M* and any added compound. Freshly neutralized ascorbic acid was added to the side arm. Final pH's after reaction were 7.0 ± 0.2. This pH was selected because of the greater solubility of many of the compounds tested as activators and the close to maximal enzyme activity at this pH.

After five minutes equilibration, the side arm contents were tipped into the main compartment. After a further brief equilibration, the vessels were closed off and readings taken at five-minute intervals or otherwise as desired. No alkali was required in the center well, and except where otherwise noted, air was used as the gas phase.

Glass redistilled water was used in all solutions and glassware rinsing. This apparently reduced the trace metal ion contamination so that usually less than 5 μl of oxygen were consumed in a 30 min experimental period.

In the spectrophotometric method adopted from the report of RACKER[15], AAO was added immediately prior to mixing and timing the reaction. The 3.00 ml final volume in the Beckman cell contained 0.0100 *M* phosphate buffer at pH 7.2 and the indicated concentration of ascorbic acid stabilized with approximately 10^{-6} *M* EDTA. After a rapid mixing, readings were taken at 30-second intervals at 265 mμ and the rate determined from the reduction in optical density between the one-minute and five-minute period during which the fall in optical density is linear as indicated in Fig. 1. The rate is expressed as the decrease in optical density per minute for this linear period.

Materials

C.P. Chemicals and biochemicals of the highest quality purchasable were used throughout. The source of special chemicals is indicated in appropriate references in the results. It was observed that sodium ascorbate gave large oxygen uptakes in the absence of enzyme, presumably owing to metal ion impurities. Accordingly C.P. ascorbic acid was used in all experiments.

The ascorbic acid oxidase (AAO) preparations were obtained from the Reheis Co., and the Nutritional Biochemicals Co. A valuable additional preparation was also obtained through the generosity of Dr. CHARLES R. DAWSON. The essential features of the various enzyme preparations are summarized in Table I. It must be emphasized that it is recognized that the properties of even the purest AAO preparations appear to alter slowly on standing despite storage in an iced Dewar maintained in a cold room. No ready solution to this storage problem seems available, since AAO loses considerable activity on lyophilization. The stability of AAO activity in the diluted state is considered in a subsequent section. However, it should be emphasized that while samples differed quantitatively, no significant qualitative differences between the four preparations cited here and earlier preparations have been noted.

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RESULTS

Activation and protection of AAO

Inert proteins and other so-called activating agents maintain AAO activity over a 30-minute experimental period. This is illustrated in Figs. 2–4. In Fig. 2, activator was added to the main compartment containing the enzyme 10–15 minutes prior to reaction initiation. After 30 minutes the rate of AAO oxidation in these vessels which did not contain gelatin had become negligible. No change in rate was observed for those systems in which activator was present. Fig. 3 shows that gelatin protection is maximal when gelatin is present prior to the addition of substrate. Its protective power diminishes as unprotected enzyme is allowed prior contact with substrate. These results become understandable if we assume that a highly reactive intermediate contributes to the enzyme's inactivation or if we assume that active enzyme undergoes some structural change which makes it more sensitive to denaturation. For either case inert protein such as gelatin might be expected to protect the AAO protein from inactivation.

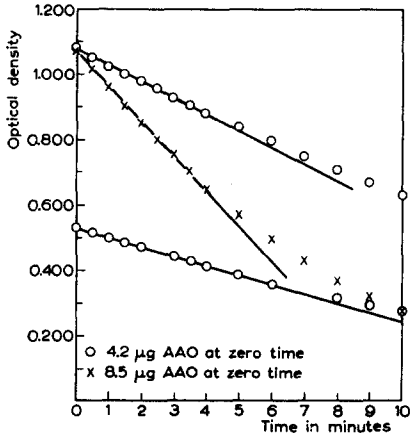

Fig. 1. Spectrophotometric determination of AAO activity. 4.2 and 8.5 μg AAO-4 were added at zero time. The reaction mixture contained 0.010 *M* phosphate buffer pH 7.2, $1.00 \cdot 10^{-6}$ *M* EDTA and either $7.0 \cdot 10^{-4}$ *M* or $3.5 \cdot 10^{-4}$ *M* freshly neutralized ascorbate. The optical density was measured at 265 mu.

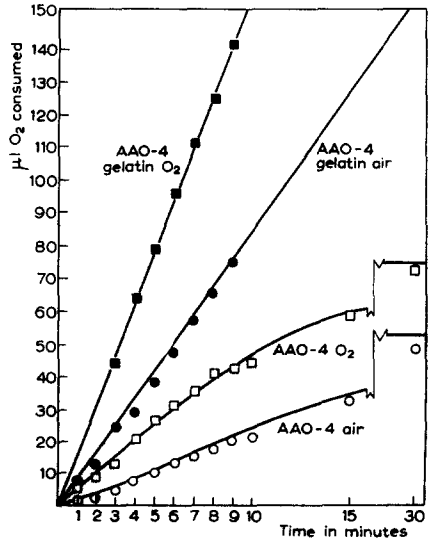

Fig. 2. Comparative effects of gelatin on the rate of ascorbate oxidation in air and oxygen. Each vessel contained 0.010 *M* phosphate buffer, pH 7.2, 0.020 *M* ascorbate, gelatin 1.0 mg/ml, and AAO-4, 5.0 units per vessel.

Effect on initial rate

Is the initial rate of ascorbate oxidation as catalyzed by AAO affected by the presence of gelatin or other activator? As indicated in Figs. 2 and 3, the Warburg method suggests a positive answer to this question. In either air or oxygen, the initial rate appears to be increased by gelatin as well as the maintaining of AAO activity. A similar experiment using the spectrophotometric method over a ten-minute period gave similar data with a definite increase in initial rate when the activator EDTA

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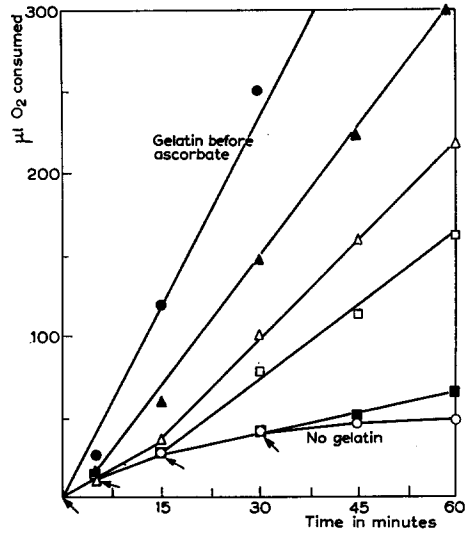

Fig. 3. Effect of time of adding gelatin on the activation of AAO. A total of 3 mg gelatin was added before ascorbate, and 1, 5, 15 and 30 min after ascorbate as indicated by arrows. Conditions are as described in Fig. 2.

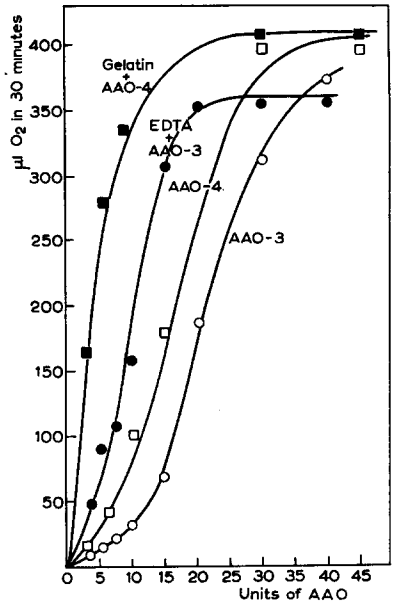

Fig. 4. Effect of AAO concentration on the rate of ascorbate oxidation. The gelatin concentration was 1.0 mg/ml and EDTA was $1.0 \cdot 10^{-4} M$ under conditions as described in Fig. 2.

was used at $10^{-4} M$. It must be emphasized here that in the spectrophotometric method, AAO is added only a few seconds before zero time.

Air and oxygen as the gas phase

In Fig. 2, oxygen and air are compared as the gas phase for AAO oxidation with and without activator. The greater rate with oxygen suggests that oxygen can be a limiting factor in this system. However, the increase in rate is not in proportion to the five-fold increase in oxygen pressure. It is of interest to note that the cupric ion-catalyzed oxidation of ascorbate shows a similar oxygen effect[16,17].

These observations are supported by the fact that AAO activity in air is somewhat sensitive to rates of shaking of the Warburg vessels[3] while activity in oxygen is relatively unaffected. Therefore when air was used as the gas phase, shaking rates were carefully standardized at 120 ± 5 oscillations per minute. However, it is believed that the similarities in the shapes of the respective curves suggests the essential identity of the action of AAO in air and in oxygen.

Stability of AAO activity

One of the problems encountered with the AAO system is the difficulty in obtaining reproducible data even using the same sample of enzyme. POWERS, LEWIS AND DAWSON[14] reported that highly diluted solutions of purified AAO, such as are prepared for activity measurements, rapidly lost their activity on standing. They also found that the loss in activity was minimized by making the dilution in the presence of an inert protein. As can be seen from Fig. 4, there are regions of enzyme activity

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which are very sensitive to small differences in enzyme concentration. Since the enzyme is very concentrated in its usual storage form, sampling variation was encountered. Thus it was important to test the stability of the non-activated and activated AAO activity in a diluted state.

As indicated in Fig. 5, we have found that diluted AAO solutions with as little as 69 μg per ml retain their activated activity when these solutions are stored at 0° C for 28 days. In some experiments, activity was maintained as long as three months. At 30° C, or when frozen and stored at —15° C, AAO activity in the presence of 10^{-4} M EDTA diminished appreciably in several days. The susceptibility of AAO to maximal activation apparently occurs despite the loss of non-activated enzyme activity. Non-activated AAO activity could not be maintained under any of these conditions. It is also possible that storage of the enzyme in the presence of an activator would protect the non-activated AAO activity.

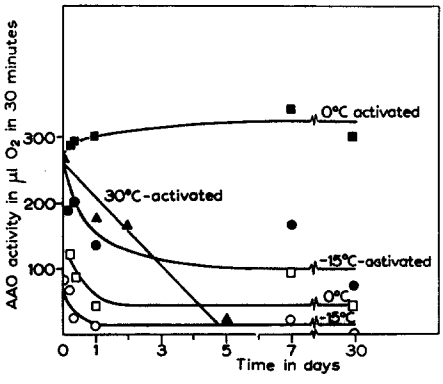

Fig. 5. Stability of AAO under various storage conditions. AAO-1 was stored in the dilution 69 μg/ml and kept at —15° C, 0° C, or 30° C respectively. Activity tests were performed as described in Fig. 1 with $1.0 \cdot 10^{-4}$ M EDTA used in the activated experiments and 6.9 μg AAO per vessel.

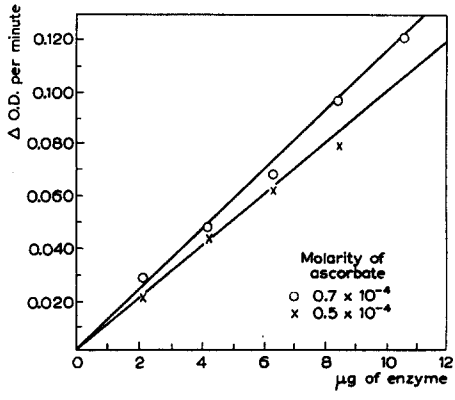

Fig. 6. Effect of AAO on the rate of ascorbate oxidation as determined by the spectrophotometric methods. The reaction mixture contained 0.01 M phosphate buffer, pH 7.2, $1.0 \cdot 10^{-6}$ M EDTA and either 0.5 or $0.7 \cdot 10^{-4}$ M ascorbate and the indicated amount of AAO in 3.0 ml. The change in optical density at 265 mu was determined from the linear portion of curves plotted as in Fig. 1.

The data for several AAO preparations are presented in Table I. The results show a qualitative similarity but some quantitative differences. In all the work on the activation of AAO, enzyme samples were accordingly prepared either from highly concentrated solutions or from diluted enzyme stored at 0–2° C. While such preparations are suitable for activation studies, they were not considered suitable for studies of native enzyme activity. It should be noted that several different AAO preparations exhibited similar stability properties.

Effect of enzyme concentration

The effect of enzyme concentration on the rate of ascorbate oxidation is shown in Fig. 4 for two different enzyme preparations. The presence of a maximally effective concentration of EDTA and gelatin gives an almost linear curve in contrast with the S-shaped curve obtained in their absence. By choosing 10 ± 5 units for these studies,

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TABLE I

SUMMARY OF SOME PROPERTIES OF THE ASCORBIC ACID OXIDASE PREPARATIONS USED IN VARIOUS EXPERIMENTS

Enzyme designation	*Source and date*	*Copper* %	*Initial activity** Units/mg × 10^3	*Non-activated AAO activity*** $\mu l\ O_2/30\ min/mg$ × 10^3	*Activated AAO activity*** $\mu l\ O_2/30\ min/mg$ × 10^3
AAO 1	Reheis Co. 6–53	0.24	1.72	34	119
AAO 2	Nutritional biochem. Corp. 8–54	0.16	1.80	9.0	63
AAO 3	Reheis Co. 9–54	0.14	1.40	12.6	27
AAO 4	Dawson 9–54	0.26	2.05	10.3	105

* Activity as given by source expressed in Lovett-Janison units, where one unit is the amount of enzyme in presence of gelatin, 1 mg/ml; oxidizing ascorbate at pH 5.6, so that $\mu l\ O_2$ is consumed per minute.

** Activity as determined under the conditions described in methods of this paper; activated AAO activity rests included 10^{-4} *M* EDTA.

intense enhancement of AAO activity can be observed. Above 30 units, activation becomes relatively insignificant. This apparent self-protection at higher enzyme concentrations is well known in enzymology and may indicate that surface phenomena may be involved in the inactivation of AAO. Enzyme concentration was also found to affect the concentration of activator necessary for maximum activation.

With the spectrophotometric method, the rate proved to be a linear function of AAO concentration, as indicated in Fig. 6. This method employs much less ascorbate per unit of enzyme and does not involve agitation during the brief reaction period of approximately five minutes; therefore, it is possible that some of the conditions which might yield the data as in Fig. 4 may not be present when the spectrophotometric method is used. The presence of 10^{-6} *M* EDTA, necessary to stabilize the ascorbate, was not found to activate AAO to any appreciable extent at this low dilution.

Effect of ascorbate concentration

As pointed out by SINGER AND KEARNEY[2], a Michaelis constant for ascorbate in the AAO system has never been reported. Fig. 7 and 8 show the effect of ascorbate concentration on the rate of ascorbate oxidation. With or without activator, similar shaped curves were obtained when the rates of ascorbate oxidation were studied with the Warburg apparatus. All data show the phenomena of high substrate inhibition, similar to those described by LINEWEAVER AND BURK[18]. Estimates of the K_m based on the Lineweaver-Burk treatment suggest a value of about $5 \cdot 10^{-3}$ *M*. However, in the spectrophotometric method, as in Fig. 8, a much lower K_m of $3.9 \cdot 10^{-5}$ *M* ascorbate is obtained. This 100-fold difference in K_m emphasizes the difference in the two methods for determining AAO activity. Rather large differences in K_m determinations using the Warburg and spectrophotometric technique have been encountered with other enzymes. Optimal ascorbate concentrations of 0.020 *M* and $1.00 \cdot 10^{-4}$ *M*, using the Warburg and spectrophotometric technique respectively, were used in most of the experiments reported in this paper.

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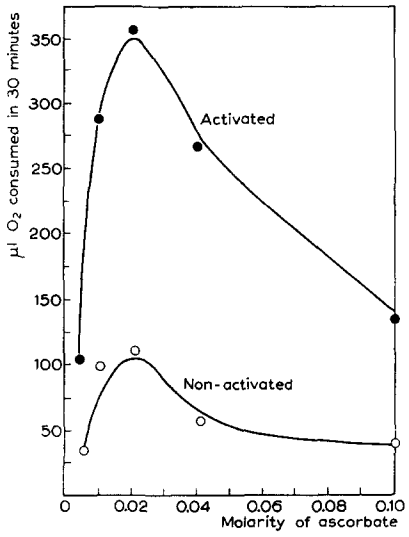

Fig. 7.

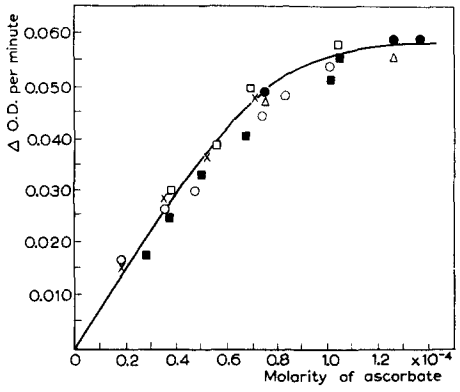

Fig. 8.

Fig. 7. Effect of ascorbate concentration on AAO activity using the Warburg technique. The reaction mixture also contained 0.010 M phosphate buffer, pH 7.2, and AAO-4, 8 μg/ml.

Fig. 8. Effect of ascorbate concentration on the rate of ascorbate oxidation as determined by the spectrophotometric method. The reaction mixture contained 0.010 M phosphate buffer, pH 7.2, $1.0 \cdot 10^{-6} M$ EDTA, and 4.2 μg AAO-4 in 3.0 ml.

It is possible that the inhibition of ascorbate oxidation at higher substrate concentrations is due to a more rapid formation of a highly reactive intermediate mentioned earlier. Since the amount of enzyme used is the same, if the rate of inactivation is also a function of the rate of formation of the intermediate, greater inactivation of AAO will obtain. An alternative explanation rests in the possible release of a greater amount of cupric ion during the early phases of the reaction resulting in greater enzyme inhibition. Nor should the possibility of the most frequent explanation for high substrate inhibition be overlooked. When the active enzyme substrate complex involves the binding of the substrate at a minimum of two different sites, high concentrations of the substrate will inhibit when two substrate molecules interact with the enzyme at one of the two required sites.

CHEMICAL REAGENTS AND ASCORBIC ACID OXIDASE

AAO, like other copper enzymes, shows marked sensitivity to copper chelators and complexing agents. The action of such compounds on AAO may be an important clue to the role of copper and to the mechanism of the catalyzed reaction. Chemical compounds which activate and/or protect AAO may be divided into two categories. The largest group comprises those compounds which produce activation at increasing concentrations, but do not inhibit AAO activity at any concentration. Prominent among these are EDTA, thyroxine, cysteine, and most proteins. Many examples of this type of activation are given in subsequent sections of this paper, including Figs. 9 and 10 and Tables III and IV.

Several compounds also activate AAO at low concentrations but inhibit the enzyme at higher concentrations. This behavior has been previously described for cyanide ion[6] and has also been observed by diethyldithiocarbamate and 8-hydroxy-

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quinoline. The inhibitors observed at high concentrations of these reagents, all of which are powerful copper complexers, may be due to the ability to remove copper from the enzyme molecule. The ability of cyanide to remove copper from another copper oxidase, polyphenol oxidase, thereby inactivating the enzyme, has been shown by KUBOWITZ[19]. Diethyldithiocarbamate activates at 10^{-7} *M* and inhibits AAO at 10^{-5} *M*. 8-Hydroxyquinoline activates at 10^{-6} *M* and shows inhibition at 10^{-4} *M*. As previously reported[6], cyanide ion activates AAO at 10^{-6} *M* and reduces initial AAO activity at about 10^{-4} *M*. Inhibition of crude AAO by these and other copper reagents such as potassium ethyl xanthate and sodium sulfide has been reported previously by STOTZ *et al.*[20]. It is therefore possible that the inhibition produced by higher concentrations of copper reagents is due to the actual removal of copper ion from AAO.

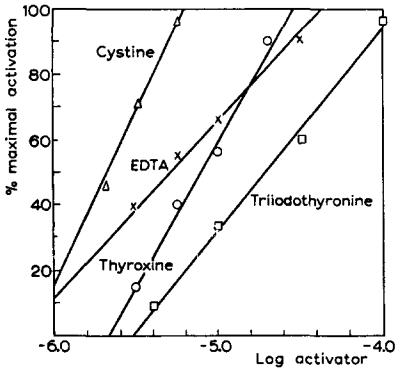

Fig. 9. Activation of AAO by several powerful copper chelators. Maximal activation was taken as that amount of activation achieved with 1.0 mg/ml gelatin. Activation was measured in 0.010 *M* phosphate buffer pH 7.2, with 6.0 μg AAO-1 per Warburg vessel and the indicated concentration of cystine, EDTA, thyroxine and triiodothyronine.

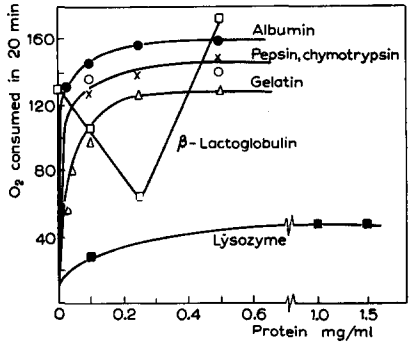

Fig. 10. The effect of some proteins on AAO activity. Activation was measured in 0.010 *M* phosphate buffer, pH 7.2, with 2.5 μg AAO-4 and the final concentration of protein in 3.0 ml Warburg vessels.

TABLE II

EFFECT OF CATALASE AND EDESTIN ON AAO ACTIVITY

Concentration of protein	*μl oxygen consumed in 30 minutes* with*	
mg/ml	*catalase*	*edestin*
0	40	40
0.0010	98	99
0.010	288	228
0.10	284	236
0.25	157	256
0.5	—	164
1.5	—	164

* The activated rate with gelatin 1.0 mg/ml was 269 μl in 30 minutes. The reaction mixture contained 0.010 *M* phosphate buffer, pH 7.2, 0.020 *M* ascorbate, 3.0 μg. AAO-4 in addition to protein as indicated.

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TABLE III

ACTIVATION OF ASCORBIC ACID OXIDASE BY AMINO ACIDS, THYROXINE ANALOGS AND NUCLEIC ACID COMPONENTS

Compounds	*Molarity**	*% maximal activation***
Glycine	10^{-2}	82
	10^{-3}	41
Glycylglycine	10^{-2}	102
	10^{-3}	2
D- or L-Phenylalanine	10^{-2}	94
	10^{-3}	21
L-Proline	10^{-2}	95
	10^{-3}	9
L-Histidine	10^{-4}	78
	10^{-5}	41
L-Tyrosine	10^{-2}	84
	10^{-3}	42
L-Tryptophane	10^{-3}	95
	10^{-4}	25
Aspartic acid	10^{-2}	65
	10^{-3}	42
Arginine	10^{-2}	91
	10^{-3}	10
Serine	10^{-2}	53
	10^{-3}	34
Thyroxine analogs		
3.5-Diiodo-DL-thyronine	$8.0 \cdot 10^{-4}$	97
	10^{-4}	29
3,5-Diiode-L-tyrosine	10^{-2}	64
	10^{-3}	5
O-Benzyl-3,5-diiodo-DL-tyrosine	10^{-4}	96
	10^{-5}	27
N-acetyl-O-benzyl-3,5-diiodo-DL-tyrosine	$3.0 \cdot 10^{-3}$	6
3,5-Diiodo-4-(3',5'-diiodo-4'-hydroxy-phenoxy) benzoic acid	10^{-3}	—3
Nucleic acid components		
Adenine	10^{-3}	64
	10^{-4}	14
Guanine	10^{-3}	104
	10^{-4}	17
Adenylic acid	10^{-3}	45
	10^{-4}	5
Guanylic acid	10^{-3}	85
	10^{-4}	30
Inosinic acid	10^{-4}	inhibitory
Cytidylic acid	10^{-3}	inhibitory
	10^{-4}	5
Uridylic acid	10^{-4}	inhibitory
DPN	10^{-3}	70
	10^{-4}	16
RNA	10^{-3}	85
	10^{-5}	15

* Unless otherwise indicated, all concentrations are 1.00 times the indicated molarity.

** Maximal activation is defined as that amount of activation obtained with 1.0 mg/ml gelatin. The reaction mixture contained 0.010 phosphate buffer, pH 7.2, 0.020 *M* ascorbate, and 5–10 units of various preparations.

Activation of AAO by proteins

Activation of AAO by proteins has been considered in some detail by POWERS, LEWIS AND DAWSON[14]. Using the Warburg technique, a number of different proteins were studied for their activating influence as summarized in Fig. 10. Included among these were several crystalline proteolytic enzymes. It was observed that except for lactoglobulin and lysozyme, these proteins gave essentially the same effect; the variations noted are due to small variations in AAO sampling. The fact that chymotrypsin did not reduce any of the activated AAO activity at pH 7.2 indicates little or no proteolysis. It was further observed that incubation of AAO with chymotrypsin for 48 hours did not modify activated AAO activity. The lack of over 40% activation by lysozyme even at much higher concentrations may indicate some important structural difference in this protein. The apparently variable effect of lactoglobulin may indicate the presence of some inhibiting impurity.

Subsequently the effect of crystalline catalase and edestin in this system was also studied. Sample data is presented in Table II. It is seen that these proteins show intense activation as lactoglobulin and also show some subsequent reduction in activation. However, little or no unique effect of catalase was found in these

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experiments. Since a satisfactory explanation for these protein effects is not readily available, this phenomenon is worthy of further study.

Activation by amino acids

Activation of AAO by amino acids has been briefly reported[5]. Some of the data are summarized in Table III. From these data it is seen that appreciable activation occurs for most amino acids at 10^{-2} *M*, but only a few amino acids such as histidine, cysteine and tryptophane activate appreciably below 10^{-2} *M*. The latter amino acids are, of course, those that might be expected to interact more effectively with copper ion than the typical α-amino acid. These observations emphasize the relatively non-specific nature of AAO activation and stimulated many of the subsequent studies on activation.

Effect of thyroxine and analogs

Activation of AAO by thyroxine was first reported by GEMMILL[4]. FRIEDEN[5] also noted that a number of thyroxine analogs showed this activity. Data on several thyroxine analogs are presented in Table III. Fig. 9 also included data with thyroxine and triiodothyronine.

It is clear from this data that the ability to activate AAO is not necesarily related to thyroxine-like activity. O-Benzyl-3, 5-diiodotyrosine activates AAO and complexes cupric ion readily[8] but serves as a thyroxine antagonist[21]. 3',3,5-Triiodothyronine is the most potent naturally occurring thyroid substance but is less effective than thyroxine as an AAO activator and as a cupric ion complexer[8]. The data suggests that the free amino group is an important requirement in the ability of compounds to activate AAO, since when this functional group is blocked or absent, activation power disappears. The diiodophenol group is of limited importance in activation power, although it can be shown that diiodophenols form complexes with cupric ion[22].

The AAO system may be used to observe interactions between thyroxine and various cations. For cations which are inert to AAO, the diminution of the ability of thyroxine to activate AAO is interpreted as indicating interaction between thyroxine and such cations. Accordingly, strong interactions between thyroxine at $1.00 \cdot 10^{-5}$ *M* and Co^{++} at 10^{-4} *M*, Mg^{++} at 10^{-3} *M*, and Mn^{++} at 10^{-3} *M*, have been observed using this system. LARDY has also reported complexes between various metal ions and thyroxine[23].

Effects of nucleic acid and components

On investigating the effects of nucleic acid, nucleotides and other fragments, it was observed that many of these compounds could also activate AAO. In the data included in Table III it is seen that RNA and several of its various fragments activate AAO at concentrations in the range of 10^{-2}–10^{-4}. However, three nucleotides which contain one or more keto groups are inhibitory. In their activating effect, nucleotides do not represent a departure from the thesis that all compounds which activate AAO also interact with copper. Intense interactions of nucleic acid and components with cupric ion have been observed and will be described in a separate communication[16].

Effects of metal ions

It has been previously noted that such metal ions as Cu^{++}, Ag^{+}, Zn^{++}, and Ni^{++}

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inhibit AAO activity[6]. However, detailed data were presented only for Cu^{++}. Included in Table IV is data indicating the relative greater effectiveness of Hg^{++} when compared with Ni^{++} or Zn^{++}. Some inhibition was also observed with large amounts of arsenate and iodoacetate.

TABLE IV

EFFECT OF METAL IONS ON ASCORBIC ACID OXIDASE

Compound	*Molarity*	*% Maximal activation*
Al^{+++}	$1.0 \cdot 10^{-4}$	30
	$1.0 \cdot 10^{-3}$	105
Ca^{++}	$1.0 \cdot 10^{-4}$	18
Mg^{++}	$1.2 \cdot 10^{-3}$	73
	$1.0 \cdot 10^{-4}$	3
Ba^{++}	$1.0 \cdot 10^{-3}$	19
	$1.0 \cdot 10^{-3}$	28
		% Inhibition
Cu^{++}	$1.0 \cdot 10^{-7}$	3
	$2.5 \cdot 10^{-7}$	50
	$5.0 \cdot 10^{-7}$	98
Hg^{++}	$5.0 \cdot 10^{-6}$	3
	$1.0 \cdot 10^{-5}$	40
	$1.0 \cdot 10^{-4}$	95
Ni^{++}	$1.0 \cdot 10^{-5}$	36
	$1.0 \cdot 10^{-4}$	56
Zn^{++}	$1.0 \cdot 10^{-5}$	35
	$1.0 \cdot 10^{-4}$	51
	$1.0 \cdot 10^{-3}$	57
Sodium arsenate	$1.0 \cdot 10^{-3}$	0
	$8.3 \cdot 10^{-3}$	28
Sodium iodoacetate	$1.0 \cdot 10^{-3}$	0
	$8.3 \cdot 10^{-3}$	47
Co^{++}	$1.0 \cdot 10^{-3}$	0
Fe^{+++}	$1.0 \cdot 10^{-3}$	0

* Reaction conditions are similar to those in Table III.

The inhibition by Cu^{++} has been confirmed by BENHAMOU, MAGEE, AND DAWONS[24] and is shown in some detail in Fig. 11. Here it is seen that a plot of AAO activity against enzyme concentration at two different Cu^{++} concentrations produced modified "irreversible" or "pseudoirreversible" inhibition curves related to those predicted by the method of ACKERMANN AND POTTER[25]. Essentially, the enzyme is titrated by the increasing amounts of cupric ion added.

Activation of AAO was surprisingly exhibited by such metal ions as Al^{+++} and Ca^{++} with possibly some effects of other ions such as Mg^{++} and Ba^{++}. It is possible that this activation may be a non-specific surface or "hydroxide" effect similar to what has been observed for succinoxidase[26].

The possible sulfhydryl nature of AAO

On the basis of the previous data and additional information it was suggested that despite the presence of copper in the protein, AAO, might be a sulfhydryl-dependent enzyme[5]. AAO is sensitive to the usual spectrum of metal ions including Cu^{++} and Hg^{++}[6] and to the organic mercurials[5,8]. The enzyme is activated by cysteine, glutathione and related agents[5]. The inhibition of AAO by phenylmercuric chloride is

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subject to reversal by cysteine and glutathione as shown in Fig. 12. FRIEDEN AND NAILE[8] reported earlier on the reversal of phenylmercuric chloride inhibition by thyroxine at less molarity. We have also observed that various other activating agents such as the polyfunctional amino acids and nucleic acid components can prevent or suppress inhibition by phenylmercuric chloride as shown in Fig. 12 and Table V. Frequently, as indicated in Table V and Fig. 12, prevention or reversal of phenylmercuric chloride inhibition was only partially complete. The reversal of organic mercurial inhibition of AAO by non-sulfhydryl compounds[9] has a number of important implications, which will be discussed later.

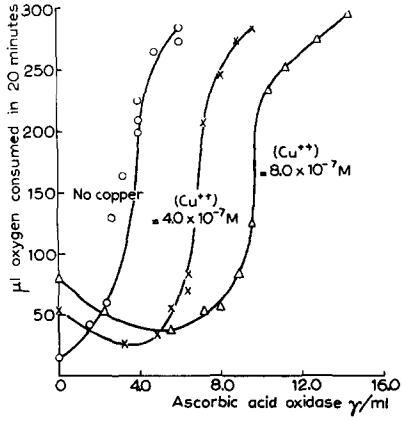

Fig. 11. Titration of AAO by Cu^{++}. Cu^{++} was added to the main compartment prior to AAO-1, following which ascorbate was tipped in.

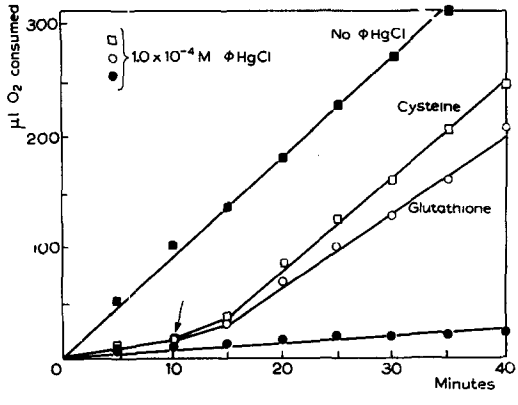

Fig. 12. Reversal of phenylmercuric chloride inhibition of AAO-1 by cysteine and glutathione Cysteine and glutathione at $1.00 \cdot 10^{-3}$ *M* were added as indicated at 10 min. $1.00 \cdot 10^{-4}$ *M* Φ HgCl (phenylmercuric chloride) was added to the compartment prior to the addition of AAO.

TABLE V

PREVENTION OF PHENYLMERCURIC CHLORIDE INHIBITION OF ASCORBIC ACID OXIDASE BY AMINO ACIDS AND NUCLEOTIDES

Compound and molarity	*Phenylmercuric chloride molarity*	*Oxygen consumed in 30 min* µl*
None	0	270
None	$1.0 \cdot 10^{-4}$	48
Histidine $1.0 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	150
Histidine $1.0 \cdot 10^{-4}$	0	315
Tyrosine $1.0 \cdot 10^{-3}$	0	307
Tyrosine $1.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-4}$	294
Tryptophane $3.3 \cdot 10^{-4}$	0	297
Tryptophane $3.3 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	273
Tryptophane $1.0 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	77
RNA $1.0 \cdot 10^{-4}$	0	292
RNA $1.0 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	284
RNA $1.0 \cdot 10^{-4}$	$8.0 \cdot 10^{-4}$	41
Guanylic acid $2.0 \cdot 10^{-4}$	0	197
Guanylic acid $2.0 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	222
Adenylic acid $1.0 \cdot 10^{-3}$	0	280
Adenylic acid $1.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-4}$	78

* Reaction conditions are as previously described. The amino acid or nucleic acid component was mixed with phenylmercuric chloride at least 10 min prior to the addition of AAO-1.

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A direct attack on this problem by amino acid analysis has not been possible in these laboratories because of insufficient amounts of enzyme protein.

Possibility of deinhibition of AAO

Many of the properties of AAO reported here might be satisfactorily explained by assuming that there is an inhibiting metal ion present in AAO preparations, which is removed from the locus of the protein. In order to check this possibility, we have tried to prepare AAO which would no longer be subject to activation, *i.e.* it would constitute an already activated enzyme.

A deinhibited AAO could not be prepared even after exhaustive dialysis against redistilled water, dialysis against a solution of an activator such as EDTA, cystine, or thyroxine, and dialysis against activator followed by redistilled water. The enzyme is subject to activation after passage through a cation-absorbing ion-exchange column such as described by JOSELOW AND DAWSON[7]. The possibility that copper ion is released during the course of the reaction is discussed later.

DISCUSSION

Activation of AAO seems to be a general property of copper ion chelators or complexers. In many instances, there is a correlation of the ability of a compound to activate AAO at low concentrations and its power to interact with cupric ion at low concentrations. For example, thyroxine, EDTA and many proteins are powerful AAO activators and also effective copper-ion binders. A point of difference appears in that some copper-ion binders, *e.g.* cyanide, diethyldithiocarbamate, and 8-hydroxyquinoline, appear to inhibit AAO at high concentrations following activation at lower levels. A tentative explanation for this may be that perhaps this type of compound can facilitate the removal of copper ion from the enzyme at sufficiently high concentrations. Presumably all other compounds only interact with the copper in the protein and do not remove it from the protein. It is possible that the activator assists in the formation of a copper protein-ascorbate-oxygen complex as suggested by DAWSON[27] or perhaps speeds the reoxidation of the assumed cuprous ion intermediate.

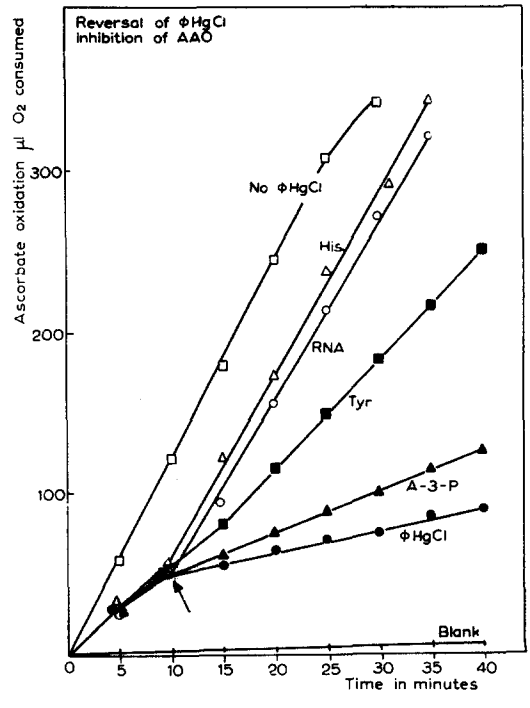

Fig. 13. Reversal of phenylmercuric chloride inhibition of AAO-1 by various compounds. The Φ HgCl concentration was $1.00 \cdot 10^{-4}$ M and the reversing compounds at a final concentration of $1.00 \cdot 10^{-3}$ M were added at 10 min.

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The only exceptions to the requirement of copper ion chelation for AAO activation so far encountered are the activations reported for Al^{+++} and Ca^{++}. It is possible that Al^{+++} and Ca^{++} activation is of a different type involving the non-specific protection of AAO similar to that observed for the succinoxidase system[26]. In fact, there is an interesting number of similarities between AAO and the succinoxidase system including their activation by Al^{+++}, Ca^{++}, thyroxine and inhibition by cupric ion and organic mercurials. There are of course some important differences, especially the difference in prosthetic group and catalytic action.

The data summarized in this paper and previous reports suggests some new possibilities to account for the many chemical responses of AAO. The scheme depicted in Fig. 14 postulates the release of copper ion during AAO activity as suggested by JOSELOW AND DAWSON[7], who showed that radioactive cupric ion exchanged with the copper of AAO during activity. It is not inconceivable that these copper ions might be especially effective in catalyzing adjacent sulfhydryl groups in AAO producing inactivated forms of AAO. (Reactions 2 and 3). This would account for the progressive inactivation of AAO during activity as well as the powerful inhibition of AAO by cupric ion. The protection of AAO might proceed through reaction (4), since the binding of the copper ion will reduce or prevent reactions (2) and (3). Activation of AAO by copper-binding agents might accrue by reducing the extent of migration of copper ion from the enzyme site.

An alternative scheme which accounts for many of these phenomena is outlined in Fig. 15. The formation of a free radical intermediate of ascorbate is depicted in reactions (5) and (6). The intermediate could also come from the Cu-catalyzed oxidation of ascorbate as suggested by WEISSBERGER AND LUVALLE[17] or from the action of hydrogen peroxide, although this view is not supported by BENHAMOU, MAGEE AND DAWSON[24]. Inactivation of AAO might proceed by the reduction of this intermediate at the expense of enzyme sulfhydryl groups to form an inactivated enzyme in reaction (7). The possibility of the "active" ascorbate interacting with enzyme sulfhydryl groups should not be overlooked. Protection of AAO might occur as in (8) where the copper ion complexer protects adjacent sulfhydryl groups by its interaction with the enzyme. Again, activation of AAO might proceed through the formation of highly active enzyme–copper chelator–ascorbate complexes. It must be emphasized that these two hypotheses are not mutually exclusive, but might occur simultaneously, though on different AAO molecules.

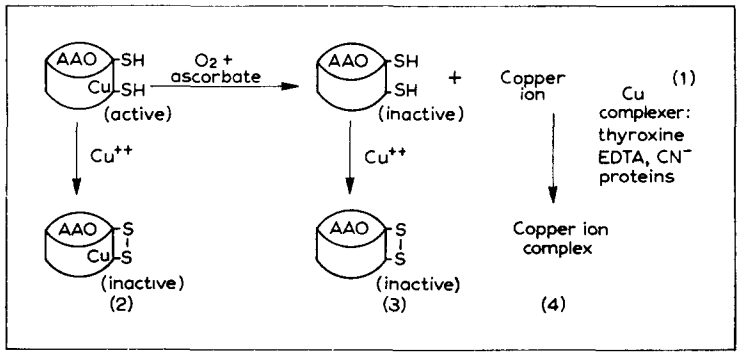

Fig. 14. A tentative scheme for the explanation of the properties of AAO.

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The many interesting interactions between phenylmercuric chloride and various biologically important substances deserves comment. Probably the most convincing evidence for the sulfhydryl dependence of an enzyme, particularly those in the crude state, is an enzyme's sensitivity to organic mercurial inhibition and the reversal of this inhibition with sulfhydryl compounds. However, the ability of other biologically important compounds to reverse phenylmercuric chloride inhibition suggests some reservations to this test for sulfhydryl dependency. Interactions between organic mercurials and various compounds have been shown by FRIEDEN AND NAILE[8,9]. It has been recognized from the start, when HELLERMAN[28] first emphasized the use of the organic mercurials, that groups other than sulfhydryl within proteins might interact. But the subtlety of some of these reactions should be more fully appreciated.

However, the various interactions in the AAO system, as a particular case, may still be explainable in terms of the possible sulfhydryl nature of this enzyme. In Fig. 16 a mechanism explaining the prevention and reversal of phenylmercuric chloride inhibition of AAO by many compounds, which are also copper-ion complexers is shown. The prevention of organic mercurial inhibition in reactions (9) and (10) might arise from the actual protection of the AAO by the copper-ion binder because of the proximity of the copper ion and sulfhydryl groups on the enzyme. In reaction (10) the copper complexer might remove the phenylmercuric ions by compound formation. The reversal of phenylmercuric ion inhibitions as pictured in (11) might

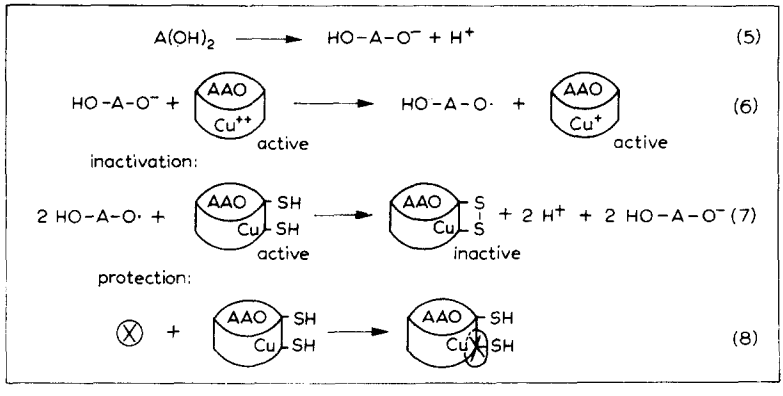

Fig. 15. Alternative scheme for the explanation of the properties of AAO.

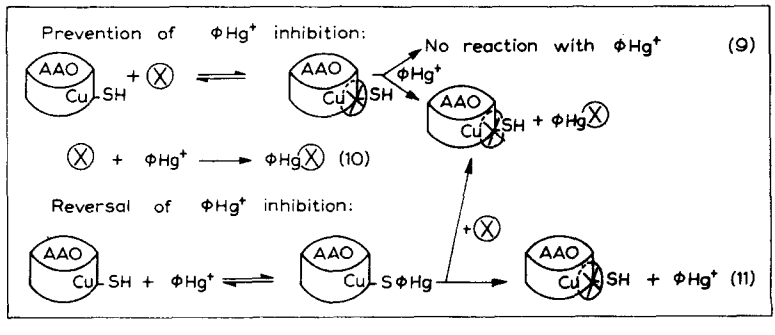

Fig. 16. Possible reactions of Φ HgCl in the AAO system.

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be a displacement reaction in which the reversing group displaces either the enzyme or the phenylmercuric ion. This argument does not preclude other groups in proteins from undergoing similar reactions.

SUMMARY

The properties of several different preparations of highly pure ascorbic acid oxidase (AAO) have been studied using both the Warburg and spectrophotometric methods. Activating agents were found to affect both the initial rate and maintenance of AAO activity. The enzymic catalysis is increased when oxygen replaces air as the gas phase. AAO activity, in the presence of activator, was found to be stable even when the enzyme was stored at 0° C in a diluted state, but was unstable at —15° C or 30° C. Estimated K_m for ascorbate was $5 \cdot 10^{-3}$ M and $3.9 \cdot 10^{-5}$ M as determined by the Warburg and spectrophotometric technique respectively.

Activators of AAO proved to be of two general types. The largest group comprises substances which activate AAO but never inhibit at any concentration. Most of these substances are powerful copper chelators and include representative proteins, amino acids, thyroxine analogs, and nucleic acid components. Activation was also observed with Al^{+++} and Ca^{++}. Activation at low concentrations but inhibition at high levels was obtained with cyanide, diethyldithiocarbamate, and 8-hydroxyquinoline. Irreversible inhibition was observed with Cu^{++} and several other metal ions. A deinhibited enzyme, however, could not be prepared.

The possible sulfhydryl nature of AAO was suggested by the above data and the observation of the reversal of the phenylmercuric chloride inhibition of AAO by sulfhydryl compounds. The interpretation of the data is complicated by the ability of non-sulfhydryl compounds also to reverse and prevent the phenylmercuric chloride inhibition of AAO. The implications of these observations is considered.

Several schemes are presented to account for the properties and interactions of AAO involving the release of inhibitory Cu^{++} from the enzyme and the production of a highly reactive intermediate in ascorbic acid oxidation.

REFERENCES

1 C. R. DAWSON AND W. B. TARPLEY, in J. B. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. II, Part 1, Academic Press, Inc., New York, p. 455–498.
2 T. P. SINGER AND E. B. KEARNEY, in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. II, Part A, Academic Press, Inc., New York, 1954, p. 135–159.
3 W. H. POWERS AND C. R. DAWSON, *J. Gen. Physiol.*, 27 (1944) 181.
4 C. L. GEMMILL, *J. Biol. Chem.*, 192 (1951) 749.
5 E. FRIEDEN, *Federation Proc.*, 11 (1952) 215; 12 (1953) 205.
6 E. FRIEDEN, *Biochem. Biophys. Acta*, 9 (1952) 696.
7 M. JOSELOW AND C. R. DAWSON, *J. Biol. Chem.*, 191 (1951) 1, 11.
8 E. FRIEDEN AND B. NAILE, *Arch. Biochem. Biophys.*, 48 (1954) 448.
9 E. FRIEDEN AND B. NAILE, *Federation Proc.*, 13 (1954) 213.
10 M. KERN AND E. RACKER, *Arch. Biochem. Biophys.*, 48 (1954) 235.
11 W. D. WOISLOIT, A. NASON AND A. J. TERRELL, *J. Biol. Chem.*, 206 (1954) 277.
12 A. NASON, W. D. WOISLOIT AND A. J. TERRELL, *Arch. Biochem. Biophys.*, 48 (1954) 233.
13 M. B. MATHEWS, *J. Biol. Chem.*, 189 (1951) 695.
14 W. H. POWERS, S. LEWIS AND C. R. DAWSON, *J. Gen. Physiol.*, 27 (1944) 167.
15 E. RACKER, *Biochem. Biophys. Acta*, 9 (1952) 577.
16 E. FRIEDEN AND J. ALLES, unpublished observations.
17 A. WEISSBERGER AND J. E. LUVALLE, *J. Am. Chem. Soc.*, 66 (1944) 700.
18 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
19 F. KUBOWITZ, *Biochem. Z.*, 299 (1939) 32.
20 E. STOTZ, C. J. HARRER AND C. G. KING, *J. Biol. Chem.*, 119 (1937) 511.
21 E. FRIEDEN AND R. J. WINZLER, *J. Biol. Chem.*, 179 (1949) 423.
22 J. L. DOLPHIN AND E. FRIEDEN, unpublished observations.
23 H. A. LARDY, *Brookhaven Symposia in Biol.*, 7 (1954) 90.
24 N. BENHAMOU, R. J. MAGEE AND C. R. DAWSON, *Abstracts, 126th National Meeting Am. Chem. Soc.*, 45 C.
25 W. W. ACKERMANN AND V. R. POTTER, *Proc. Soc. Exptl. Biol. Med.*, 72 (1949) 1.
26 B. L. HORECKER, E. STOTZ AND T. R. HOGNESS, *J. Biol. Chem.*, 128 (1939) 251.
27 C. R. DAWSON, in W. D. MCELROY AND B. GLASS, *A Symposium on Copper Metabolism*, Johns Hopkins Press, Baltimore, 1950, p. 18–47.
28 L. HELLERMAN, *Physiol. Rev.*, 17 (1937) 454.

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