CLXX. THE OXIDATION OF SULPHYDRYL COMPOUNDS BY HYDROGEN PEROXIDE.

I. CATALYSIS OF OXIDATION OF CYSTEINE AND GLUTATHIONE BY IRON AND COPPER.

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THE oxidation of thiol compounds by oxygen and methylene blue has, in recent years, been very extensively studied, while their oxidation by hydrogen peroxide has suffered almost complete neglect. Abderhalden and Wertheimer [1923] found that cysteine, like other mercaptans, was instantaneously oxidised by hydrogen peroxide in neutral solution, and that this oxidation was unaffected by cyanide. At neutrality an oxidation of the thiol group would be complicated, at any rate in the presence of iron, by the Fenton reaction. Quastel, Stewart and Tunncliffe [1923] have indeed prepared an oxidation product of this type from glutathione. In acid solution and in the presence of the theoretical amount of hydrogen peroxide the reaction proceeds smoothly with the production of pure oxidised glutathione [Pirie, 1931].

In this paper the results of a study of the oxidation of cysteine and glutathione to the corresponding disulphides will be given while in a later one a more deep-seated oxidation will be described. The former action is catalysed by iron, copper and thiocarbamide while the latter is catalysed by vanadium, titanium and molybdenum. The following elements have, in acid solution, either no action or a very slight one: Ag, As, Be, Co, Cr, Hg, Mn, Ni, Pb, U, W, Zn.

EXPERIMENTAL.

The course of the oxidation was followed polarimetrically since the ordinary methods which have been used for following oxidations are clearly inapplicable. Polarimeter tubes were made of heavy-walled glass tubing, 4 mm. internal diameter and 20 cm. long, with a side-tube through which they were filled. The ends (end plates for the ordinary type of polarimeter tube) were fixed on with de Khotinsky cement. Except for the part over the bore of the tube the end plates were blackened. When not in use the tubes, and all the other vessels used in this work, were kept filled with 20 % hydrochloric acid. Before use they were rinsed out five or six times with glass-distilled water and dried by connecting to a mechanical pump having an efficient sulphuric acid absorption tube.

"Metal-free" cysteine hydrochloride was prepared according to Dixon's method by recrystallisation four times from metal-free 20 % hydrochloric acid; metal-free glutathione was obtained by the method already described [Pirie, 1930] the vessels used for washing and decomposing the cuprous compound being washed with acid before use. Merck's "perhydrol" has a very low metal content and was used in all cases diluted to give a 0.44 *N* solution. At this concentration it is stable for some days but if diluted much further it tends to decompose.

Glass apparatus was used throughout, and, presumably because all the fluids used were acid, no trouble was experienced through metals being adsorbed by the glass.

The oxidation of cysteine.

In the absence of added metal the rate of oxidation of cysteine is very small at p_H 2.1 (the p_H of a dilute solution of cysteine hydrochloride) but is markedly increased by small amounts of iron or copper. These results are shown in Fig. 1. In each case the appropriate amount of a dilute solution of ferrous sulphate (freshly prepared each day) or copper sulphate was added to 1 cc. of a 1 % solution of cysteine hydrochloride, the amount of metal-free water necessary to make up the volume to 4.5 cc. was run in from a burette and finally 0.5 cc. of 0.44 *N* H_2O_2 was added. The mixture was then poured into a dry polarimeter tube and the rotation measured for mercury green light. It is clear that in each case the rate of oxidation is proportional to the amount of metal added over a fairly wide range. The temperature coefficient of the reaction was calculated from the velocities at 10° and at 20° in the presence of 0.0013 mg. Cu and of 0.002 mg. Fe and was found to be 2.0 ($\pm$ 0.1) in the first case and 2.2 in the second.

The catalytic effects of iron and copper are sharply differentiated from each other by the different effects of variations in the concentrations of cysteine and H_2O_2 . The rate of oxidation in the presence of copper is proportional to the peroxide concentration and independent of the amount of cysteine, whereas in the presence of iron it depends on cysteine and is independent of peroxide. (Curves illustrating this phenomenon in the case of iron are given later when the effect of phosphate is considered.) This effect is somewhat different from that studied by Elliott [1930] in the aerobic oxidation of cysteine; in this case the rate of oxidation depended on the cysteine concentration for both iron and copper catalysis. Elliott also found that, with 8 mg. of cysteine hydrochloride and at p_H 7.3, 0.0042 mg. Cu caused maximum rate of oxidation. It is apparent from Fig. 1 that there is no such limit in the case of oxidations at a lower p_H .

Effect of p_H . The effect of an increase in p_H on the catalytic oxidation is complicated by the fact that the rate of oxidation in the absence of added metal is also greatly increased and, at neutrality, is much too rapid to be followed conveniently by this technique. I have worked mainly at p_H 4.6.

1 cc. of 1 % cysteine hydrochloride was conveniently buffered at this p_H by 0.3 cc. of $M/3$ metal-free ammonium acetate, which was prepared by neutralising redistilled acetic acid with distilled ammonia. It is clear from Fig. 2 that this change in p_H has affected the rate of oxidation in the absence of added metal or in the presence of iron to a greater extent than that in the presence of copper. This suggests that the unavoidable oxidation is due to the presence of traces of iron. It is not of course permissible to compare these rates of change of rotation directly, for the specific rotations of cystine and cysteine vary with p_H . Cysteine in acid solution has a small positive rotation whereas

Fig. 1.

Fig. 2.

Fig. 1. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2.1.

1.	1 cc. 1 % cysteine-HCl;	0.02 mg. Cu;	0.5 cc. 0.44 N H_2O_2 .
2.	"	0.05 mg. Fe;	"
3.	"	0.003 mg. Cu;	"
4.	"	0.001 mg. Fe;	"
5.	"	0.001 mg. Cu;	"
6.	"	0.0005 mg. Fe;	"
7.	"	No metal	"

Fig. 2. Volume made up to 5 cc. in each case. Temperature 20°. p_H 4.6.

1.	1 cc. 1 % cysteine-HCl;	0.001 mg. Fe;	0.3 cc. $M/3$ ammon. acetate;	0.5 cc. 0.44 N H_2O_2 .
2.	"	0.0003 mg. Fe;	"	"
3.	"	0.001 mg. Cu;	"	"
4.	"	No metal;	"	2.0 cc. 0.44 N H_2O_2 .
5.	2 cc. cysteine-HCl;	0.6 cc. ammon. acetate;	1 cc. H_2O_2 incinerated etc. then 1 cc. cysteine-HCl;	0.3 cc. ammon. acetate and 0.5 cc. H_2O_2 .
6.	1 cc. 1 % cysteine-HCl;	0.3 cc. $M/3$ ammon. acetate;	0.5 cc. 0.44 N H_2O_2 .	

Bergmann and Michalis [1930] found that cysteine itself was slightly laevo-rotatory in aqueous solution. I find that this shift occurs at about p_H 4, and it is shown by a 0.04° change in the initial rotations in Figs. 1 and 2. Cystine undergoes a change in rotation at about the same p_H . Andrews [1925] found that the specific rotation of cystine, dissolved in hydrochloric acid, increased with the dilution. Lutz and Jirgensens [1930] showed that, with the same concentration of cystine, the rotation increased as the concentration of the acid or alkali used to dissolve it diminished. On account of its insolubility the rotation of cystine was not measured by these workers at neutrality. Supersaturated solutions of cystine are however relatively stable and, as Fig. 2 shows, the rotation of a very highly supersaturated solution of cystine does not alter for at least 40 minutes. By this method the specific rotation

of cystine for mercury green light and in 0.2 % solution has been determined at varying p_H .

p_H	2.1	$[\alpha]_{5461}^{20}$	$- 276^\circ \pm 2$	p_H	5.5	$[\alpha]_{5461}^{20}$	$- 312$
	2.5		$- 279$		7.7		$- 297$
	3.1		$- 286$		8.3		$- 227$
	4.6		$- 312$		9.0		$- 156$

In a later section when the effects of various inhibitors are considered the reason for the fairly high rate of oxidation in the absence of added metal will be discussed more fully. It has already been shown that the effect of p_H on the rate suggests that it is a case of iron catalysis. Curve 4, Fig. 2, shows however that at least the greater part of it is not due to iron, since a fourfold increase in H_2O_2 concentration increases the rate threefold, whereas control experiments show that at p_H 4.6, as at p_H 2.1, the rate of oxidation in the presence of iron is independent of the concentration of H_2O_2 .

A similar conclusion was reached by testing the reagents separately for catalytic power after incineration. The following method was found to be the most reliable. A silica test-tube (10×1.5 cm.) was heated with a very small oxy-coal gas flame at a point a little to one side of the bottom and then drawn out with a piece of silica rod to form a horn 2 mm. long and 1.5 mm. broad at the base. The tube was clamped in a sloping position with the horn downwards and heated with a micro-burner giving a flame about 1.5 mm. broad. If care were taken that only the tip of the horn was touched by the flame the incineration proceeded smoothly without any bumping. Sulphuric acid and ammonia were freed from metal by distillation although ordinary pure ammonia (sp. gr. 0.880) is generally sufficiently free from metals already. The results of these experiments are somewhat inconclusive on account of the danger of contamination during the fairly lengthy incineration which is necessary with such a low metal concentration. 0.1 cc. of concentrated sulphuric acid was used and one drop of strong ammonia solution (sp. gr. 0.93) was added before the mixture was heated, this reduced the time necessary for complete incineration to about 15 minutes. The mixture was cooled, and 2 cc. of water were added followed by slight excess of ammonia. The excess ammonia was removed by cautiously boiling till ammonium sulphate crystallised. 1 cc. of cysteine hydrochloride solution was now added and, after a minute or two, 0.3 cc. of $M/3$ ammonium acetate, 0.5 cc. of 0.44 N H_2O_2 and the volume made up to 5 cc. Curve 5 on Fig. 2 is the result of incinerating 2 cc. of cysteine hydrochloride, 1 cc. of H_2O_2 and 0.6 cc. of ammonium acetate in this way. Similar experiments on the reagents separately showed that the greater part of this metal came from the cysteine hydrochloride. Ammonium sulphate at this concentration is without effect on the rate of oxidation either in the presence or the absence of added metal, though higher concentrations, as for example when 0.6 cc. of sulphuric acid is used for the incineration, inhibit the oxidation in both cases.

At p_H 1.6 the rate of oxidation in the presence of copper is only slightly less than at p_H 2.1, whereas the oxidation due to iron is greatly diminished. At this p_H cystine is fairly soluble, the effect of added cystine was therefore determined. Elliott [1930] has shown that, at p_H 7.3, cystine catalyses the copper but not the iron catalysis of cysteine oxidation. It is apparent from Fig. 3 that it catalyses neither type of oxidation appreciably at p_H 1.6. Metal-free cystine hydrochloride was made by precipitation with hydrogen chloride as described by Gerwe [1931]. A 1 % solution of this in $N/9$ HCl was used. In plotting the rotations measured in the presence of cystine, 0.86° , the rotation due to the cystine, has been subtracted in each case. Curves

Fig. 3.

Fig. 4.

Fig. 3. Volume made up to 5 cc. in each case. Temperature 22° . p_H 1.6.

1. 1 cc. 1 % cysteine-HCl; 1 cc. $N/9$ HCl; 0.01 mg. Fe; 0.5 cc. $0.44 N H_2O_2$.
2. " " 1 cc. $N/9$ HCl; 0.001 mg. Cu; 0.5 cc. $0.44 N H_2O_2$.
3. " " 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0.001 mg. Cu; 0.5 cc. $0.44 N H_2O_2$.
4. " " 1 cc. 1 % cystine-HCl in $N/9$ HCl; 0.001 mg. Fe; 0.5 cc. $0.44 N H_2O_2$.
5. " " 1 cc. $N/9$ HCl; 0.001 mg. Fe; 0.5 cc. $0.44 N H_2O_2$.

Fig. 4. Volume made up to 5 cc. in each case. Temperature 19° . p_H 2.1.

1. 2 cc. 1 % cysteine-HCl; 0.002 mg. Fe 0.88 mg. KH_2PO_4 ; 0.5 cc. $0.44 N H_2O_2$.
2. 1 cc. 1 % cysteine-HCl; 0.002 mg. Fe; no phosphate; "
3. " " " 0.44 mg. KH_2PO_4 ; "
4. " " " 0.88 mg. KH_2PO_4 ; "
5. " " " 1.0 cc. $0.44 N H_2O_2$.
6. " " 0.001 mg. Fe; 0.44 mg. KH_2PO_4 ; 0.5 cc. $0.44 N H_2O_2$.
7. " " 0.002 mg. Fe; 2.2 mg. KH_2PO_4 ; "

showing the effect of cystine on iron catalysis in the presence of phosphate are given later.

Effect of phosphate. Copper catalysis is unaffected by even $M/100$ phosphate both at p_H 2.1 and 4.6. Iron catalysis on the other hand is strongly inhibited at both reactions. This is shown in Fig. 4. The stock phosphate solution was $M/62$ KH_2PO_4 , and even 0.2 cc. *i.e.* $M/1550$, was strongly inhibiting. As curves 1, 4 and 5 show, the rate of oxidation in the presence of iron, even when it is partially inhibited by phosphate, depends on the cysteine concentration and is independent of the concentration of H_2O_2 . The slight inhibition shown in curve 5 has been obtained several times and becomes large if a large amount of H_2O_2 is added. In the presence of $0.00064 M$ phosphate the rate of oxidation, during the first hour at any rate, is no longer proportional to the iron present. In the presence of phosphate, therefore, cysteine behaves like thioglycollic acid [Elliott, 1930] so far as iron catalysis is con-

cerned. The situation is, however, complicated by the autocatalytic shape of the curves. Dixon and Tunnicliffe [1922] observed this autocatalysis in the methylene blue oxidation of .SH groups at p_H 7.6 but not at p_H 6. Fig. 5 shows that it is not due to catalysis by the cystine formed for, in the presence of iron and at p_H 1.6, the oxidation is autocatalytic when phosphate is present but is not catalysed by cystine. The autocatalysis does not seem to be due to a difference between ferric and ferrous phosphate for the curves are the same whether the iron and cystine are first mixed together and allowed to stand for 10 minutes before the addition of the phosphate and peroxide, or the iron and peroxide mixed first and then the other reagents added. It is possible, though from a quantitative point of view unlikely, that the increase in the

Fig. 5.

Fig. 6.

Fig. 5. Volume made up to 5 cc. in each case. Temperature 22°.

- 1 cc. 1 % cysteine-HCl; 1 cc. 1 % cysteine-HCl in $N/9$ HCl; 0.01 mg. Fe; 0.66 mg. KH_2PO_4 ; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 1 cc. $N/9$ HCl; 0.01 mg. Fe; 0.66 mg. KH_2PO_4 ; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0.62 mg. Fe; 0.001 mg. Cu; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 6 mg. sodium pyrophosphate; 0.62 mg. Fe; 0.001 mg. Cu; 0.5 cc. 0.44 N H_2O_2 . The reagents being added in this order.
- 6 mg. sodium pyrophosphate; 0.62 mg. Fe; 0.5 cc. 0.44 N H_2O_2 ; 1 cc. 1 % cysteine-HCl. The reagents being added in this order.

Fig. 6. Volume made up to 5 cc. in each case. Temperature 22°. p_H 4.6.

- 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.0005 mg. Fe; 0.5 cc. 0.44 N H_2O_2 .
2. " ; " ; 1.6 mg. KH_2PO_4 ; " ; "
3. " ; " ; 2.2 mg. KH_2PO_4 ; no metal; " ; "

rate of oxidation as the oxidation proceeds is due to the disappearance of H_2O_2 since the latter exerts a slight inhibitory effect. If this were the explanation, the initial rate of oxidation of a mixture, made up as in curve 4, Fig. 4, but with only 0.4 cc. of H_2O_2 , should be the same as the rate after 20 minutes with 0.5 cc. Actually the two curves are identical.

At p_H 4.6 the inhibitory effect of phosphate is, as might be expected from the work of others, less marked. A pair of curves illustrating this is given in Fig. 6 and also a control curve in the presence of 0.0032 M phosphate. A comparison of this curve with the similar one in Fig. 2 shows that the inhibition is hardly outside the experimental error; this suggests, in agreement with the incineration results, that only a small part of the unavoidable oxidation can be due to iron.

Effect of pyrophosphate. Copper catalysis is unaffected by pyrophosphate unless the latter is present in very high concentration. Catalysis by iron on the other hand is inhibited quantitatively by pyrophosphate even when there is present only slightly more than one molecule of pyrophosphate for each molecule of iron. When there is a large amount of iron present (0.02 mg.) it can be inhibited by less than one molecule of pyrophosphate to one of iron, but a precipitate forms after a few minutes and makes the fluid in the tube opaque. No precipitate is formed in the presence of excess pyrophosphate. Fig. 7 shows the effect of pyrophosphate on iron catalysis at p_H 4.6, and it is clear

Fig. 7. Volume made up to 5 cc. in each case. Temperature 20° . p_H 4.6.

1. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.001 mg. Fe; 0.5 cc. 0.44 N H_2O_2 ; 0.0032 mg. $Na_4P_2O_7$; 10 H_2O .
2. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.001 mg. Fe; 0.008 mg. pyrophosphate; 0.5 cc. H_2O_2 .
3. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.062 mg. Fe; 0.0002 mg. Cu; 0.8 mg. pyrophosphate; 0.5 cc. H_2O_2 .
4. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.001 mg. Fe; 0.011 mg. pyrophosphate; 0.5 cc. H_2O_2 .
5. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.001 mg. Fe; 0.016 mg. pyrophosphate; 0.5 cc. H_2O_2 .
6. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; no metal or pyrophosphate; 0.5 cc. H_2O_2 .
7. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.062 mg. Fe; 0.8 mg. pyrophosphate; 0.5 cc. H_2O_2 .

that copper may be detected by this technique in the presence of 300 times its weight of iron. The curves for iron in the presence of a substantial excess of pyrophosphate show that the latter has only a very slight effect on the "blank." Experiments with pyrophosphate but without added iron show that not more than 15 % of the unavoidable oxidation can be due to iron. The curves in Fig. 7 show that there is always some rapid oxidation at the beginning of each experiment. Two curves are given in Fig. 5 which indicate that this is due to a difference between ferric and ferrous pyrophosphate since it is not obtained when the cysteine is added last. Fig. 5 also shows that, in

the presence of 620 times its weight of iron, copper is somewhat inhibited by pyrophosphate.

Effect of HCN. Metal-free HCN was prepared by distilling a mixture of sodium cyanide and sulphuric acid and collecting the distillate in water. The results are summarised in Fig. 8. By comparison with Fig. 1 it is apparent that cyanide activates iron at p_H 2.1 while it has a hardly appreciable inhibitory action on copper. At p_H 4.6 on the other hand it inhibits small amounts of copper completely and has a slight inhibitory effect on iron. The difference between curve 2 on Fig. 2 and curve 1 on Fig. 8 is however not

Fig. 8. Volume made up to 5 cc. in each case. Temperature 22°.

- 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; 0.0003 mg. Fe; 0.27 mg. HCN; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Fe; 0.54 mg. HCN; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Cu; 1.0 mg. HCN; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.3 $M/3$ ammon. acetate; 0.003 mg. Cu; 0.54 mg. HCN; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; no metal; 0.54 mg. HCN; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ ammon. acetate; no metal; no HCN; 0.5 cc. 0.44 N H_2O_2 .

Fig. 9. Volume made up to 5 cc. in each case. Temperature 20°. p_H 2.1.

- 1 cc. 1 % cysteine-HCl; 0.002 mg. Fe; 0.4 mg. ferrocyanide; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.002 mg. Fe; 1.0 mg. ferrocyanide; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Cu; 1.0 mg. ferrocyanide; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Cu; 1.0 mg. ferricyanide; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Cu; 0.2 mg. ferricyanide; 0.5 cc. 0.44 N H_2O_2 .
- 1 cc. 1 % cysteine-HCl; 0.001 mg. Cu; 1.0 mg. ferricyanide; 0.5 cc. 0.44 N H_2O_2 .

marked. A curve is also given in Fig. 8 showing that HCN has no effect on the rate of oxidation in the absence of added metal.

Effect of ferro- and ferri-cyanide. No attempt has been made to analyse fully the somewhat intricate systems to which these reagents give rise. They are slightly catalytically active themselves in the absence of added metal and, since the rate of oxidation seems to increase as the cyanide becomes more dilute, this is probably due to the decomposition of the cyanide. Fig. 9 shows that ferrocyanide activates iron at first and that this effect later gives place to an almost complete inhibition. Ferricyanide gives no such activation. Ferricyanide in the same way seems to inactivate copper almost completely at p_H 2.1 while ferrocyanide has first to be oxidised to ferricyanide (*cf.* curves 4 and 6 in Fig. 9). At p_H 4.6 the catalytic effect of these compounds masks any inhibitory effect which they may exert.

A number of other substances has been tested, using concentrations of about 1 mg. in 5 cc., as inhibitors for copper or iron. Carbon monoxide, urea, urethane, glutamine, alanine and histidine have no effect on copper; glutamic acid and potassium thiocyanate do not affect iron. The various reagents¹ introduced by Eichholtz [1929] and Krah [1930] were without effect on either metal when used in the maximum concentration possible with this technique. Owing to the colour of the reagents and to their insolubility in acid solution the concentration was generally 0.1 mg./cc. Sulphuretted hydrogen was converted immediately to free sulphur by the H_2O_2 present.

It was at first thought that low results which were often got with iron after incineration might be due to inhibition by pyrosulphate. The addition of pyrosulphate, however, inhibits neither iron nor copper at p_{H} 2.1 or 4.6. For some reason, therefore, the method of incineration used is not satisfactory if one wishes to estimate iron although it works perfectly in the case of copper.

Catalysis by copper derived from haemocyanin.

It is well known, from the work of Roche [1930] and others, that haemocyanin is not stable in acid solution. Haemocyanins from three species, *Helix pomatia*, *Limulus polyphemus* and *Maia squinado*, have been examined and in each case the copper appears to be completely liberated in catalytically active form at p_{H} 2.1. This, in the case of *Helix* is shown in Fig. 10, where the rates of oxidation in the presence of serum and of the same amount of serum after incineration are compared. This comparison has been made repeatedly and there has never been any difference between serum or dialysed serum and incinerated serum so far as catalytic activity is concerned. In only one sample of serum was there any detectable difference between the rates of oxidation in the presence and in the absence of pyrophosphate, the iron content of the sera of these three animals is therefore less than 1/20 of the copper content.

At p_{H} 4.6 neither *Helix* nor *Limulus* haemocyanin has any catalytic activity at all. In curve 6, Fig. 10, the crosses give the rate of oxidation in the absence of haemocyanin and the line gives two determinations of the rate in the presence of some snail haemocyanin. Curve 2 shows the effect of adding the same amount of haemocyanin to the cysteine hydrochloride before the ammonium acetate instead of after it. It makes no difference if the acetate is added 10 minutes after the haemocyanin instead of 20 seconds after, *i.e.* the decomposition at p_{H} 2.1 is complete in less than 20 seconds.

Maia haemocyanin is less stable than the other two and has a slight catalytic activity at p_{H} 4.6. Curves 3, 4 and 5 in Fig. 10 illustrate the decomposition which it undergoes in 3 minutes at p_{H} 3.9 and 3.65.

Since it is perfectly stable at p_{H} 3.3 this was the p_{H} used for the oxidation with *Helix* haemocyanin, the decomposition resulting from 3 minutes at

¹ 8-Hydroxyquinolinesulphonic acid, 1-amino-8-naphthol-4-sulphonic acid, 1-amino-2-naphthol-6-sulphonic acid, 5-nitro-4-chloro-2-aminophenol.

p_H 2.4, 2.7 and 2.9 is shown in Fig. 11. In these and all other cases the amount of ammonium acetate necessary to give any required p_H was found from a titration curve. $M/3$ ammonium acetate was run into 0.2 % cysteine hydrochloride solution and the p_H measured with a hydrogen electrode. Curves 1 and 2 on Fig. 11 show that, as might be expected, the rate of oxida-

Fig. 10.

Fig. 11.

Fig. 12.

Fig. 10. Volume made up to 5 cc. in each case. Temperature 21°.

1. ——— 0.047 cc. of *Helix pomatia* serum; 1 cc. 1 % cysteine-HCl; 0.5 cc. 0.44 N H_2O_2 .
- o—o— 0.047 cc. of *Helix pomatia* serum incinerated; 1 cc. 1 % cysteine-HCl; 0.5 cc. 0.4 N H_2O_2 .
2. 1 cc. 1 % cysteine-HCl; 0.01 cc. *Helix* serum; 0.3 cc. $M/3$ amon. acetate; 0.5 cc. 0.44 N H_2O_2 .
3. } 0.03 cc. of *Maia* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate and the H_2O_2 were then added
4. } { p_H 3.65
5. } { p_H 3.9
6. } { p_H 4.6
6. 1 cc. 1 % cysteine-HCl; 0.3 cc. $M/3$ acetate; 0.01 cc. *Helix* serum; 0.5 cc. 0.44 N H_2O_2 .
- × × Extent of oxidation of controls after 50 minutes.

Fig. 11. Volume made up to 5 cc. in each case. Temperature 20°.

1. 1 cc. 1 % cysteine-HCl; 0.067 cc. *Helix* serum; 0.5 cc. 0.44 N H_2O_2 .
2. 0.017 cc. *Helix* serum;
3. } 0.017 cc. *Helix* serum left for 3 minutes with a mixture of 1 cc. cysteine-HCl and acetate at the p_H stated. The rest of the acetate, and the 0.5 cc. of 0.44 N H_2O_2 were then added.
4. } { p_H 2.4
5. } { p_H 2.7
6. } { p_H 2.9
6. Control at p_H 3.3 with no haemocyanin.

Fig. 12. Temperature 20°. In each case the volume is 5 cc. and the final p_H is 4.6. An amount of *Limulus polyphemus* serum containing 0.0032 mg. of copper was added to each after the addition of the amount of ammonium acetate solution necessary to bring 1 cc. of 1 % cysteine-HCl to the required p_H . After a suitable time the rest of the ammonium acetate was added and then 0.5 cc. of 0.44 N H_2O_2 .

1. 3 minutes at p_H 2.1.
2. 3 minutes at p_H 2.9.
3. 10 minutes at p_H 3.3.
4. 3 minutes at p_H 3.3.
5. 20 seconds at p_H 3.3.
6. 3 minutes at p_H 3.75.
7. 3 minutes at p_H 4.6.
8. Control at p_H 4.6.

tion at p_H 2.1 is proportional to the amount of haemocyanin. The effect of variations in the concentration of haemocyanin on the extent of its decomposition when the decomposition is only partial has not been measured.

In Fig. 12 curves are given illustrating the effect of p_H on the stability of *Limulus* haemocyanin. In 3 minutes a barely perceptible decomposition takes

place at p_H 3.75, but at lower p_H the decomposition is rapid. Three curves at p_H 3.3 show the effect of different times of exposure to acid. In all these experiments the actual oxidation takes place at p_H 4.6. Figs. 11 and 12 both show that when the oxidation is allowed to proceed to completion in the presence of decomposed haemocyanin the final rotation observed is more than 0.1° higher than that of the cystine formed. This extra rotation is due to the degraded haemocyanin. Natural haemocyanin has a small negative rotation which is doubled or trebled on the addition of acid, the addition of a corresponding amount of alkali does not reduce the rotation again to its old level. For example:

Rotation of 2 dm. of a solution of dialysed <i>Helix</i> haemocyanin containing 0.005 mg. of copper per cc.	= - 0.08°
Rotation after the addition of 0.2 cc. of $N H_2SO_4$ to 10 cc. of haemocyanin. p_H now 1.8	= - 0.18°
Rotation after adding 1 cc. of $N/5 NaOH$ to this	= - 0.16°

This last value was the mean of several readings made by two observers, since on neutralising a solution of haemocyanin which has been acid a very considerable turbidity develops. This was observed in all the experiments in which the stability of haemocyanin at varying p_H was measured, but, if the decomposition were not carried more than half-way, it is fairly slight.

Catalysis by haematin and haemoglobin.

The catalysis which is observed with these two substances is due, in part at any rate, to inorganic iron liberated under the conditions of the experiment. A few minutes' contact with H_2O_2 in the presence of cysteine hydrochloride is sufficient to discharge the colour of haemoglobin and, after this treatment, haematin will no longer give a haemochromogen with pyridine; both, on the other hand, now give an iron test with potassium thiocyanate. This decomposition takes place at both p_H 2.1 and 4.6.

The oxidation of glutathione.

In the presence of copper the oxidation of glutathione is very similar to that of cysteine. The rate of oxidation depends on the concentrations of copper and of hydrogen peroxide but it is independent of the concentration of glutathione. Thus curves 3 and 4 in Fig. 13 have the same gradient although the initial rotations are different since one represents only half as great a concentration of glutathione as the other. This rate of oxidation is doubled in curve 2, which has twice the H_2O_2 concentration, and in curve 1, with 0.006 instead of 0.002 mg. of copper, it is trebled. Meldrum and Dixon [1930] found that, for the aerobic oxidation of glutathione, small quantities of a third factor were necessary. Mason [1931] suggested that this was cysteinylglycine. This factor is of only secondary importance in the copper-catalysed oxidation by hydrogen peroxide. The sample of glutathione used in this work was very

pure, having been recrystallised from aqueous solution at about the freezing point. It gave theoretical analytical figures. A few cc. of the 2 % solution used were heated at 100° in a steam-bath for 35 minutes and, after cooling, used for oxidation experiments. The rate of oxidation in the presence of 0.006 mg. of copper or in the absence of added metal at p_H 4.6 was unaltered by this treatment although a considerable amount of decomposition is known to occur.

When the oxidation of glutathione is catalysed by iron it differs markedly from the oxidation of cysteine. The rate of oxidation is no longer proportional to the metal concentration but decreases suddenly when there is less than 1 part of iron for each 2000 of glutathione. Elliott [1930] found a very similar state of affairs in the iron-catalysed aerobic oxidation of thioglycollic acid.

Fig. 13.

Fig. 14.

Fig. 13. Volume made up to 5 cc. in each case. Temperature 21°.

1. 1 cc. 2 % reduced glutathione; 0.006 mg. Cu; 0.5 cc. 0.44 N H_2O_2 .
2. 1 cc. 2 % reduced glutathione; 0.002 mg. Cu; 1.0 cc. 0.44 N H_2O_2 .
3. 1 cc. 2 % reduced glutathione; 0.002 mg. Cu; 0.5 cc. 0.44 N H_2O_2 .
4. 0.5 cc. 2 % reduced glutathione; 0.002 mg. Cu; 0.5 cc. 0.44 N H_2O_2 .
5. 1 cc. 2 % reduced glutathione; no metal; 0.3 cc. $M/3$ ammon. acetate; 0.5 cc. 0.44 N H_2O_2 .
6. 1 cc. 2 % reduced glutathione; no metal; 0.5 cc. 0.44 N H_2O_2 .

Fig. 14. Volume made up to 5 cc. in each case. Temperature 21°.

1. —.—.— 1 cc. 2 % reduced glutathione; 0.01 mg. Fe; 0.5 cc. 0.44 N H_2O_2 .
—○—○— 1 cc. 2 % reduced glutathione; 0.01 mg. Fe; 1.0 cc. 0.44 N H_2O_2 .
2. 1 cc. 2 % reduced glutathione; 0.01 mg. Fe; 0.5 cc. 0.44 N H_2O_2 ; 0.18 mg. KH_2PO_4 .
3. 1 cc. 2 % reduced glutathione; 0.008 mg. Fe; 0.5 cc. 0.44 N H_2O_2 .
4. 0.5 cc. 2 % reduced glutathione; 0.01 mg. Fe; 0.5 cc. 0.44 N H_2O_2 .
5. 1 cc. 2 % reduced glutathione; 0.005 mg. Fe; 0.5 cc. 0.44 N H_2O_2 .

As with cysteine the rate of oxidation is independent of the peroxide concentration but is, as would be expected, an inverse function of the glutathione concentration. These facts are illustrated in Fig. 14.

A preliminary heating of the glutathione to 100° seems to increase the rate of oxidation in the presence of iron. The effect is small however and, since any decomposition will result in a lowering of the concentration of glutathione and this has already been shown to cause an increased rate of oxidation, it may well be unconnected with the production of cysteinylglycine.

Inhibitors, so far as they have been studied, have a similar effect on both oxidations. Cyanide inhibits copper partially at p_H 2.8 while phosphate is without effect. Phosphate inhibits iron strongly and at certain concentrations ($M/4000$) the curve is slightly inflected (curve 2, Fig. 14).

DISCUSSION.

The nature of the oxidation occurring at p_H 4.6 cannot be considered to be definitely settled but it seems very probable that all the oxidation at this p_H is not due to traces of metals. It behaves in a manner unlike iron catalysis so far as variations in H_2O_2 concentration are concerned and it is not inhibited by phosphate or pyrophosphate. It behaves on the other hand unlike copper catalysis towards variations in p_H and it is not inhibited by cyanide. Furthermore, if an experiment similar to that given in curve 5 of Fig. 2 is carried out, but with the addition of 0.1 mg. of pyrophosphate, the rate of oxidation is identical with that of the "blank" in the presence of pyrophosphate. This indicates that what metal there is, is iron. In a later paper I hope to describe the catalysis of this reaction by thiocarbamide. This oxidation will proceed rapidly in the absence of added metal and the effect of metal is, over a certain range at any rate, simply additive. The existence of this latter example of a "metal-free" oxidation by hydrogen peroxide lends colour to the idea that the present oxidation also can proceed in the absence of metal. At Prof. Haldane's suggestion the temperature coefficients of the copper- and iron-catalysed oxidations and of the oxidation without added metal were measured. In each case the coefficient was 2 ± 0.2 . No distinction between the three types of oxidation can therefore be drawn in this way.

At p_H 6 the oxidation of cysteine, without the addition of metal, is complete in a few minutes, the final rotation being 0.95° as in the case of the metal-catalysed oxidation. The final rotation when the oxidation occurs at p_H 4.6 cannot be determined since the cystine begins to crystallise after about 2 hours, *i.e.* before the reaction has gone to completion. Even in the presence of a very substantial excess of peroxide (1 cc. of 0.064 *M* cysteine hydrochloride or 1 cc. of 0.066 *M* glutathione mixed with 0.5 cc. of 0.44 *N* H_2O_2) the rotation of an acid solution of either cystine or oxidised glutathione will remain constant for over 12 hours, while the expected amount of hydrogen peroxide remains in the solution and can be estimated by titration. In acid solution therefore, in the presence of iron and copper and in the case of glutathione of the decomposition products formed by boiling for a short time, hydrogen peroxide can only react very slowly, if at all, to bring about further oxidation. On the alkaline side further decomposition readily takes place. In this connection the specific rotation of oxidised glutathione has been measured at several different reactions. Mercury green light was used and the solution, initially 1 %, was diluted somewhat at the higher p_H since in all 1.5 cc. of *N*/5 sodium hydroxide were added to 6 cc. of glutathione solution.

p_H 2.85	$[\alpha]_{5461}^{21} - 115^\circ \pm 1$	p_H 7.0	$[\alpha]_{5461}^{21} - 133$
5.0	- 125	8.6	- 150
6.0	- 126	9.6	- 176
			100—2

Glutathione resembles cysteine in showing a slight increase in specific rotation as the p_H increases from about 3 to 5. Unlike cysteine, however, it shows a further increase as the p_H rises above 5.

It is obvious that this catalytic reaction offers a very convenient method for estimating small amounts of copper, if necessary in the presence of iron. The sensitivity is about the same as that of Warburg's method [1927] but the technique is easier and, since all the solutions are acid, vessels simply need to be rinsed out 3-4 times with glass-distilled water after use. With the polarimeter available in this laboratory readings are accurate to 0.01° (in general the observed points have only been put on those curves which are non-linear; if the points on the graphs had been given a diameter corresponding with this probable error they would all have touched the lines which have been drawn) quantities of copper of the order of 0.001 mg. may therefore be estimated with an error of about 5 %. Metals such as vanadium will of course interfere with this method of estimation.

SUMMARY.

In the presence of copper the rate of oxidation of cysteine by hydrogen peroxide at p_H 2.1 is proportional to the concentrations of copper and peroxide but independent of that of cysteine.

In the presence of iron it is proportional to the concentrations of iron and cysteine and is slightly inhibited by an increase in H_2O_2 .

At p_H 4.6 there is some metal-free oxidation and the rate of oxidation in the presence of iron is much increased while that in the presence of copper is less markedly affected.

Figures are given for the specific rotations of cysteine and glutathione between p_H 2.1 and 9.0.

Phosphate inhibits iron and gives an inflected oxidation curve when the inhibition is incomplete. Pyrophosphate inhibits iron stoichiometrically.

At p_H 2.1 cyanide activates iron and hardly affects copper, at p_H 4.6 both are inhibited. Ferricyanide inhibits copper and iron, but ferrocyanide activates iron and only inhibits copper when it has been oxidised.

Haemocyanin has no catalytic effect but at p_H 2.1 it is decomposed completely giving catalytically active copper. The stability of three different haemocyanins at varying p_H has been measured.

Haematin and haemoglobin are not stable under the conditions used.

The rate of oxidation of glutathione in the presence of copper is proportional to the concentrations of copper and H_2O_2 and independent of that of glutathione. In the presence of iron there is a sort of auto-inhibition and the rate of oxidation is an inverse function of the glutathione concentration; it is independent of the H_2O_2 .

This reaction is suggested as a convenient method for estimating small amounts of copper.

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