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Investigations in Serum Copper

IV. Effect of Different Anions on the Enzymatic Activity of Coeruloplasmin

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In the previous paper [1] of this series we have presented evidence for the assumption that coeruloplasmin is an oxidase with copper in its active group. It has many properties in common with laccases of plant origin.

When we compared the enzymatic activity per atom of copper in serum and in purified preparations of coeruloplasmin in presence of paraphenylene diamine (ppd) in the same buffers and at the same pH, we found that pure preparations showed less than half the activity of serum. In tracing the cause of this difference, we have made some observations which are of interest for the understanding of the kinetics of the oxidation of ppd by this enzyme. This paper will deal with some observations made in the course of these studies.

To explain the discrepancy between the experiments with crude and pure coeruloplasmin, two explanations may be thought of. Either the isolation procedure has a detrimental effect on coeruloplasmin, or there is present in serum a supplementary substance necessary to promote the full activity of the enzyme. In order to find out which might be the case, pure coeruloplasmin was at first dialyzed against serum. Such dialysis increased the catalytic activity of the pure preparation to the same level as that of serum. Later, different dialyzable components of serum were tested, and the active substance of serum could be identified as the Cl ion.

The experiments described in this paper have been made in order to find out under what conditions and in what way the Cl ion and other anions affect the catalytical activity of coeruloplasmin.

EXPERIMENTAL

Coeruloplasmin has been prepared according to a method published in an earlier paper [2]. The preparations used had a copper content of about 0.35 per cent.

Copper was determined with sodium diethyl dithiocarbamate after wet ashing, protein with the biuret method.

Paraphenylene diamine (ppd) c.p. (Coleman and Cell Co.) was used.

The enzymatic activity was determined with the Warburg technique, temperature 37° C. The consumption of oxygen per unit time during the period of the most rapid oxidation has been used as measure for the velocity of the oxidation. We have thus not used the initial reaction velocity in cases where there has been an induction period before the rapid oxygen consumption starts (discussion see below).

pH was measured with glass electrodes.

Influence of Cl ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s

In order to study the effect of Cl ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s the following experiments were performed. Ppd was acidified with HCl to the pH wanted. The saltfree enzyme solution was treated in the same way. Warburg experiments were performed, in which NaCl was added to this system in varying amounts. The buffering capacity in these systems is naturally poor. During the oxidation of ppd there is a slow shift in pH towards the acid side. By making pH measurements we have convinced ourselves that the shift in pH during the initial phase is very small. It is therefore possible to compare the initial reaction velocities in these experiments.

At pH 7.5 we found that the amount of Cl ions added in order to neutralize the solutions was suboptimal, by adding NaCl, higher reaction velocities could be reached. Even at pH 6.8 this was the case. At pH 6, however, the oxidation velocity could not be increased by adding NaCl. Fig. 1 shows this effect at three different pH:s. From these experiments it can be concluded that the Cl ion accelerates the oxidation of ppd by coeruloplasmin.

From Fig. 1 it can also be seen that *there is an optimal concentration of Cl ions which varies with pH*. If the Cl ion concentration is increased over this optimum, an increasing inhibition results. If an experiment is performed at pH 5.5, the amount of HCl necessary to acidify the substrate is so big that the optimal Cl ion concentration at this pH is considerably surpassed. The addition of even a small amount of NaCl therefore causes an inhibition. From Fig. 1 *it is also evident that the inhibitory effect of Cl ions is dependent on pH.*

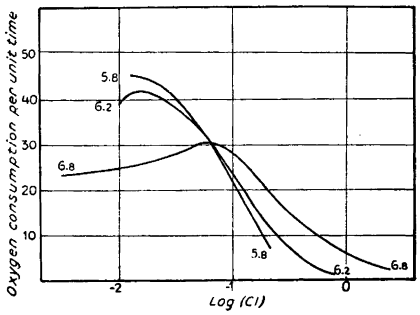

Fig. 1. Influence of chloride ions on the oxidation of ppd in the presence of coeruloplasmin at different pH:s (5.8, 6.2, and 6.8). Ppd was acidified with HCl to the pH wanted. NaCl was added in varying amounts. Abscissa: Logarithm of the chloride concentration (M). Ordinate: Initial reaction velocity (oxygen consumption per unit time). Substrate concentration: 1.8 · 10⁻². Enzyme concentration: About 1 · 10⁻⁶ M. Volume: 2 ml.

An effect similar to that of the Cl ion, *i.e.* acceleration in small concentrations, inhibition when the concentration was increased, has also been found for the following ions: Br^-, NO_3^-, CH_3COO^-, $HCOO^-$, SCN^-. With iodine the results are complicated, probably because this ion is oxidized in the system. F^- has not been investigated in detail, but preliminary experiments indicate that this ion has no accelerating effect.

Fig. 2 shows the acceleratory and inhibitory effects of NO_3^-, $HCOO^-$, SCN^-, and Cl^- ions at pH 6.8 and 5.8 respectively. At pH 6.8 the optimal effect of NO_3^-, $HCOO^-$, and Cl^- ions occurs at approximately the same concentrations, whereas the optimal effect of SCN^- is reached at a lower concentration. The inhibitory effects occur in the following order: $SCN^- > Cl^- > NO_3^- > HCOO^-$. Both

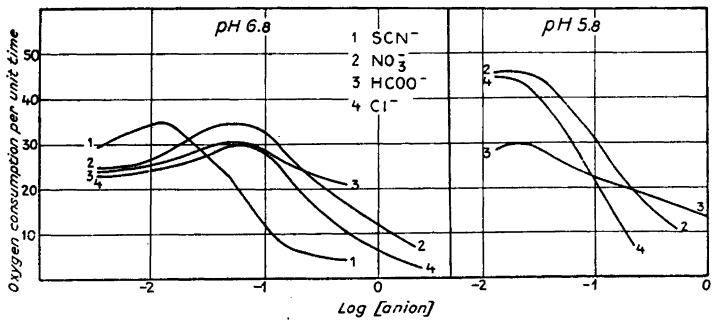

Fig. 2. Influence of different monovalent anions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8 and 5.8. Ppd was acidified with nitric, formic, or hydrochloric acid. The corresponding sodium salts were added in varying amounts. In the experiments with the rhodanide ion the acidification was performed with hydrochloric acid, and the chloride concentration has not been included in the anion concentration in this case. Abscissa: Logarithm of anion concentration. Ordinate: Initial reaction velocity. Substrate concentration: 1.8 · 10⁻². Enzyme concentration: About 1 · 10⁻⁶ M. Volume: 2 ml.

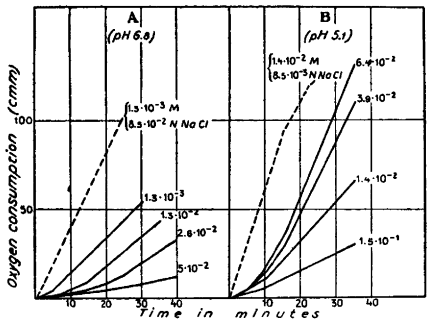

Fig. 3. Influence of phosphate ions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8 and 5.1. Ppd was acidified with H_3PO_4, and varying amounts of phosphate buffer were added. Abscissa: Time. Ordinate: Oxygen consumption. The broken lines show the reaction when an optimal concentration of NaCl was added to the ppd solution, which had only been adjusted to the pH wanted with H_3PO_4. The figures at the end of each line tell us the molarity of phosphate in each experiment. Substrate concentration: $2.2 \cdot 10^{-2}$. Enzyme concentration: About $2.4 \cdot 10^{-6}$ M. Volume: 2 ml.

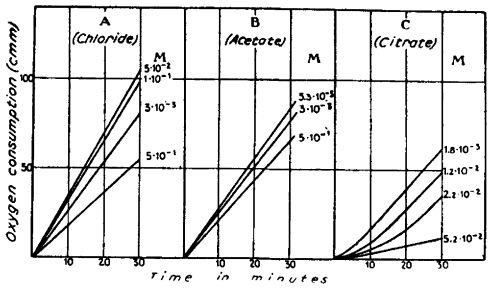

Fig. 4. Influence of chloride, acetate, and citrate ions on the oxidation of ppd in the presence of coeruloplasmin. Abscissa: Time. Ordinate: Oxygen consumption. Ppd was acidified in series A with HCl, in B with HAc, and in C with citric acid. Varying concentrations of the corresponding sodium salts (buffers) of the same pH (6.8) were added. The total anion concentration in M is given at the end of each line. Substrate concentration: $2.2 \cdot 10^{-2}$. Enzyme concentration: About $1.6 \cdot 10^{-6}$ M. Volume: 2 ml.

the accelerating and the inhibitory effects are clearly dependent on pH. At a lower pH, smaller amounts of ions are needed to produce these effects.

The investigation of the anions of polyvalent acids is more complicated, as these acids exist as different anions at different pH:s. The divalent phosphate ion (HPO_4^{--}) has been studied at pH 6.8. No accelerating effect was found, only an increasing inhibition. This inhibition differs in type from the one caused by large amounts of Cl ions (Br^-, CH_3COO^- a.s.o.). The inhibition is in the case of divalent phosphate, most pronounced during the initial phase of the reaction, and so gives rise to an induction period. Fig. 3 A.

The effect of monovalent phosphate ($H_2PO_4^-$) can not be tested in detail, as this has to be done at an acid pH where a comparatively large amount of phosphoric acid must be added in order to correct the pH of the ppd solution. An experiment performed at pH 5.1 is recorded in Fig. 3 B. As in the case of chloride, increasing concentrations of $H_2PO_4^-$ results in increasing reaction velocities, until an optimal concentration of $H_2PO_4^-$ has been reached. If the optimal concentration is surpassed, the reaction velocity decreases. The

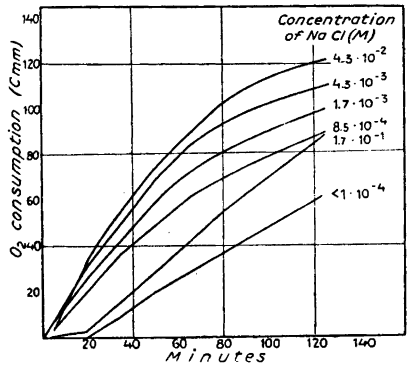

Fig. 5. Influence of chloride ions on the oxidation of ppd in phosphate buffer (M/60) in the presence of coeruloplasmin at pH 6.2. Abscissa: Minutes. Ordinate: Oxygen consumption. The lowest chloride concentration is given as less than $1 \cdot 10^{-4}$. In this case no sodium chloride has been added, but small amounts of chloride ions deriving from substrate, buffer, and enzyme solution can not be excluded. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

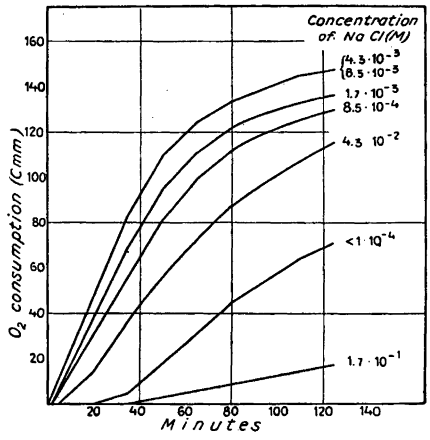

Fig. 6. Influence of chloride ions on the oxidation of ppd in citrate buffer (M/20) in the presence of coeruloplasmin at pH 5.4 Abscissa: Minutes. Ordinate: Oxygen consumption. The lowest chloride concentration is given as less than $1 \cdot 10^{-4}$. Substrate concentration $4.6 \cdot 10^{-3}$. Enzyme concentration: About $15. \cdot 10^{-6}$ M. Volume: 2 ml.

inhibitory effect of high $H_2PO_4^-$ concentrations are less pronounced than in the case of chloride. The concentration of this ion needed for optimal activity is greater than for Cl^-.

Some experiments were performed at pH 6.8 with minimal amounts of different acids. The acids used were hydrochloric acid, sulphuric acid, phosphoric acid, oxalic acid, nitric acid, acetic acid, and citric acid. In all cases fairly good activities were reached, best with nitric acid and hydrochloric acid. The smallest activity was noted with oxalic acid. It amounted to about 50 per cent of the activity measured with nitric acid. No clear induction period could be noted in these experiments. If the concentration of chloride, nitrate, or acetate ions in such experiments is increased within certain limits, the result is an increasing reaction velocity. If these ions are present in still higher concentrations, an inhibition is noted, but no induction period appears. If on the other hand the ions of citric, phosphoric, sulphuric, and oxalic acid are increased, the result is always a lower reaction velocity. In this case an

induction period becomes apparent. The length of this induction period is a function of the ion concentration. Some typical experiments illustrating these phenomena are recorded in Figs. 3 A and 4 (phosphate /3 A/, chloride /4 A/, acetate /4 B/, and citrate /4 C/).

The promotion of an induction period is, however, not only typical of polyvalent anions. A similar effect has, for instance, been noted with hippuric acid.

Effects of the combination of two different anions on the enzymatic activity of coeruloplasmin

If coeruloplasmin is inhibited by an anion as, for instance, the divalent phosphate ion, this inhibition can be broken by adding a small amount of one of the accelerating anions such as Cl^-, Br^-, CH_3COO^-, or NO_3^-. If such ions are added in sufficient amounts, the induction period disappears (Figs. 5 and 6). This effect can only be explained by a competition between the accelerating and the inhibiting ions. Fig. 7 shows clearly the competition between chloride and citrate ions.

Determination of pH optimum for the oxidation of ppd by coeruloplasmin

Before we knew that different anions have specific effects on the oxidation of ppd by coeruloplasmin, we tried to determine the pH optimum of the enzyme (Fig. 8). For this determination phosphate buffers were used between pH 6 and 7.5. Buffer concentration in the final mixture 1/60 *M*. Acetate and citrate buffers were used between pH 4 and 6 (final concentration in both cases 1/20 *M*).

As induction periods of different magnitude occurred in these experiments, the activities were computed from the consumption of oxygen during the most rapid phase of the reaction. In some cases where the buffer capacity was insufficient, there was some drift in pH towards the acid side during the experiments. When this has been the case, determination of pH has been made twice, at the beginning and at the of the experiment. In Fig. 8 this has been marked by combining two points with a straight line. This figure shows clearly that different buffers have different effects on the oxidation regardless of pH. The greatest velocity was observed in acetate buffers between pH 5 and 6. In this region citrate obviously gave an inhibition. A sharp decline in the catalytical effect was observed in changing from acetate to phosphate buffer at pH 6, indicating that phosphate, too, has an inhibitory effect.

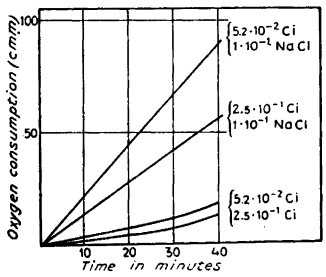

Fig. 7. Experiments showing the competitive effects of chloride and citrate ions on the oxidation of ppd in the presence of coeruloplasmin at pH 6.8. Abscissa: Time. Ordinate: Oxygen consumption. Substrate concentration: $2.2 \cdot 10^{-6}$. Enzyme concentration: About $1 \cdot 10^{-2}$ M. Volume 2 ml.

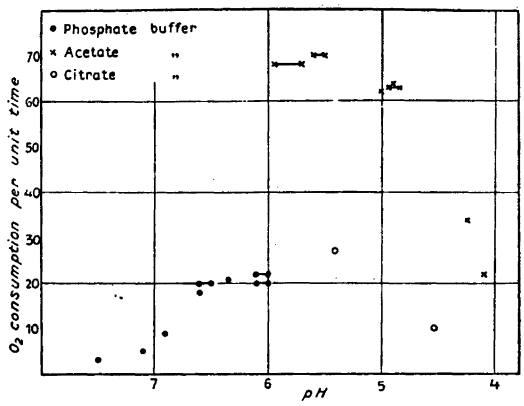

Fig. 8. Oxidation velocity of ppd in the presence of coeruloplasmin in different buffers at different pH:s. Abscissa: pH. Ordinate: Oxygen consumption per unit time. Concentrations of buffer: Phosphate M/60, acetate M/20, and citrate M/20. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

By adding NaCl in optimal concentrations to the buffers at each pH, another pH activity curve can be constructed. This has been done and the results are shown in Fig. 9. The pronounced accelerating effect given by Cl ions in phosphate and citrate buffers is very clear when Figs. 8 and 9 are compared. This curve comes very near to the ideal pH activity curve. Between pH 4 and 5 and 6.5 and 8 the real optimal activities are a little better than is suggested by the figures. In the first-mentioned region, the amount of acetate ions used gives some inhibition. In the second region, the amount of Cl ions necessary to break the phosphate inhibition is great enough to cause some Cl ion inhibition. The errors are, however, relatively small.

Do the anions investigated act by combining with coeruloplasmin or with ppd?

As ppd is a weak base, it occurs in neutral and slightly acid solutions partly uncharged and partly as a monovalent cation (ppd H^+). The possibility must be kept in mind that the anions might act by combining with ppd H^+ and not with the enzyme. The experimental attack on this problem presented

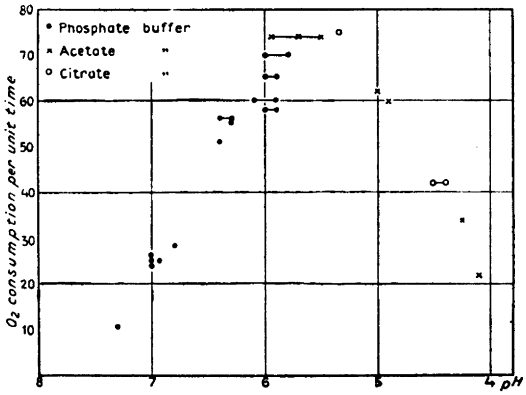

Fig. 9. Oxidation velocity of ppd in the presence of coeruloplasmin in different buffers and with optimal chloride concentration at different pH:s. Concentration of buffer: Phosphate M/60, acetate M/20, and citrate M/20. Abscissa: pH. Ordinate: Oxygen consumption per unit time. Substrate concentration: $4.6 \cdot 10^{-3}$. Enzyme concentration: About $1.5 \cdot 10^{-6}$ M. Volume: 2 ml.

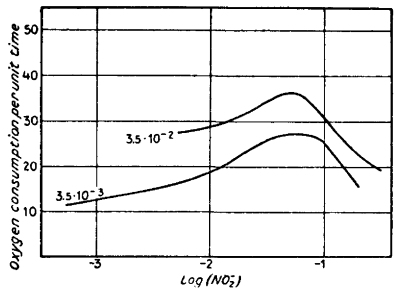

Fig. 10. Influence of nitrate ions on the oxidation velocity of ppd in the presence of coeruloplasmin at different substrate concentrations ($3.5 \cdot 10^{-2}$ and $3.5 \cdot 10^{-3}$). Ppd was acidified with nitric acid in both cases to pH 6.8. Varying amounts of sodium nitrate of the same pH were added. Abscissa: Logarithm of nitrate concentration (M). Ordinate: Oxygen consumption per unit time. Enzyme concentration: About $1 \cdot 10^{-6}$ M. Volume: 2 ml.

some practical difficulties. After some unsuccessful attempts, the problem was attacked in the following way.

1. Keeping the enzyme concentration constant, two series of Warburg experiments were performed at pH 6.8. In the first series, the concentration of ppd was kept at $3.5 \cdot 10^{-2}$ *M*, and in the second series, at $3.5 \cdot 10^{-3}$ *M*. In each series the concentration of the anion chosen (NO_3^-) was varied over a broad interval. The results of these experiments are presented in Fig. 10. From this figure it is evident that optimal reaction velocity is reached at the same concentration of NO_3^- ions in both series. That this is the case seems to us to indicate clearly that the nitrate ions act by combining with the enzyme and not with the substrate. (The differences in absolute activity in both series are due to the fact that the substrate is suboptimal in the second series [1]).

2. The effect of chloride ions on the oxidation of ppd and catechol was compared at pH 6.9. Catechol was chosen as it differs from ppd in being practically undissociated at this pH. These experiments show that the chloride ion has both accelerating and inhibiting effects also in the coeruloplasmin-

catechol system. The chloride concentration at optimal activity was the same in both systems.

These experiments therefore support the assumption that monovalent anions act on the enzyme and not on the substrate.

SUMMARY AND CONCLUSIONS

We have in this paper presented evidence for the occurrence of three different anion effects on the oxidation of ppd in the presence of coeruloplasmin.

Experiments have been performed which show that these anion effects are due to an interaction of enzyme and anion.

Firstly, many monovalent anions as for instance the rhodanide, the nitrate, the chloride, the bromide, the formiate, and the acetate ions, have an accelerating effect on this enzymatic process. Secondly, these same ions, when present in higher concentrations, show an inhibitory effect. Both these effects increase with decreasing pH. Thirdly, some polyvalent ions such as phosphate, citrate, sulphate, and oxalate ions, show an inhibitory effect, which differs from the effect of monovalent ions in being most pronounced in the initial stages of the reaction, and, thus, in causing an induction period. The inhibition and induction caused by polyvalent ions can be eliminated by adding monovalent ions in suitable concentrations.

It is impossible, at the present state of our knowledge, to give a full explanation of the different salt effects here described. We propose, however, to advance a working hypothesis which seems to fit the experimental facts hitherto collected.

Let us start with the inhibition caused by monovalent ions. With reference to the inhibitory effect, the ions investigated by us can probably be grouped in the following order: $SCN^- > Cl^- > NO_3^- > HCOO^- > CH_3COO^-$.

There is some difficulty in interpreting exactly the curves showing this inhibition owing to the fact that the inhibitory and the accelerating effects show different degrees of overlapping. It is therefore possible that further investigations might show that smaller alterations in the order will be necessary.

This arrangement of ions evidently shows similarities to the Hoffman series and to the series published by Klotz and Urquhart [3] and Scatchard and Black [4] relating to the binding of different anions to serum albumin. It seems probable, therefore, that these anions inhibit when they are bound to some cationic groups on the surface of the enzyme molecule. It has been proposed that the inhibitory effect of the anions in the Hoffman series on

different enzymes are due to an aggregation of the protein molecules. With coeruloplasmin this can not be the case. Unpublished experiments made by K. Pedersen at the Institute of Physical Chemistry, Upsala, have not revealed any differences in the sedimentation constant of coeruloplasmin when the concentration of NaCl in the solution was varied over a broad interval.

Let us then proceed to the accelerating effect of these same ions. Here it is still more difficult to group the different ions according to their affinity. The optimal effect is reached with practically the same concentrations of Cl^-, $HCOO^-$, NO_3^-, and CH_3COO^- ions, whereas SCN^- ions seem to reach this activity at a lower concentration. If this effect is also due to a binding between the anion and cationic groups in the enzyme, the cationic groups responsible for the acceleration must have a higher affinity for all the anions investigated than those responsible for the inhibition. It should be kept in mind that Scatchard *et al.* [5,6] have interpreted their finding as regards the binding of Cl^- and SCN^- ions to serum albumin by assuming two sets of groups with widely differing affinities for these anions.

What we assume is that two types of cationic groups exist on the enzyme surface. One of these types has a high affinity for different anions. When these groups are blocked by monovalent anions, the result is generally an increased enzymatic activity. The other type of cationic group has a lower affinity for anions. Blocking of these groups leads to inhibition.

How can now the observations on the specific inhibition of the enzyme by polyvalent anions be made to fit into this hypothesis? As there is evidently a competition between these ions and the monovalent anions, they, too, are probably attached to the cationic groups with high affinity for anions. Contrary to the monovalent ions they must in some way interfere with the activity of the enzyme. Experiments showing whether this is due to aggregation have not yet been performed.

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