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THE OXIDATION OF ASCORBIC ACID IN THE PRESENCE OF COPPER

By

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The part which the study of various inhibiting and activating factors play in the investigation of the nature and action of enzymes is of considerable importance. The experiments here may follow two courses- the first group deals with the influence of factors which exhibit strong action, such as KCN, CO, etc., all of them foreign to the living organism. This group gives clear effects of mainly inhibitory character in many catalytic processes and serves as a very valuable method of elucodation especially in the field of haemcatalyses.

The second group is concerned with the influence of substances which are normally present in the living organism, but apparently do not participate in the course of enzymic reactions. Here the experiments are of a much more complicated character as the effects obtained are not so strong and often it is very difficult to define the predominating influence among many simultaneously occuring reactions.

This group deals in the first place,with the study of the influence of various inorganic salts, proteins and their derivatives on enzymic processes.

Many experiments have already been done in this field and though sometimes it may be difficult to infer from the effects obtained in vitro whether a given factor plays any definite role inside the organism,much light haw been thrown on the subject by this method of investigation.

During the years 1937-1939 the author has began a series of experiments on the influence of some simple substances, normally present in the living organism, on enzymic processes. As an example of this kind of influence may be quoted the work of the author on the amylase in muscle extract (E.M.Mystkowski,1937). In this extract two enzymic systems are present - amylolytic and glycolytic. The glycolytic system requires phosphates for its action. In the absence of phosphates only amylase acts, causing the hydrolytic decomposition of glycogen, but this takes place only if Cl- salts are present. In the presence of phosphates the amylolytic system is completely suppressed and the glycolytic decomposition of glycogen takes place. This may be considered as a competition for the same substrate between two enzymic systems, regulated by the presence of such simple substances as sodium chloride and phosphates. Many examples of such competition regulated by the presence of simple metabolites might be quoted and they probably play a great part in metabolism.

In these experiments the influence of chlorides seemed to us to be of some interest.Their influ-

ence/

ence on various oxidative processes has been examined by many authors. Wurster (I889 , cit. by Baudisch,I920) found that hydrogen peroxide and the solution of α-naphtylamine in acetic acid produced a dye only in the presence of sodium chloride. The same author found that the oxidation of ammonium hydroxide to nitric acid by hydrogen peroxide was greatly activated by the presence of sodium chloride. These findings were confirmed by Baudisch.

In our experiments it was found(Mystkowski & Lasocka,I939, unpublished) that aldehyde dehydrogenase isolated from milk and inactivated by copper sulphate may be reactivated by sodium chloride. The same was true for amylase. The last fact led us to consider whether the normal activation of amylase did not depend on the same influence of sodium chloride. The experiments, however, were not of decisive character.

Another example where the influence of chlorides and other factors was examined was the oxidation of ascorbic acid in the presence of copper salts. The first part of this work was published in I939 (Mystkowski & Lasocka,I939).

The inhibition of ascorbic acid oxidation by sodium chloride was first described by de Caro and Giani (I934) and Kellie and Zilve (I935). Their statement however was not followed by any detailed examination of this influence. In our experiments it was found that among all salts examined (NaCl , KCl,$CaCl_2$,$MgCl_2$, $(CH_3COO)_2$ Mg , $MgSO_4$,Li_2SO_4 ,Na_2SO_4 ,KNO_3 ,NaF) only

only chlorides have an inhibitory influence on the oxidation of ascorbic acid.This inhibition takes place irrespective of the cation. These results were confirmed by Mapson (I94I).

The action of sodium chloride appears to be related to the catalytic action of copper, as was shown by the experiments in which the ralative concentrations of copper sulphate and sodium chloride were varied.The greater the concentration of copper sulphate, the greater was the concentration of sodium chloride necessaryto inhibit the catalytic action of copper. Amino acids and proteins exert a similar inhibitory action. Their action depends on their binding power for copper. Two points, however, must be emphasised here, namely that their binding power is limited and that copper-protein complexes retain a certain catalytic activity. This limited binding power of proteins causes an almost uninhibited oxidation of ascorbic acid in a protein containing solution if a great excess of copper sulphate is present. Natural copper- protein complexes such as are obtained from serum thus act on the one hand as feeble catalysts on the other hand they are still able to bind a certain amount of copper and can in this way act as an inhibitory factor in the oxidation of ascorbic acid.

In the present paper we give further results obtained in the oxidation of ascorbic acid under the influence of sodium chloride,proteins and tyrosine in various systems. The action of copper as an inorganic catalyst/

talyst and as a component of certain enzymes was here examined. During the course of these experiments we had to examine several other catalytic activities of copper not connected directly with the oxidation of ascorbic acid and these results are given here in brief.

Methods

The systems contained phosphate buffers in final concentration 0.I - 0.I5 M., ascorbic acid 3-4 mgr, substances whose influence was to be examined up to the volume of I5 or 20 mls. The detailed composition of the systems is given in the appropriate tables.

The systems were left in open conical flasks at 24° C ,if different temperatures were used it is indicated in the corresponding tables. After a given time samples were taken and after acidification with acetic acid, the remaining amount of ascorbic acid was determined by titration with a solution of 2,6-dichloro-phenol-indophenol. In the experiments where the influence of temperature was examined the systems were kept for a given period in water bath and then after rapid cooling determinations were carried out.

In the experiments with proteins,potato extract,cucumber juice, the deproteinization before the titration was effected by a solution containing 8% of trichloracetic acid and 2% metaphosphoric acid.

To prepare an extract potatoes were either sliced with a knife or put through a meat mincer and then ground in a mortar with double their volume of water.

After/

TABLE 1.

	0				NaCl				$CuSO_4$				$CuSO_4$ - NaCl				$Fe_2(SO_4)_3$				$Fe_2(SO_4)_3$ - NaCl			
	30'		4 h.		30'		4 h.		30'		4 h.		30'		4 h.		30'		4 h.		30'		4 h.	
pH	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
5.7	57.1	-	88.5	+55.0	5.7	-90.0	14.3	-75.0	74.3	+30.0	100.0	+75.0	60.0	+5.0	91.4	+60.0	57.1	0	88.5	+55.0	11.4	-80.0	17.1	-70.0
6.0	62.8	+10.0	88.5	"	8.5	-85.0	20.0	-65.0	74.3	"	100.0	"	60.0	"	91.4	"	62.8	+10.0	88.5	"	17.1	-70.0	20.0	-65.0
6.4		+20.0	88.5	"	8.5	"	20.0	"	74.3	"	100.0	"	62.8	+10.0	91.4	"	62.8	"	88.5	"	17.1	"	20.0	"

5he systems contained in 20 ml. : 5 mls. phosphate buffers M/5
3.5 mgr. of ascorbic acid
NaCl 0.14 M.
$CuSO_4$ and $Fe_2(SO_4)_3$ 2.5×10^{-5} M.

1. - per cent decomposition
2. - per cent activation (+) or ibhibition (-).

After a given time (as indicated in the corresponding tables) the solid particles were centrifuged off and the supernatant fluid was used in experiments. A variable amount of extract was used according to the type of experiment in each system, in controls extract was replaced by the same amount of water or solution of phosphates. Cucumber juice was prepared according to Stewart and Meiklejohn (1941).

EXPERIMENTAL

I. Inhibition of ascorbic acid oxidation by sodium chloride

The oxidation of ascorbic acid in the presence of copper sulphate, ferrous sulphate, ferric sulphate and ferric chloride under the influence of sodium chloride was examined. The results of one typical experiment are given in table I.

Table I

This experiment shows great activation of the oxidation by copper sulphate. The increase of oxidation in the presence of copper sulphate amounts to 30-75% .The activation by ferric sulphate is quite insignificant and never goes beyond limits of experimental error. In the systems containing only the copper normally present in didtilled water, inhibition caused by sodium chloride amounts to 65-90 % .Sodium chloride also decreases distinctly the activation caused by added copper sulphate, this decrease beeing from 30 % to 5 % after 30 mins and from 75 % to 60 % after 4 hrs. This decrease is/

is proportional to the concentration of sodium chloride and inversely proportional to the concentration of copper sulphate, as it was shown in our previous paper and has been confirmed in this work.

In the presence of sodium chloride no catalytic activity of Fe-salts was found. The inhibition by sodium chloride occurs here as in systems without any activators. In systems with Fe salts in concentrations greater than those indicated in the table I a small activation of ascorbic acid oxidation was found and of all Fe salts examined, ferric chloride showed the largest activation. This activation is not due to the copper impurities in corresponding salts, as the degree of activation caused by them did not change after several recrystallizations.

The increase in temperature of the reaction has comparatively little effect on the increase of the rate of ascorbic acid oxidation. In our experiments the oxidation at 24° and 97° was compared. Although the increase of the velocity of reaction at 97° was not a great one, the inhibition by sodium chloride was smaller (13.6-25 %) in comparison with the inhibition at 24° (88.2%)% The activation by added copper sulphate was at the same level (13.0 - 25.0 % as compared with 11.7 % at 24°).

2. Correlation between appearance of cuprous oxide and oxidation of ascorbic acid.

In experiments with the usual concentration of copper sulphate (2.5×10^{-5} M.) the red precipitate of cuprous oxide appears after a few minutes of ascorbic acid/

TABLE 2.

			45 mins.			3 h. 30 mins.			3h. 30 mins.	
	$CuSO_4$ concentr.	NaCl concentr.	1	2	3	1	2	3	mls. of $KMnO_4$	p.c. inhib.
1.	5×10^{-4} M	0	1.3	32.5	-	2.7	67.5	-	2.9	-
2.	"	0.13 M.	1.2	30.0	7.7	2.4	60.0	11.1	1.7	41.3
3.	"	0.4 M.	0.6	15.0	53.8	1.9	47.5	44.4	1.6	44.8
4.	"	0.65 M.	0.4	10.0	69.2	1.0	25.0	62.9	1.5	51.7

pH = 6.5 Composition of systems in 30 mls. : 3 mls. of phosphate buffers
4 mgr. of ascorbic acid.

1. - mgrs. of oxidized ascorbic acid.
2. - per cent decomposition.
3. - per cent inhibition.

acid oxidation at 97°. In the system containing sodium chloride this precipitate does not appear.

The amounts of cuprous oxide produced in ordinary experiments were to small to be determined quantitatively so in a series of experiments much higher concentrations were used. At the concentration of copper sulphate 5 x 10^{-4} M. the precipitate of cuprous oxide appears even at 24°.

To detrmine the amount of cuprous oxide formed, the last stage of the Bertrand method for sugar estimation was used. Cuprous oxide formed during the reaction was centrifuged off, washed with distilled water dissolved in a solution of ferric sulphate in concentrated sulphuric acid and then titrated with N/50 potassium permanganate. The results in table 2 are given in mls of potassium permanganate used in titration.

Table 2.

In this experiment performed at 24° ,cuprous oxide precipitate was visible after one hour.No strict parallel between the amount of ascorbic acid oxidized and cuprous oxide formed was found. Nevertheless, in general throughout the experiments the greater the rate of ascorbic acid oxidation, the greater was the amount of cuprous oxide formed. The inhibition of oxidation of ascorbic acid by sodium chloride was always accompanied by the diminution or complete absence of cuprous oxide formation. Thus the inhibition of the oxidation of ascorbic acid is accompanied by a similar inhibition/

TABLE 3.

Salt used	Conc. of $CuSO_4$	% oxidation after 5 hr.	% oxidation in the presence of 5.7×10^{-3} NaCl after 5 hr.
$CuSO_4 .5 H_2O$	1.9×10^{-5} M	0	0
"	9.5×10^{-5} M	63.6	27.2
Anhydrous $CuSO_4$	3.8×10^{-5} M	40.9	0
"	1.9×10^{-4} M	100.0	45.4

Concentration of ascorbic acid 8.5×10^{-4} M

After 5 hr., the unchanged ascorbic acid was estimated by dichlorphenoindophenol titration in a 2 ml. sample diluted by addition of 10 ml. of 0.5% acetic acid.

inhibition of Cu^{ii} - Cu^{i} transformation. This effect is in analogy to the results obtained by Barron et al. (1936) who found that the copper catalysis of ascorbic acid by molecular oxygen is inhibited by CO. They conclude that the metal in the course of oxidation undergoes a cyclic change from Cu^{ii} to Cu^{i} and that carbon monoxide by combination with Cu^{i} form inhibits the reaction.

3. The influence of sodium chloride on copper catalysis in non-aqueous media.

The catalytic action of copper and the inhibitory influence of sodium chloride on it are not limited to aqueous systems. As all the components of the systems examined are soluble in absolute alcohol a series of experiments in the latter medium has been perfomed. The results of these experiments were published in Nature (1942).

It was found in these experiments that water present in alcoholic systems in concentration up to 1.0% has no influence on the reaction. However to prevent the possibility of interference from this factor the catalytic action of $CuSO_4.5\ H_2O$ with the action of anhydrous copper sulphate was compared. The results are given in the following table.

Table 3.

This experiment shows that in absolute alcohol copper sulphate has the same catalytic effect in oxidation of ascorbic acid as in water. This oxidation is proportional to the concentration of the catalyst.

Also/

Also the influence of sodium chloride is analogous here to that in water systems.

This oxidation which must involve a direct electron transfer,is also accompanied by the formation of cuprous oxide. In all the systems with sufficiently great concentration of copper sulphate the red precipitate of cuprous oxide appears, which however must undrgo further changes as it disappears after several hours. This last stage of reaction has not beeen followed quantitatively.

4.The influence of sodium chloride on the peroxidase and catalase activities of copper.

The action of copper considered in the previous chapter deals with its only one catalytic manifestation ~ the oxidation of ascorbic acid by atmospheric oxygen. This reaction must therefore be considered as a type of oxidase catalysis in which copper acts as an oxidase model. This type of copper catalysis is characteristic not only for oxidation of ascorbic acid,but belongs to the large group of well known metal catalyses. This group comprides in the first place iron catalyses, and secondly copper catalyses, the second group being much less known.The much weaker catalytic activity of copper in most reactions of this type as compared with iron has caused that not many biological reactions are known in which copper plays as important part as iron. Nevertheless many catalytic reactions with copper have been described, especially oxidation of fructose(Meyerhof/

hof and Matsuoka,I924) and other sugars (Krebs,I927), cysteine and other sulphur containing compounds(Warburg,I927-Voegtlin,I926-Elvehjem,I930 and others).

The role of copper in the oxidation of ascorbic acid must be considered quite separately in view of its uniquness in many respects,chiefly because of its greater activity in this reaction in comparison with iron and secondly because of its participation in the structure of the enzymes oxidising ascorbic acid both directly and indirectly.Whether copper should be considered as a model of a "true" oxidase or rather of dehydrogenase, will not be discussed here.It seems that the most appropriate explanation of this type of reaction was given by Szent-Gyorgyi(I938) whosw view will be mentioned later in this work.

In the following chapter two other catalytic activities of copper will be discussed,namely peroxidase and catalase ones.

The oxidation of ascorbic acid in the presence of hydrogen peroxide occurs with a much greater velocity than the oxidation with molecular oxygen.But, on the other hand,in systems containing hydrogen peroxide it may be expected that the catalase action of copper may interfere with the peroxidase one and thus influence the oxidation of ascorbic acid.In the following series of experiments the mutual relationship of these two reactions and the influence of sodium chloride on them has been examined. This last reaction seemed to be of a particular interest, as it could elucidate whether the inhibitory/

TABLE 4.

The systems contained phosphates in concentration 0.I.M.

$CuSO_4$ - 0.0002 M. H_2O_2 - 0.175 N

NaCl - 0.4 M. pH 6.5

The systems have been kept at 60° for 60 mins. After this time the remaining hydrogen peroxide was determined by titration with $KMnO_4$ 0.I.N.

Concentration of $CuSO_4$ Mols	Concentration of NaCl Mols	% decomposition of hydrogen peroxide
0.0002	0	48.6
0	0.4	60.0
0.0002	0.4	68.6
0	0	0

bitory action of sodium chloride,found in oxidation of ascorbic acid by molecular oxygen,is of a general character and concerns all the catalytic activities of copper or whether it is limited only to this particular type of reaction.

a)the catalase action of copper sulphate and sodium chloride.

The experiments showing the influence of copper sulphate and sodium chloride on the decomposition of hydrogen peroxide were carried out by the titration after a given time of the remaining H_2O_2 with the aid of 0.I N potassium permanganate.

Table 4.

During 60 mins at 60° no perceptible decomposition of hydrogen peroxide occurred in the control system containing phosphates and hydrogen peroxide. In system with copper sulphate 48.6 % of hydrogen peroxide has been decompsed, in system with sodium chloride - 60.0% .The highest decomposition was found in system containing copper sulphate and sodium chloride,although the activation here is smaller than the sum of the activations caused by each of these factors taken separately.

This experiment indicates that not only copper sulphate has a catalytic effect on the decomposition of hydrogen peroxide,but also that sodium chloride shows the same influence in the absence of added copper. This action of NaCl does not consist in activation of the traces of copper present in water used in these experiments. If it were so, the activation by sodium chloride in the presence of added copper would be of a much

TABLE 5.

The systems contained phosphates in concentration 0.I M

$CuSO_4$ 0.001 - 0.0025 M. H_2O_2 - 0.175 N

NaCl 0.4 M pH - 6.5

The systems have been kept at 37° or 70°. After the time given in the table the remaining hydrogen peroxide was determined by titration with $KMnO_4$ 0.I.N.

Concentration of $CuSO_4$ Mols	Concentration of NaCl Mols	% decomposition of H_2O_2 37° 24 hrs.	70° 30 mins.
0.0010	0	40.0	48.6
0.0025	0	42.8	65.8
0.0010	0.4	74.3	62.9
0.0025	0.4	80.0	65.8

higher order,than it was actually found.

The decomposition of hydrogen peroxide increases greatly with the rise of temperature,as is shown in the following table

Table 5.

At 37° the decomposition of hydrogen peroxide even in the presence of copper sulphate is slow in comparison tith the same reaction at 70° . During 24 hrs at 37° and the concentration of $CuSO_4$ 0.0I M. 40.0% of hydrogen peroxide was decomposed, about the same amount was decomposed at 70° during 30 mins. A difference of the same order was found in the presence of both activators, copper sulphate and sodium chloride. Here again their joined influence is amaller than the sum of the activations taken separately.

Thus in the second type of catalytic action of copper, the role of sodium chloride is entirely different. In the oxidase action copper sulphate was inhibited by sodium chloride, here it is difficult to speak about any influence of NaCl on $CuSO_4$ because the former shows by itself the same activity as copper.In all the circumstances examined the joined effect of these two activating substances has been found smaller than the sum of their action taken separately.

b)the catalase action of copper sulphate in the presence of an oxidizable substance.

The object of the following experiments was to examine the action of copper as catalase in the presence/

presence of substances which would readily undergo oxidation in these experimental conditions. Thus it was intended to establish the mutual influence of these two simultaneous reactions. In connection with this firstly the experiments of Karczag (I92I) were repeated. This author found that in the presence of hydrogen peroxide certain dyes undrgov an oxidative decoloration under the influence of copper and iron salts.This reaction showed a great dependence on the temperature , especially when copper salts were used as catalyst.

systems with methyl red

The experiments of Karczag were repeated here with the difference that buffered systems were used (pH range 5.0- 6.5) At the same time the influence of sodium chloride on this reaction has been examined.

In these experiments two catalytic actions of copper may be expected.As a model of peroxidase copper will catalyse the decoloration of methyl red, as catalase - the decomposition of hydrogen peroxide .The difficulty to find a suitable method which would allow to observe both reactions simultaneously has caused that the following experiments have rather a semi-qualitative character.

The catalase activity of copper may be followed by observing the evolution of bubbles of oxygen in systems containing phosphate buffers,copper sulphate, sodium chloride and hydrogen peroxide. This, however, may be oserved only at higher temperatures. As was shown before(table 5) at 37^{o} the decomposition of hydrogen peroxide/

oxide is slow, only above 50° it was possible to observe a very slow escape of bubbles of gas,but still to slow to follow it in an experiment. Above 70° the evolution of gas is quick enough to compare the reaction in particular systems.

When a solution of methyl red (final concentration in the system about o.I%) was added to the buffered $CuSO_4$ - H_2O_2 system and the whole was brought to 75° , no visible decomposition of hydrogen peroxide took place. Control systems without methyl red showed in the same time a violent decomposition of hydrogen peroxide.

In systems with methyl red, instead of gassing the dye undergoes decomposition, which is complete in I5 mins. This decoloration is activated by sodium chloride.The complete decoloration in systems with copper sulphate and sodium chloride occurs 2-3 times as quickly as in the absence of NaCl (5 mins instead of I5 mins).Sodium chloride alone does not show any appreciable activation in this reaction.

Thus the presence of an acceptor for oxygen(methyl red) inhibits the evolution of oxygen from hydrogen peroxide or,in other words, the reaction of oxidation of the dye inhibits the catalase action of copper and shifts it towards a peroxidase one.

Systems with tyrosine.

Similar results were obtained with tyrosine as a substrate for oxidation.These experiments were important/

TABLE 6.

The systems contained:

phosphates 0.1 M — NaCl 0.2 M

$CuSO_4$ 2×10^{-5} and 4×10^{-5} M — tyrosine - 1 mgr. per system

H_2O_2 0.25 N — total volume 15 mls.

Colour changes.

	at 37°			at 85°		
	30 mins	1 h 30 mins	3 hr.	20 mins	40 mins	60mins
Control	0	±	+	++	+++	++
$CuSO_4$ 2×10^{-5} M	+	++	+++	++++	0	0
" 4×10^{-5} M	++	++++	++++	0	0	0
NaCl	0	+	++	+++	+++	0
$CuSO_4$ 2×10^{-5}M+ NaCl	+++	++++	++++	0	0	0

Evolution of gas at 85°

	systems with tyrosine			systems without tyrosine		
	20 mins	40mins	60mins	20mins	40mins	60mins
Control	0	±	±	0	±	+
$CuSO_4$ 2×10^{-5} M	±	±	+	±	++++	+
" 4×10^{-5}M	+++	++	0	+	++++	+
NaCl	±	±	++	+	+++	±
$CuSO_4$ 2×10^{-5}M+NaCl	++++	+	0	++	++++	0

portant also in view of the results obtained with ascorbic acid oxidation in potato.

The systems examined in this series of experiments contained phosphate buffers, copper sulphate, sodium chloride, hydrogen peroxide and tyrosine. After a given time the colour caused by the products of tyrosine oxidation was observed and the results were expressed in + , + corresponding to faint pink colour, ++++ to dark brown almost black, while ++ and +++ correspond to the intermediate stages in colour.

In the experiment given below, the systems were left for 3 hrs in an incubator at 37^{o}, after which time the flasks were placed in the water bath at 85^{o} .The changes in colour were observed during the first period and again at intervals during the following period of intensive heating. The results are given in the table below.

Table 6.

The stronegst development of colour was observed in systems with copper sulphate, and copper sulphate plus sodium chloride. The difference in $CuSO_4$ plays here an important role, the higher it was the more rapid was the rate of colour changes.Control systems showed comparatively very slow changes in colour.During the subsequeht period of heating the systems passed their maximum of colour development and became colourless again. At 80^{o} system containing copper sulphate in concentration 4×10^{-5} has developed its maximum of colour before the first/

first comparison was made - in the table the colour intensity is marked 0 from the beginning of the experiment. These changes in colour follow the order observed in the first part of these experiments, the strongest coloured systems become colourless first. At the same time the intensity of escape of bubbles of gas was observed in this experiment. During the period when the systems were kept at 37° no evolution of gas was observed. During the subsequent heating period the intensity of the decomposition of hydrogen peroxide is in close relation to the colour changes in that, that the appearence of bubbles of gas begins as a rule after the system has passed its maximum of colour. Whenever the reaction of oxidation of tyrosine is still in progress the decomposition of hydrogen peroxide does not take place. Only after this reaction has been finished the catalase one begins. The control system which never has reached the intensity of colour achieved in systems containing catalysts, shows only a small degree of decomposition of hydrogen peroxide. That the presence of tyrosine inhibits the second catalytic function of copper- catalase one- is also shown by the comparison of the intensity of gassing in the corresponding systems of identical composition but without tyrosine. The decomposition of hydrogen peroxide in these systems, where there is no substrate for oxidation, is of a much greater intensity.

The following conclusions may be drawn from these experiments. At 37° copper sulphate shows a peroxidase/

xidase action , the substrate tyrosine being oxidized proportionally to the concentration of the catalyst. The catalase action at this temperature is very slow and its manifestations are not perceptible. This is in agreement with the previous experiments where by titration with $KMnO_4$ it was found that at this temeperature no perceptuble decomposition of hydrogen peroxide should take place during the time the observations in this experiment have been made. At higher temperatures this peroxidase action is greatly increased, but simultaneously the catalase action increases also and to such extent that finally it becomes manifest . This, however takes place only in systems which have completed or are approaching the completion of peroxidase stage of reaction. Sodium chloride activates the peroxidase action of copper, as for its influence on the catalase action ,it is known from the previous experiments that it exerts this action by itself, so in this type of experiments it is impossible to draw any conclusions as to its influence.

These experiments with tyrosine as a substrate for oxidation are in complete agreement with the previous experiments with methyl red.In both cases their presence inhibits distinctly the catalse action of copper.

On the other hand in systems where the concentration of copper sulphate was considerably greater than in experiments quoted above no regular colour changes/

changes were observed at higher temperatures and only aviolent decomposition of hydrogen peroxide was the characteristic feature of the reaction. This type of experiment cannot be considered as typical, because the colour development depends on the concentration of copper sulphate, and the rapid changes occurring in systems make impossible any correct observations.

To summarize the resulta of this series of experiments it may be said that in systems containig an oxidizable substrate the peroxidase action of copper prevails and diminishes greatly the catalase action. Whether it is a complete inhibition or only a great diminution of catalase action it is impossible to decide in these experimental conditions. Contrary to the results obtained with the oxidation of ascorbic acid,sodium chloride shows an opposite effect in the reactions examined in this chapter. The peroxidase type of oxidation of methyl red and tyrosine is activated by NaCl, the catalase action of copper is slightly increased in the presence of this substance but cannot be considered as activated by it, as NaCl itself shows a similar influence. This shows that although the inhibitory influence of sodium chloride is not of a general character,nevertheless its particopation in redox systems with copper as catalyst is very interesting.

5.The oxidation of ascorbic acid in the presence of hydrogen peroxide .

Two types of sodium chloride influence on oxidation/

TABLE 7.

Systems without H_2O_2								Systems with H_2O_2							
systems without tyrosine				systems with 0.5 mgr. of tyrosine				systems without tyrosine				systems with 0.5 mgr. of tyrosine			
40'		90'		40'		90'		10'		20'		10'		20'	
1.9	63.3	2.4	80.0	1.1	36.6	1.7	56.3	6.	1.0	33.3	1.8	60.0	0.1	3.3	0.9	30.0
2.7	90.0	2.7	90.0	1.9	63.3	2.6	86.6	7.	2.7	90.0	2.7	90.0	1.3	43.3	2.0	63.3
2.7	90.0	2.7	90.0	2.4	80.0	2.7	90.0	8.	2.7	90.0	2.7	90.0	2.6	86.6	2.7	90.0
0.8	26.6	1.5	50.0	1.6	53.3	2.3	76.6	9.	0.6	20.0	1.5	50.0	0.4	13.3	1.1	36.6
0.9	30.0	1.8	60.0	1.7	56.3	2.6	86.6	10.	2.4	80.0	2.7	90.0	2.5	83.3	2.7	90.0

systems contained in 15 ml.: 1.5 ml. phosphate buffers
3 mgr. ascorbic acid.

1. mgms. of oxidized ascorbic acid in the whole system.
2. percentage of oxidized ascorbic acid.

ontrol.
uSO_4 4×10^{-5} M
uSO_4 2×10^{-4} M
aCl 0.2 M
uSO_4 4×10^{-5} M, MaCl 0.2 M.

on oxidation with copper as catalyst were found in the previous chapter. One - oxidation of ascorbic acid by molecular oxygen was inhibited by sodium chloride, the second- oxidation of methyl red and tyrosine by hydrogen peroxide was activated by NaCl.These results suggested therefore that in all reactions of peroxidase type the activating influence of sodium chloride should be expected. To examine this possibility a series of experiments was performed in which the oxidation of ascorbic acid in the presence of hydrogen peroxide was examined. The results of a typical experiment are given in table 7.

Table 7.

As it was mentioned before. the oxidation of ascorbic acid by hydrogen peroxide is a much quicker process than that by atmospheric oxygen.In control systems with hydrogen peroxide 60 % of ascorbic acid was oxidized during 20 mins, the corresponding control without peroxide showed 63.3% oxidation after 40 mins. This oxidation depends on the concentration of hydrogen peroxide, which in these experiment must be sufficiently low to avoid a to quick reaction in order to give suitable figures for comparison.In systems containing twice as large concentration of hydrogen peroxide as in experiment above, no ascorbic acid was found after IO mins in any of the systems.

Copper sulphate activates greatly this oxidation. In the presence of H_2O_2 after IO mins 90% of/

90 % of ascorbic acid has disappeared in systems containing 4 xIO^{-5} M. of copper sulphate as compared with 33.3% oxidation in system without added copper.

Similarly to the systems without hydrogen peroxide, sodium chloride inhibits this oxidation although here this inhibition is smaller. This is shown especially in systems where besides copper sulphate also sodium chloride was present. In systems without hydrogen peroxide NaCl lowers the oxidation by copper sulphate from 90 % to 30 %, causing a true inhibition as compared with the control system(63.3 % oxidation). In system without hydrogen peroxide only a small decreaseof oxdiation was found, without NaCl the oxidation reached 90 %, with NaCl- 80 % . There was no true inhibition in comparison with control system (33.3 % oxidation). Only a small inhibition was found in system with NaCl but without added copper sulphate,the oxidation here was 20%,in control 33.3%.

In our previous paper(Mystkowski&Lasocka,l.c.) it was found that amino acids inhbit the oxidation of ascorbic acid by copper sulphate.The same effect shows tyrosine in systems without hydrogen peroxide.Its presence lowers the oxdiation to 36.6 % as compaared with 63.3% in systems without tyrosine.The same inhibition was found in systems with added copper sulphete, although here it was much smaller.

In the presence of tyrosine sodium chloride shows none of its ususal inhibition of the oxidation

dation of ascorbic acid either in systems with hydrogen peroxide as also in those without it.

In the series without hydrogen peroxide sodium chloride increases the rate of oxidation from 36.6% to 53.3 %.Thes same activating effect was found in systems containing hydrogen peroxide.

The following conclusions may be drawn from these experiments,

a)tyrosine inhibits the oxidation of ascorbic acid in all the systems examined (with O_2 and with H_2O_2),

b)sodium chloride inhibits the oxidation of ascorbic acid by hydrogen peroxide,similarly to the oxidation by atmospheric oxygen,

c)tyrosine and sodium chloride present together show a smaller degree of inhibition than the sum of inhibition caused bythese two factors taken separately,and in some cases

d)in the presence of tyrosine the influence of sodium chloride is reversed - activation of ascorbic acid oxidation instead of inhibition.

It has been shown before that sodium chloride activates the oxidation of tyrosine by hydrogen peroxide. Here in the same type of reaction-with copper as preroxidase model but with different substrate,an opposite effect has been met. Thus with the change of the substrste,the different effect of NaCl influence makes this reaction similar to the oxidation of ascorbic acid by molecular oxygen.Another reversion of the

influence/

TABLE 8.

The systems contained phosphates 0. I M

$CuSO_4$ 2×10^{-5}

Ascorbic acid 1 mgr. per system NaCl 0.2 M.

Tyrosine 1 mgr. per system.

Total volume of each system 15 mls. pH 5.7 - 6.4.

Colour intensity in (after 17 hrs. at 37°)

pH	Control	$CuSO_4$	$CuSO_4$ + ascorb. acid	Ascorb. acid.	NaCl + ascorb. acid.
6.4	0	0	++++	+	++
6.2	0	0	++	+	++
6.1	0	0	+	+	++
5.7	0	0	+	+	++

molecular oxygen could be proved.

The formation of hydrogen peroxide and its instantaneous decomposition by copper during the oxidation of ascorbic acid was first postulated by Barron,Klemperer and deMeio (I935).Also Robeznicks(I938) suggested that in the system ascorbic acid oxidase-ascorbic acid hydrogen peroxide is formed and served to the further oxidation of the substrate. Now it is generally accepted that during the oxidation of ascorbic acid,hydrogen peroxide actually is formed(Hand &Chase Greisen, I942).

A series of experiments was made to prove that tyrosine undergoes oxidation in systems ascorbic acid-tyrosine-atmospheric oxygen with copper sulphate as the catalyst. In these experiments the appearence and development of coloured products of tyrosine oxidation was observed.The results of one of such experiments are given in the following table.

Table 8.

In this experiment the production of colour at different pH values(5.7 - 6.4)was observed. No oxidation of tyrosine was found in control systems containing phosphates and tyrosine. The addition of copper sulphate to this system had also no effect. In the presence of ascorbic acid the colour appeared and was greatly increased in intensity by addition of copper sulphate.The activation took place also in the presence of sodium chloride

This reaction is very slow, only after I0 hrs at 37° it was possible to compare the intensity of/

of colour in the systems under examination. This very slow rate of the reaction,which could not be increased by changing the experimental conditions(pH,changes in concentration of particular components) caused that the postulated influence of the products of tyrosine oxidation on the oxidation of ascorbic acid in the absence of hydrogen peroxide must be considered as hypothetical and should be examined again with the aid of different methods.

6.The oxidation of ascorbic acid in the presence of enzymic systems.

In the previous chapters only the aspects of inorganic catalysis of ascorbic acid oxidation were considered and the influence of various agents on this process examined. In the following chapter the influence of these factors on the enzymic oxidation of ascorbic acid has been examined.

Szent-Gyorgyi(I93I) was first to establish that in cabbage leaf exists an enzyme capable of oxidizing ascorbic acid.Stotz,Harrer and King(I937)have proved that the catalytic effect of plant juices on the oxidation of ascorbic acid depends on copper-protein catalysis.In the following years two different mechanisms dealing with oxidation of ascorbic acid in plants were recognized. One of them is an enzyme causing direct oxidation -ascorbic acid oxidase, the second comprises a number of enzymes which are responsible of oxidaizing ascorbic acid but only indirectly,through substances

which/

which first undergo oxidation under the influence of corresponding enzymes and then in turn oxidize ascorbic acid. Johnson and Zilve(I937) have found that in cabbage,cauliflower,cucumber and marrow the enzyme oxidizes ascorbic acid directly, thus belonging to the ascorbic acid oxidase type, whereas in apple and potato phenolases,through their intermediate action are responsible for this oxidation.

Silverblatt and King(I938) reported strong evidence of the copper-protein nature of enzymes oxidizing ascorbic acid.Lovett-Janison and Nelson found that ascorbic acid oxidase from summer squash is a copper protein compound containing 0.I5 % of copper.Also Ramasarma,Data and Doctor(I940) found that copper is an active constituent of ascorbic acid oxidase.The existance of ascorbic acid oxidase as a copper-protein compound in cucumber juice has been confirmed by Meiklejohn and Stewart(I94I).

To the second type of enzymes oxidizing ascorbic acid belong many copper containing systems. Here belongs system described by Keilin and Hartree(I938) in which cytochrome is oxidized by indophenol oxidase, and the former in turn oxidizes catechol,hydroquinone, p-phenylendiamine,o-phenylendiamine and ascorbic acid. Another system described by Keilin and Mann(I938) and found in mushroom-Agaricus campestris- oxidizes ascorbic acid rapidly in the presence of traces of catechol.

Also/

Also laccase(Keilin and Mann,1939) is able to oxidize ascorbic acid in the presence of p-phenylendiamine but not of catechol. Kubowitz (1938) showed that polyphenolase from potato oxidaizes ascorbic acid similarly to the system described by Keilin and Mann in mushroom. Tauber (1936) also postulated the presence of carrier capable to undergo oxidation to quinone form in the oxidation of ascorbic acid by peroxidase-peroxide system in plants.

Both types of enzymes mentioned in this short review contain copper , and it was interesting to see whether any similarities exist between catalytically active sopper of inorganic character and enzymic copper. Already Stotz,Harrer and King(l.c.) examined a number of compounds which inhibit the catalytic action of copper. They found that both types of catalyses- by inorganic copper in the presencce of proteins and enzymic copper are inhibited by added "copper inhibitors". However, in cauliflower, and cabbage juices this inhibition was smaller than in other systems. Experiments by Barron et al(1938) have shown that cabbage juice and squash juice were not inhibited by o-hydroxyquinoline in their enzymic activity. E.Stotz (1940) has shown that glycine at pH 6.0 inihibits completely tha action of non-enzymic copper, but does not show any influence on the copper in the enzyme.

In the following experiments two plant extracts/

extracts have beam used- potato exract as typical for indirect enzymic oxidation of ascorbic acid and cucumber juice as typical for direct oxidase activity.

a)Potato extract

At least two systems dealing with the oxidation of ascorbic acid were found in potato.First of them causes a very rapid disappearence of almost all of the ascorbic acid after the structure of the potato has been destroyed by grinding.

An experiment showing this quick disappearance was performed as follows. Four pieces of freshly peeled potato,each weighing 10 grams were ground in amortar

a) with 20 mls of 10% trichloracetic acid

b) with 20 mls of distilled water

c) with 10 mls of distilled water

d) with 20 mls of 5.0% sodium chloride solution.

To c) Io mls of 20.0% trichloacetic acid were added 2 mins after grinding.

All samples were centrifuged immediately after grinding and the ascorbic acid was determined in the supernatant fluid.The following amounts were found

a) I.12 mgr of ascorbic ac. c) 0

b) 0 d) 0

Only a trace of ascorbic acid was found in the first sample after the second washing and none at all in all the remaining samples.

This experiment shows that during

the time of grinding(3- 5 mins usually) the whole of ascorbic acid disappears from potato. In the system c) trichloacetic acid added 2 mins after the end of grinding in a concentration equal to this in system a) was not able to prevent this rapid disappearance -the whole reaction has been finished during the short period before.

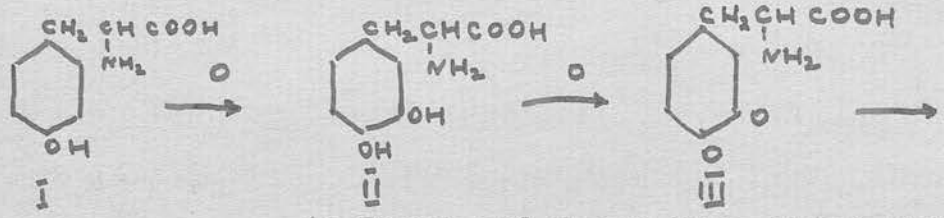
Only when the grinding is effected in a strong solution of trichloracetic acid,ascorbic acid is protected from this rapid oxidation. Sodium chloride does not prevent this oxidation, which suggests that the mechanism of the reaction must be different from the oxidation by inorganic copper and molecular oxygen.

Many facts speak in favour of the conception that the quinones play the most important part in the oxidation of ascorbic acid in potato, although the picture is not yet completely clear.

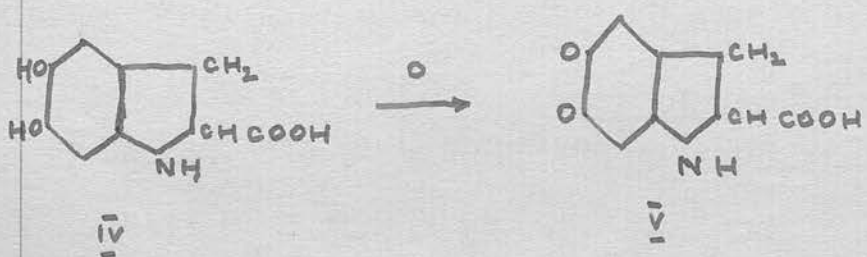
The formation of quinones in potato during and after its grinding is a very quick reaction. Already in few minutes after the grinding has been finished the pink colour of potato pulp appears. In these experiments ,however, when trichloracetic acid was present during the grinding or added shortly after the grinding (systems a) and c) in the above experiment), no pink colour has appeared, nevertheless in system c) the whole of ascorbic acid has been oxidaized.This fact indicates that the oxidation of ascorbic acid has been finished in system c) before the "red substance" from tyrosine/

tyrosine oxidation has been formed.

According to Raper(I(32) the first stage inin the oxidation of tyrosine is the formation of 3.4--dihydroxyphenylalanine (II) with subsequent oxidation to 3.4- quinone of phenylalanine (III)-dopa quinone



As Raper and Evans have shown this quinone must be considered as a strong oxidizing agent for ascorbic acid. The "red substance" which represents the further step in this oxidation is due to an intramolecular change, again with the subsequent oxidation to quinone form



As the whole ascorbic acid disappears before the red substance comes into view the suggestion arose that these quinones are responsible for the oxidation of ascorbic acid. This has been proved by many experiments,im which the oxidation of ascorbic acid by quinones inhibits formation of the red substance.Abderhalden (I934,I936) first noticed that ascorbic acid inhibits the formation of dopa-quinone.Schaaf(I935) and Evans/

Evans and Raper(1937) have shown that this inhibition does not depend on the influence on the enzyme but on the reduction from the stage of quinone. Similarly Tauber(1936) found that on addition of p-phenylendiamine to the horse-radish extract the coloured stage of the oxidation did not appear untill all the ascorbic acid was oxidized. In none of these papers ,however, the relation between the disappearance of ascorbic acid and the production of colour was followed quantitatively.

In connection with this problem the following experiments have been done.

Three pieces of potato,each weighing 10 gram were ground in the following media

a) with 20 mls phosphate buffers, 0.2 M, pH 6.5

b) with 20 mls solution of ascorbic acid in phosphate buffers at the same pH as above,containing 6.25 mgr of ascorbic acid

c) with 20 mls of buffered solution of ascorbic acid containing 12.5 mgr.

After 5 mins the colour in a) was distinctly pink,b) and c) were colourless. After 30 mins system a) was of a dirty reddish colour, b) was faintly pink, c) showed only a trace of pink -almost colourless.This experiment was done at 16°.

Another similar experiment was done with the filtered water extract of potato. 30 grms of freshly peeled potato were ground with 60 mls of phpsphate buffers/

TABLE 9.

	mgms. ascorbic acid left.		% oxidation.
	30 mins.	1 h. 15 mins.	1 h. 15 mins.
1.	0	0	0
2.	1.6	1.04	83.4
3.	2.3	1.52	87.8

buffers 0.2 M,pH 6.5 and filtered quickly through glass wool. From this extract threesystems were made,viz.

a) I5 mls of extract and 5 mls phosph.buffers
b) I5 mls of extract and 5 mls of buffered solution of ascorbic acid containing 6.25 mgrs
c) I5 mls of extract and 5 mls of buffered solution of ascorbic acid containing i2.5 mgrs

All systems were left in conical flasks at I7^{o} .After this time ascorbic acid was determined in 5 mls samples after the additioh of 2 mls of 20% trichloracetic acid. The results are given in the table

Table 9

The control system ,without ascorbic acid was distinctly pink after 3 mins,the colour gaining in strenght gradually. A trace of pink colour appeared in system c),containing I2.5 mgrs of ascorbic acid after I hr I5 mins when about 88.0% of ascorbic acid was oxidized. After 4 hrs both systems b) and c) were pinkish and only a trace of reduction has been found in them. After 24 hrs all systems were black in colour,no trace of reduction was found anywhere. These experiments show that the oxidation of tyrosine in a ground potato tissue and in potato extract is inhibited by ascorbic acid, and only after almost all the ascorbic acid has been oxidized the "red substance" begins to form.

The experiments given at the beginning of this chapter have shown that during the grinding

TABLE 10.

Potato extract - 200 grms. of potato to 640 ml. -

Phosphates 0. I M.

NaCl 0.2 M.

the colour development after 3 hrs. at 16°

Without NaCl		With NaCl	
Ph		Ph	
6.1	+++	6.1	++++
5.8	++	5.7	+++
5.4	+	5.5	+++

ding the oxidation of ascorbic acid is not inhibited ba sodium chloride. The more detailed analysis of the influence of sodium chloride on these process was made in the extracts from potato. First the influence of NaCl on the development of red substance has been examined.

Table IO

This experiment shows that in a carefully beffered extract the oxidation of tyrosine is increased by the presence of sodium chloride. It is necessary to emphasize the need for a very careful buffering in these experiments, because of the great importance of pH, small changes of which may lead to results which could not be compared with the corresponding systems containing NaCl.

This enzymic system behaves similarly to those with inotganic copper in the presence of hydrogen peroxide as concerns the influence of sodium chloride. In both cases sodium chloride has an activating influence on the oxidation of tyrosine.

Also in a potato extract containing sodium chloride and ascorbic acid the former substance has an activating influence on the development of colou Its influence on the rate of ascorbic acid oxidation is very difficult to ascertain.

In the following experiment the oxidation of ascorbic acid in potato extract was compared with/

TABLE 11.

Potato extract 200 gr. - 640 ml.

Volume of each system 30 mls.

Phosphates 0.1 M.

NaCl 0.2 M.

Ascorbic acid 5 mgms. per system.

	Genuine extract.			Boiled extract.		
	pH	mgr.Asc. acid left	% oxidation	pH	mgr.Asc. acid left	% oxidation
without NaCl	6.2	2.6	48.0	6.3	4.5	10.0
	5.6	2.9	42.0	5.7	4.4	12.0
with NaCl	6.2	2.0	60.0	6.1	4.7	6.0
	5.5	3.2	36.0	5.7	5.0	0

Estimations made after 1 hr. 30 mins.

with the corresponding reaction in the same extract but boiled for 20 mins and made up to the original volume afterwards.

Table II

The considerable dilution of the extract is responsible for a rather slow rate of both reactions,i.e. colour development and oxidation of ascorbic acid. This has been done purposely,because if the reaction proceeded at a quicker rate it would be impossible to follow any of them - the rapid oxidation of tyrosine and quick development of colour would obscure the whole reaction. The comparison with the boiled extract was made in order to show that the reactions occuring in the unboiled one are of the termolabile,enzymatic character. This reaction in the boiled extract is caused chiefly by the inorganic copper and probably small amount of quinones formed during the preparation of the extract.

In unboiled systems an activation of ascorbic acid oxidation was found at pH 6.2 and a slight inhibition(within the limits of experimentsl error, especially when considered that pH was slightly lower here) at pH 5.5. The oxidation of tyrosine is distinctly increased by sodium chloride.

This experiment,however, is typical only at pH near the optimum for tyrosine oxidation and the little activation by sodium chloride is probably of a secondary character,depending on the production of quinones/

nes under the influence of sodium chloride. Others experiments performed at a lower pH(4.3 - 5.8) did not show such activation. Moreover in most of them no regular relations between pH, concentration of NaCl and tyrosine and the rate of oxidation of ascorbic acid could be found. This is explained by a very complicated chain of mutual reactions influences occuring in these systems. The oxidation of ascorbic acid would depend here on the rate of the formation of quinones from tyrosine different at different pH values, on the rate of further oxidation of these quinones and on the influence of sodium chloride on both reactions.On the other hand it known here from the previous experiments that the oxidation of tyrosine is activated by sodium chloride but inhibited by the presence of ascorbic acid.

In conclusion it may be said that the enzymic system in potato shows certain characteristic features in relation to sodium chloride. Firstly the quick disappearance of ascorbic acid from potato during the grinding is not inhibited by sodium chloride. Secondly the oxidation of tyrosine is activated by this substance, influence similar to that in inorganic systems oxidizing tyrosine and composed of copper sulphate,hydrogen peroxide and NaCl. Finally it has been shown that a very advanced oxidation of ascorbic acid is essential for the oxidation of tyrosine,which reaction is beeing hold up at the stage of quinone leading to the formation of red substance by the ascorbic acid .

The existance of ascorbic acid in potato tuber shows that an intact structure is essential to prevent its oxidation. This is not an isolated fact, the existance of enzymes in living cells side by side with the corresponding substrates is a problem which has been considered by many aurthors. Whether it is due to the protection by special factors in the living cell or to the"spatial separation" including absorption on different surfaces it is difficult to decide (discussion on it -Przyłęcki, I935). Not without significance in the case of ascorbic acid is the access of oxygen. From the kitchen practice it is known that to prevent potatoes from blackening after peeling it is necessary to keep them protected by a layer of water.But even in these conditions quinones are formed and a considerable amount of ascorbic acid is oxidized (about 25 % during 24 hrs,Kramer(I942) and own experiments). In the course of these experiments tha rate of oxidation of ascorbic acid in an unshaken system was much greater in broad conical flasks,with an easy access of air, than in the narrow test tubes. This applies not only to potato xtract but also to the systems with inorganic copper.

As the second system capable of oxidizing ascorbic acid in potato were ,in this work, considered quinones formed during the second stage of tyrosine oxidation,i.e. after the disappearance of the genuine ascorbic acid from potato.Here belongs red substance and further products. Even in the extract inwhich the oxidation of tyrosine has reached its end,e.g. incubated for 24 hrs/

TABLE 12.

The systems contained in 15 mls:

Phosphates 0.1 M

Cucumber Juice (AAO) 1 ml. pH 6.1

Ascorbic Acid 4 mgms.

NaCl 0.4 M

System.	Mgms. ascorbic Acid left after 30 mins.	% Oxidation
1. With AAO	1.7	57.5
2. With AAO + NaCl	1.7	57.5
3. Without AAO	2.6	35.0
4. " " with NaCl	3.8	5.0

TABLE 13.

	0				$CuSO_4$				NaCl			
	30'		90'		30'		90'		30'		90'	
System	1	2	1	2	1	2	1	2	1	2	1	2
1.	1.2	31.6	2.1	55.2	1.7	44.7	2.7	71.1	0.4	10.5	0.7	18.4
2.	1.7	44.7	2.8	73.7	1.7	44.7	2.5	65.8	1.8	47.3	2.7	71.1
3.	0.2	5.2	0.8	21.1	1.3	34.2	2.2	57.9	0.4	10.5	0.8	21.1

System 1. water + phosphate buffers.

2. water + 2 mls. of cucumber juice + phosphate buffers.

3.water + 2 mls. of inactivated cucumber juice + phosphate buffers.

Concentration of $CuSO_4$ - 2.5×10^{-5} M

" " NaCl - 0.14 M

1. - mgrs. of oxidized ascorbic acid.

2. - per cent decomposition.

24 hrs at 37° under toluene and coloured black of melanines a slow oxidation of ascorbic acid takes place. This oxidation was not inhibitsd by sodium chloride.

b) cucumber juice

Cucumber jiuce haw been taken as an example of the system where the oxidation of ascorbic acid takes place directly undr the influence of a specific oxidase.

First the comparison of the influence of sodiun chloride in systems containing scorbic acid oxidase with systems containing inorganic copper has been made. The results are given in table I2.

Table I2

In this experiment tha rate of oxidation by oxidase is about 40 % greater tahn in system without enzyme. The inhibition by sodium chloride was found only in inorganic system, in the presence of enzyme NaCl did not show any influence.

In order to establish whether this lack of inhibition did not depend on the presence in cucumber juice of substances which might interfere with the action of sodiumchloride,an experiment was made where the oxidation in systems with active ascorbic acid oxidase was compared with systems containing the same amount of cucumber juice but inactovated by boiling.

Table I3

In this experiment the rate of oxidation in systems/

in systeme containing boiled cucumber juice is lower than that in control systems without juice. In control system the oxidation reached 55.2 % after 90 mins, in system with boiled juice - 2I.I % .

This fact can be explained by the binding of inorganic copper by the proteins presnet in boiled juice. The only factor responsible for oxidation of ascorbic acid in such systems is inorganic copper present in traces ih water. If this copper ic inactivated by the binding with proteins,the activity of the system falls considerably. It has been shown (Mystkowski & Lasocka) that this factor has a considerable influence in systems with copper sulphate. By changing alternatively the concentrations of $CuSO_4$ and preotins it was possible to increase or decrease the activity of the systems. Similarly, in the experiment above when the concentration of copper sulphate was increased,the activity of boiled juice increased also,still,however,not to the samedegree as in inorganic control system.The oxidation in inorganic system with added copper sulphate increased after 90 min from 55.2 % to 7I.I %, in system with boiled cucumber juice from 2I.I % to 57.9 % in comparison with system without added copper. In system with active oxidase the addition of copper did not increase the activity . It is quite probable that in systems containing inactivated juice,copper bound to the enzymic protein loses its properties as a result of the denaturation of the protein, and the whole system behaves /

TABLE 14.

The influence of egg albumin and NaCl on the oxidation of ascorbic acid by copper.

The systems contained phosphates in concentration 0.1 M NaCl 0.2 M albumin 0.133% ascorbic acid 4 mgms per system total volume 15 mls p++ 6.1

The estimation of remaining ascorbic acid was made in 1 ml., after deproteinisation with trichloracetic acid.

After 20 hrs. $CuSO_4$ was added to all systems in conc. 2.5×10^{-5} M and again the estimation of remaining ascorbic acid was made after 1 hr.

% oxidation of ascorbic acid.

	1 hr.45 mins	5 hrs	20 hrs	1 hr. after addition of $CuSO_4$
Control	50.0	100.0	100.0	-
NaCl	0	10.0	62.5	-
Albumin	0	12.5	22.5	42.5
NaCl + albumin	0	12.5	22.5	36.5

behaves like inorganic copper in the presence of non-specific protein.

Sodium chloride does not show any inhibitory effect either in systems with active or inactivated juice. This shows that the influence of NaCl on oxidative reactions cannot be considered as a fact concerning all forms of catalytically active copper. Here the enzymatically active copper bound to a protein is resistent to this type of influence.

It was interesting to examine whether this fact has more general significance,i.e. whether during the oxidation of ascorbic acid by copper the presence of a protein protects the catalyst against the influence of NaCl. For this purpose a series of experiments was performed,in which the oxidation of ascorbic acid was examined in systems containing phosphates,copper sulphate,sodium chloride and different proteins.

Table I4

In this experiment the oxidation is inhibited by protein to a much higher degree than by sodium chloride. The addition of sodium chloride to the systems containing protein did not increase this inhibition but if to this system,containing ascorbic acid already partially oxidized, copper sulphate was added, the inhibition caused by albumin and sodium chloride was slightly greater than in the presence of the protein alone.In all experiments of this type, also with casein,never a complete/

TABLE 15.

The systems contained:

phosphates 0.1 M — ascorbic acid 4 mgms per system

$CuSO_4$ 2×10^{-5} — pH 6.1

albumin 0.13% — total volume 15 mls.

	mgms ascorbic acid left after		% oxidation after
	30 mins	3hrs 30 mins	3 hrs 30 mins.
1. Control	2.6	1.1	72.5
2. $CuSO_4$	2.1	0.1	100.0
3. Albumin	3.8	3.6	10.0
4. Albumin + $CuSO_4$	2.5	0.9	77.5
5. Boiled albumin	4.0	3.9	2.5
6. Boiled albumin +$CuSO_4$	2.6	1.1	72.5

complete summation of inhibitions was observed. The presence of sodium chloride in fairly high concentrations,as in experiment above, only slightly increased the inhibition caused by protein. With smaller concentrations of sodium chloride,but sufficient to cause an inhibition by themselves, no increase of inhibiton in the presence of protein was observed.

Denatured proteins have even greater inactivating power on catalytically active copper.This is shown in the table below. In this experiment the comparison of inactivating power of the same protein (albumin) in native state and denatured by boiling was made.

Table I5

These experiments show that the presence of protein modifies considerably the catalytic properties of copper, firstly diminishing its catalytic activity, secondly- by making it less sensitivy to the inhibitory influence of sodium chloride.

It is interesting that even in comparatively high concentrations of proteins,such as were used in some of these experiments, copper always retains some of its activity. This is in connection with the fact that theproteins have only a limited power for binding and inactivating added copper. Ba increasing sufficiently the concentration of copper sulphate, it is possible to restore the original activity of the system.

The fact that proteins diminish sensitivity of copper to the inhibitory influence of sodium

chloride, its complete lack of influence in systems with active ascorbic acid oxidase may depend on this effect. This is substantiated by the similar lack of influence in systems with inactivated oxidase.

Aother factor which may be also responsible for it, may be the way in which copper is bound with its protein carrier in the molecule of the enzyme. This special link may protect completely the active group of the enzyme from this type ch inhibitory influence. In favour of this conception speaks the lack of influence of glycine on copper in ascorbic acid oxidase in opposition to the inhibitory effect on inorganic copper (Stotz, I940).

These facts and considerations concern only catalytically active copper present in ascorbic acid oxidase. Form the experiments described in first part of this work it is known that sodium chloride exerts quite a distinct influence on the catalytically active copper in enzymes dealin with the oxidation of tyrosine.

DISCUSSION

The biological activity of copper, like that of iron, comprises two groups of reactions.
i/ inactivating influence on enzymic processes,both hydrolytic and Redox,
ii/ catalytic action in many Redox processes.

The catalytic action of iron in

living organism is connected with its presence in a number of enzymes. The role of copper in enzymic processes is not so well established, at least only very few compounds of an importance similar to tha of iron compounds are known(Kubowitz ,1937,1938 . Meiklejohn & Stewart ,1941). Nevertheless the widespread presence of copper in tissues and its catalytic activity observed in vitro suggest that its role must be of considerable importance.

On the other hand none of these metals present in tissues have been found to have any definite role ,unless bound with some specific protein.

The catalytic action of copper is very easily influenced by many factors. Having established that certain substances normally present in the tissues have a distinct influence on the oxidation of ascorbic acid, we have examined some different aspects of the catalytic activity of copper.

The oxidation of ascorbic acid by atmospheric oxygen in the presence of copper was found to be inhibited by sodium chloride, amino acids and proteins.

In the presence of hydrogen peroxide, however, the catalytic action of copper is more complicated by the fact that copper can act as a model not only of peroxidase but also of catalase. It was found that the activity of copper depends on the kind of substrate present. Thus at higher temperatures the catalase action of copper was greatly diminished when the substrate

suitable for oxidation was available.The presence of either methyl red or tyrosine,which undergo oxidation by H_2O_2 stopped almost completely the latter s catalase function.Both reactions,decomposition of H_2O_2 and oxidation of mathyl red and tyrosine are activated by NaCl. These experiments show that the catalytic action of Cu is of two kinds and changes according to the presance of different substrates and also that the influence of NaCl is of opposite chracter in two cases.

The influence of amino acids on the catalytic action of Cu depends in the first place on their power to bind Cu.Tyrosine,however, shows another type of influence in connection with its role in redox reactions. In the presence of tyrosine the course of ascorbic acid oxidation is changed by the action of the products of tyrosine oxidation. In such systems two different reactions take place-one,inhibited by sodium chloride and tyrosine-the oxidation by O_2 and H_2O_2, the second activated indirectly by NaCl-oxidation by quinones.The third possible factor in this reaction is the competition of tyrosine with ascorbic acid for oxygen and an inhibitory influence caused by this mechanism.This factor is connected with redox potentials of different parts of the system,and the final result in such a system will depend on these potentials and the concentration of components.

The action of proteins on ascorbic acid oxidation is always of inhibiting nature. Our previous experiments have shown,how this influence depends on the/

on the concentration of copper and protein. Although some copper-protein compunds obtained in vitro may show certain properties common with enzymes(Stotz,Harrer and King,1937) their action in the oxidation of ascorbic acid is smaller than that of corresponding concentration of copper sulphate.

The relation between the structure of copper- protein complexes and their catalytic activity is not known. From these experiments it is clear that the denatured proteins have a greater inactivating power on the catalytic action of copper that then native proteins. This fact shows, that many "enzymic "properties of the copper protein complexes may depend on the colloidal state of the protein in solution.

On the other hand in these complexes the catalytic activity of copper in regard to the oxidation of ascorbic acid is not completely abolished. All similar complexes,e.g. those isolated by Keilin and Mann (1939), may be of some significance in biological catalyses,especially when their widespread occurence is considered. As an another example may be here the work by Ramasarma, Data and Doctor (1940) who also found that not all the copper present in vegetable juice is enzymic. This copper probably also may play part in catalyses of inorganic type and non-specific character.

The influence of sodium chloride on ascorbic acid oxidation in systems containing enzymes is different from that on inorganic copper. It is rather difficult/

difficult to speak about this influence in potato, as the oxidation of ascorbic acid here is of an indirect nature, and depends on the oxidation of another substance -tyrosine. Thus all factors influencing oxidation of tyrosine will at the same time influence also the oxidation of ascorbic acid. On the other hand it has been found that the presence of ascrobic acid inhibits the oxidation of tyrosine. This chain of mutual influences causes that the picture of oxdiation processes in potato is still far from being clear. The enzymic system in potato dealing with the oxidation of tyrosine is activated by sodium chloride. In cucumber juice, containing ascorbic acid oxidase, no influence of sodium chloride in either direction has been found.

The lack of influence of sodium chloride on ascorbic acid oxidation in cucumber juice, the inactivation of of added copper by enzymically active juice and the transformation of of the latter into an inorganic system after the denaturation of present proteins suggested as an explanation the difference between enzymic and inorganic copper. This difference may consist either in the specifity of the enzymic protein or in the special form of binding of copper with the protein in the molecule of the enzyme. The importance of a special form of binding, which does not exclude the first possibility. is supported bymany facts. The observations by Kubowitz(I938) on polyphenoloxidase and Meiklejohn and Stewart (L. c.) on ascorbic acid oxidase show that co-

pper/

pper present in enzyme does not undergo dialysis as long as the protein is in a genuine state, but it is possible to splitt the enzyme molecule into these two components by a suitable treatment and reunite it afterwards with a complete restoration of its enzymic activity. Mystkowski(I942) has shown that the cataly sis by copper of ascorbic acid oxidation increases many times when copper is in complex with pyridine. This increase however, takes place only in alcoholic medium, in aqueous systems pyridine inhibits this reaction and the copper-pyridine complex shows a much lesser activity than copper alone. These observationssindicate that even if a specific protein was not responsible for the different behaviour and especially greatly increased activity of copper in the molecule of the enzyme. the linkage of these two components is of greatest importance.

The relation between tyrosine and ascorbic acid in redox processes is also of a great interest. It has been known since the work of Abderhalden (l.c.) that these two substances form in vitro a redox system, which as it has been found lately (Sealock & Silberstein, I939, Levine, Marples & Gordon, I939, Rothman,I940) plays probably an imprtant role in living organisms.

These relations in vitro of redox character has been confirmed in this work, both in inorganic system as in potato, where the disappearance of ascorbic acid is a condition for the development of one of the stages in tor̊sine oxidation(red substance). It Has been also establushed that in the presence of ascorbis

bic acid a system is formed in which,with copper as catalyst,tyrosine undrgoes oxidation. The possibility of participation of hydrogen peroxide formed during the oxidation of ascorbic acid in this reaction has also been discussed.

The way in which chlorides influence the catalytic activity of copper is not known.In simple systems, where the oxidation of ascorbic acid runs parallel to Cu^{ii} - Cu^{i} transformation, the inhibitionof of the catalysis is accompanied by a diminution of curpous oxida formation. The suggestion put forward by Mapson(I942) that a formation of a copper-halide complex may be responsible for that inhibition but requires a sufficient concentration of NaCl is substantiated by our previous experiments (I939) when it was found that cupric chloride has the same catalytic effect and is similarlyinhibited by sodium chloride,in concentrations corresponding to those inhibiting similar concentrations of copper sulphate.

A more full explanation hase been suggested by Szent- Gyorgyl (I938) in his discussion in a series of papers cocerning the mechanism of metal catalyses of ascorbic acdi and catechol oxidation. This authors idea is that oxygen and substrate for oxidation are coordinated on the atom of metal,inside which a shift of electrons occurs. A third molecule,which also can enter this complex on metal,may be able to modify the properties/

properties of the complex. Especially nitrogen containing molecules are able to serve as this "third molecule".

The influence of protein on copper catalyse, the activity of copper-pyridine complexes, the behaviour of copper as catalyst in alcoholic medium as also the influence of sodium chloride in catalyses examined in this work are in good agreement with these ideas.

CONCLUSIONS.

I.The oxidation of ascorbic acid by molecular oxygen with copper as catalyst is inhibited by sodium chloride

2.This type of catalysis and also inhibition takes place also in absolute alcohol.

3.The inhibition by NaCl is parallel to Cu^{ii}-Cu^{i} transformation.

4.Copper catalyses the decomposition of hydrogen peroxide ,this reaction is not inhibited by NaCl.

5.NaCl shows an activating influence on the decomposition of hydrogen peroxide.

6.Copper catalyses the oxidation of methyl red and tyrosine by H_2O_2.NaCl activates this catalysis.

7.The decomposition of H_2O_2 in the presence of copper is inhibited by the presence of substrate suitable for oxidation by peroxidase mexhanism.

8.The oxidation of acsorbic acid by H_2O_2 is catalysed by copper, this reaction is inhibited by NaCl.

9.Tyrosine inhibits the oxidation of ascorbic acid by O_2 and H_2O_2 with copper as catalyst.On the other hand the products of tyrosine oxidation oxidaize ascorbic ac

IO.In the presence of tyrosine the influence of NaCl on ascorbic acid oxidation is reversed - activation instead of inhibition.

II. The participation of quinones in acsorbic acid oxidation in inorganic systems has been examined.

I2./

I2.In potato the quinones formed during the oxidation of tyrosine are the chief agent responsible for the oxidation of ascorbic acid.

I3.The influence of ascorbic acid on the oxidation of tyrosine has been examined

I4.Sodium chloride,probably indirectly, activates this type of ascorbic acid oxidation.

I5. Ascorbic acid oxidase is not inhibited by NaCl.

I6.Proteins inhibit the oxidation of ascorbic acid and diminish the inhibitory influence of NaCl on this process.

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