CLIX. THE OXIDATION OF SULPHYDRYL COMPOUNDS BY HYDROGEN PEROXIDE.

II. CATALYSIS OF OXIDATION OF CYSTEINE BY THIOCARBAMIDES AND THIOLGLYOXALINES.

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THE first paper of this series [Pirie, 1931] dealt with the copper- and iron-catalysed oxidation of cysteine and glutathione by hydrogen peroxide in acid solution. It was shown that the corresponding disulphides were formed. Certain differences were observed between the kinetics of the copper-catalysed and the iron-catalysed reactions and no other metal of those tried was found to catalyse this type of oxidation. In the first paper brief mention was made of the fact that this action is catalysed by thiocarbamide: the present paper will be devoted to a study of this kind of catalysis and of the similar catalysis by chemically related substances.

EXPERIMENTAL.

The same polarimetric technique has been used in these experiments as in the series already published and the reagents, 1.0 % cysteine hydrochloride and 0.4 *N* hydrogen peroxide, were prepared in the same way.

Catalysis by thiocarbamide. "Metal-free" thiocarbamide can readily be prepared by crystallisation from dilute hydrochloric acid.

The kinetics of the thiocarbamide-catalysed oxidation resemble closely those of the copper-catalysed oxidation; the rate of oxidation, for example, is proportional to the concentrations of thiocarbamide and hydrogen peroxide but independent of that of cysteine hydrochloride. Curves illustrating these relations are given in Fig. 1.

In the paper already referred to it was shown that the iron-catalysed oxidation was inhibited by phosphate and pyrophosphate and the copper-catalysed oxidation, at p_H 4.6, by cyanide. These reagents have no effect on the thiocarbamide-catalysed oxidation; it seems therefore that this is not due to traces of metal impurity. Two further lines of argument are still more convincing. Firstly, the catalytic power is unaffected by repeated recrystallisation of the thiocarbamide; secondly, the residue obtained after incinerating 10–100 mg. of thiocarbamide with sulphuric acid, by the method already described [Pirie, 1931], is without catalytic activity. Curves 2 and 3 in Fig. 1 show that thiocarbamide has about the same catalytic power as 1/340 of its weight of copper.

I have been unable to demonstrate any effect of thiocarbamide on the aerobic oxidation of either cysteine or methylene blue at p_H 7.6. A few experiments have been carried out, with hydrogen peroxide, using glutathione instead of cysteine. In all respects these were analogous and the ratio of copper activity to thiocarbamide activity was once again 340.

There are two distinct products of the oxidation of thiocarbamide by hydrogen peroxide. Barnett [1910] prepared imino-aminomethanesulphinic acid, $\text{NH}:(\text{NH}_2)\text{CSO}_2\text{H}$, by oxidation in neutral solution, while Werner [1912]

Fig. 1. Volume made up to 5 cc. in each case. Temperature 19° , p_H 2.1.

1. 1 cc. 1 % cysteine-HCl; 0.56 mg. thiocarbamide; 1 cc. 0.4 N H_2O_2 .
2. " " 0.28 mg. thiocarbamide; " "
3. " " 0.0005 mg. Cu " "
4. " " 0.28 mg. thiocarbamide; 0.5 cc. 0.4 N H_2O_2 .
5. 0.5 cc. 1 % cysteine-HCl; " " 1 cc. 0.4 N H_2O_2 .
6. 1 cc. 1 % cysteine-HCl; no catalyst; " "

obtained salts of dithioformamidine, $\text{NH}:(\text{NH}_2)\text{CS}.\text{SC}(\text{NH}_2):\text{NH}$, by oxidation in acid solution. The former has no catalytic power whereas the latter is both a catalyst and an oxidising agent.

Oxidation by dithioformamidine. Dithioformamidine dihydrochloride is readily prepared, following Werner's [1912] method, by the dropwise addition of twice the theoretical amount of sulphuryl chloride to a solution of thiocarbamide in alcohol. When washed with alcohol and ether and dried it seems to be stable indefinitely. The yield is 80 % of the theoretical.

In Fig. 2 the effect of adding the same amount of cysteine hydrochloride to varying amounts of dithioformamidine is illustrated. It is obvious that two distinct reactions can occur. The first is

while the second is most probably

To get clear-cut results it is essential to add the cysteine, with vigorous shaking, to the dithioformamidine; if the procedure is reversed some of the cysteine gets oxidised to cystine and this cannot be further oxidised at any appreciable rate. The product of reaction B, on the other hand, is almost instantly reduced to cystine by cysteine; this can easily be demonstrated by mixing half the quantity

of cysteine that was used in the experiments illustrated in Fig. 2 with one molecule of dithioformamidine, the other half of the cysteine is now added and, although reaction B takes place initially, the rotation is found to be 6.6° , *i.e.* the value corresponding to cystine. On standing for a few hours crystals of cystine separate from the reaction mixture. The cross in Fig. 2 represents the

Fig. 2. 5 cc. of 3.12 % (*M* 5) cysteine hydrochloride solution and the appropriate amount of dithioformamidine dihydrochloride solution used for each point. Volume made up to 10 cc. in each case.

rotation reached immediately when the usual quantity of cysteine hydrochloride is added to a molecule of dithioformamidine and the other molecule is added after 30 seconds. After several hours the rotation falls to the normal value for reaction B whereas the initial value is that for reaction A.

Similar results are obtained by use of the Folin and Marenzi [1929] method of cystine estimation. In this method sodium sulphite in alkaline solution is used to reduce the cystine to cysteine before the addition of the Folin uric acid reagent. Mirsky and Anson [1930] have pointed out that only half of the cystine is reduced under these conditions and Clarke [1932] has shown that the other half combines with the sulphite to form a cysteinesulphonate

It is interesting to note therefore that under these conditions no cysteine is formed from the product of reaction B. This compound, however, in the absence of sulphite, is very unstable in alkaline solution and reacts almost instantly to give a body that is estimated as cystine by the Folin-Marenzi technique¹. It seems therefore that the product of reaction B, apparently the sulphenic acid corresponding with cysteine, is stable only in acid solution whereas the complex obtained on the addition of sulphite is fairly stable in alkaline solution. This

¹ This method was used on account of its great convenience although it has frequently been shown to be by no means specific. I have, in fact, isolated from deaminated caseinogen [Dunn and Lewis, 1921] a sulphur-free compound, apparently a tyrosine derivative, which gives as much colour with the reagent as the same weight of cystine. This substance presumably accounts for the large amount of "cystine" found by Steudel and Schumann [1929] in deaminated caseinogen.

complex may be identical with that studied by Clarke. The preparation and properties of the sulphenic acid and of its alkaline decomposition products will be described fully in a later paper.

1 cc. of *M*/156 cysteine hydrochloride solution is added to each of seven flasks; these contain 0, 0.5, 0.7, 0.9, 1.0, 1.1 and 1.3 cc. of *M*/156 dithioformamidine dihydrochloride solution. The Folin-Marenzi reagents are now added, the sulphite being added first. The first flask gives twice as intense a colour as the second, the third and fourth are fainter while the fifth is very faint and the sixth and seventh give no more colour, at first, than the control. The sixth and seventh tend to darken on standing. If a large excess of dithioformamidine is added more colour develops and also a turbidity, due to the alkaline breakdown of the disulphide.

The evidence, both polarimetric and colorimetric, points therefore to a reaction of the type of B with the sulphenic acid as the most likely product. This compound has so far only been isolated in a crude form but it shows the expected properties.

Glutathione, like cysteine, reacts with dithioformamidine to give a product with a smaller rotation than oxidised glutathione.

Catalysis by compounds related to thiocarbamide. A number of compounds more or less closely related to thiocarbamide have been tried as catalysts for the oxidation of cysteine by hydrogen peroxide. These are listed in Table I.

Table I.

Substance	Method of preparation	Catalytic activity
Thiocarbamide	—	147 ± 7
<i>N</i> -Allylthiocarbamide	—	125
<i>N</i> -Phenylthiocarbamide	—	121
Ergothioneine	Pirie [1933]	126
<i>dl</i> -2-Thiolhistidine	Harington and Overhoff [1933]	127
2-Thiolglyoxaline	Marckwald [1892]	122
Ethylenethiocarbamide	Ruiz and Libenson [1930]	75
<i>N</i> -Phenyl-2-thiolglyoxaline	Wohl and Marckwald [1889]	60
<i>NN</i> -Methylphenylthiocarbamide	Gebhardt [1884, 1]	57
<i>N</i> -Ethyl- <i>N'</i> -phenylthiocarbamide	Weith [1875]	54
<i>NN</i> -Diethyl- <i>N'</i> -allylthiocarbamide	Gebhardt [1884, 2]	6.3
2-Thio-4-methylhydantoin	Johnson [1912]	10
<i>N</i> -Acetylthiocarbamide	Werner [1916]	3
<i>S</i> -Methyl- <i>pseudo</i> -thiocarbamide	Taylor [1917]	0
Diacetyl (<i>pseudo</i> ?) thiocarbamide	Werner [1916]	0
2-Thio-3-acetyl-4-methylhydantoin	Johnson [1912]	0
Thiobenzamide	Gabriel and Heymann [1890]	0

Also many other compounds such as methionine, *S*-ethylcysteine, ascorbic acid and urea are inactive.

The preparation of these compounds calls for little comment; in all cases the reactions described by the authors quoted were found to proceed smoothly and the substances were readily obtained pure. The pure substances were recrystallised from water or dilute alcohol, to which a little hydrochloric acid had been added, and were filtered off on an acid-washed glass funnel. Those substances which were catalytically active were carefully tested for metal impurity. The sample of *dl*-2-thiolhistidine used was given to me by Prof. C. R. Harington; it proved to be already free from catalytically active metals.

The figure in the third column of Table I is a measure of the catalytic power of each substance. Under the usual conditions, *i.e.* 1 cc. of 1 % cysteine

hydrochloride and 1 cc. of 0.4 *N* hydrogen peroxide made up to 5 cc., and measured in a 2 dm. tube, it is given by the expression

$$\frac{\text{Rate of change of observed rotation in degrees per hour}}{\text{Weight of catalyst in mg.}} \times \text{mol. wt. of catalyst.}$$

The conditions must be adhered to, for with these substances, as with thiocarbamide, the reaction velocity is proportional to the peroxide concentration as well as to the concentration of catalyst. It is independent of the concentration of cysteine. The temperature coefficient of the catalytic reaction is 2. Using this coefficient the values of the catalysis constant have been calculated for a temperature of 18°. The very marked differences between the constants for different substances will be discussed later.

The individual differences between the substances in Table I call for some comment. In two cases hydrogen peroxide acts on the catalyst in acid solution with the formation of colloidal sulphur. This action has, in the case of phenylthiocarbamide, been studied by Hector [1889; 1892] who showed that 3:5-diimino-2:4-diphenyl-1:2:4-thiodiazoltetrahydride was formed by the elimination of sulphur and hydrogen from two molecules of phenylthiocarbamide. In the presence of cysteine this action cannot apparently take place, but one observes colloidal sulphur in the polarimeter tube within a few minutes of the completion of the catalysed oxidation. *N*-Acetylthiocarbamide is broken down with the formation of sulphur at an early stage of the oxidation; polarimeter readings therefore are only possible for a few minutes with this catalyst. It is clear however that its catalytic power is very small. Hofmann and Gabriel [1892] have described a reaction with *NN*-methylphenylthiocarbamide very similar to that which occurs with phenylthiocarbamide. This action proceeds only in hot solution and I have not noticed the formation of colloidal sulphur under the conditions of these experiments.

Ergothioneine alone among the catalysts in Table I is optically active; this property allows one to study the ergothioneine-catalysed oxidation in some detail. The curves in Fig. 3 show the effect of adding various amounts of hydrogen peroxide to the same amount of ergothioneine. There are clearly two reactions. The first is between two molecules of ergothioneine and rather more than one molecule of hydrogen peroxide; presumably a disulphide is formed; Barger and Ewins [1911] have isolated the iodide of such a compound but I have not yet isolated the substance itself. This action is complicated by the more general breakdown, accompanied by desulphuration, which occurs with an excess of peroxide. As in the case of other thiolglyoxalines, sulphuric acid is formed. These two oxidations are catalysed by copper and iron but only to a very slight extent, *e.g.* 4γ of copper (as CuSO_4) cause no appreciable shift of curve 3 in Fig. 3; with 40γ however catalysis is noticeable. When studying the ergothioneine-catalysed oxidations it is important to use the minimum of ergothioneine so as to avoid the interference of its rotation with that of the cystine; it is also important to add the peroxide to the reaction mixture last; if it is added before the cysteine the ergothioneine will be partially desulphurised with loss of catalytic activity.

Catalysis by metals and thiocarbamides simultaneously. A few experiments have been made with copper or iron and thiocarbamide or ergothioneine acting together. When copper and iron are used together the rate of oxidation is the sum of the rates due to the two catalysts when acting independently; the same is true of thiocarbamide and ergothioneine. The catalytic effect of a mixture of copper and thiocarbamide is the sum of their independent effects for slow rates

of oxidation (0.0005 mg. Cu and 0.2 mg. thiocarbamide) only; when larger amounts of the catalysts are used the one is found not to increase the rate of oxidation due to the other; a similar state of affairs obtains with iron-thio-

Fig. 3. Volume is 5 cc. in each case. Temperature 21°. 17.2 mg. of ergothioneine hydrochloride in each experiment.

1. 0.3 cc. of 0.4 N H_2O_2 .
2. 0.2 cc. „
3. 0.5 cc. „
- 0.5 cc. of 0.4 N H_2O_2 and 0.004 mg. Cu.
4. 1.0 cc. of 0.4 N H_2O_2 .
5. 2.0 cc. „

carbamide and metal-ergothioneine mixtures. In view of the readiness with which sulphur compounds form metal complexes these results are not wholly unexpected.

DISCUSSION.

The mechanism of this catalytic oxidation seems to be fairly simple. The results given in Fig. 3 together with the earlier work of Werner and others show that compounds of the type used in these experiments are readily oxidised in acid solution by hydrogen peroxide and that this oxidation does not need a metal catalyst. It is convenient to assume therefore that the first step is the formation of a disulphide and that this is immediately reduced by the cysteine. This step has only been followed in the case of the disulphide form of thiocarbamide, dithioformamide. The formation of cyclic compounds, as in the case of phenylthiocarbamide, is probably irrelevant, for thiobenzamide behaves in this way [Hofmann and Gabriel, 1892] without being a catalyst.

It is clear from Table I that the more replaceable hydrogen atoms a thiocarbamide has the more powerful will be its catalytic effect. The thioglyoxalines however, with two replaceable hydrogens, are comparable with the thiocarbamides with three, and phenylthioglyoxaline with only one labile hydrogen is as catalytically active as a thiocarbamide with two. This enhanced activity is apparently due to the double bond, for ethylenethiocarbamide has about the activity

expected. The inactivity of the thiohydantoin is presumably due to the influence of the carbonyl group on the adjacent imino group; with two carbonyl groups the inactivity is complete. The very low value for the catalytic power of *N*-acetylthiocarbamide is however both interesting and unexpected.

Although ergothioneine occurs in blood corpuscles no function has yet been assigned to it: it is possible, though unlikely, that it acts normally as a catalyst in a manner similar to that in which it has been used in this work. The activity as an oxidising agent of the disulphide form of thiocarbamide, and also presumably of the disulphide form of ergothioneine, is however worthy of some attention. I have shown in this paper that it can oxidise cysteine to one of the many possible intermediate compounds between the cystine ingested by an animal and the sulphate excreted by it, and I hope later to describe further work on this type of oxidation.

Woodward and Fry [1932] and Benedict and Gottschall [1932] have found that, although ergothioneine is not readily oxidised by iodine, ferricyanide or arsenophosphotungstic acid in acid solution, it is oxidised by these reagents when in the presence of glutathione. With equal amounts of glutathione and ergothioneine only about 60 % of the ergothioneine is oxidised and with smaller amounts of glutathione the oxidation is very much smaller. I have tried small amounts (since both catalyst and oxidant are optically active it is difficult to interpret the results if large amounts are used) of cysteine hydrochloride (2 mg.) and glutathione (4 mg.) as catalysts for the hydrogen peroxide oxidation of ergothioneine, using the same conditions as in curve 3, Fig. 3. No catalytic effect could be demonstrated. With the exception of the work just referred to no catalysed oxidation in any way similar to that described in this paper seems to have been studied. The resemblances between this type of oxidation and an enzyme action are interesting but need not, at the present stage, be emphasised. Bersin [1932] has described the effect of a large group of substances on the autoxidation of thiolacetic acid anilide in alkaline alcoholic solution. The active substances include iodoacetic acid, creatine and thianthrene, and it is hard to see any sign of a possible common action; moreover the shapes of the curves published make it probable that some of these substances catalyse different reactions. Bersin explains some of his results by the intermediate formation of thio-ethers. I have tried methionine and ethylcysteine as catalysts for the hydrogen peroxide oxidation of cysteine and have found them quite inactive.

SUMMARY.

Thiocarbamide catalyses the oxidation of cysteine to cystine by hydrogen peroxide in acid solution.

Dithioformamidine can oxidise cysteine to a sulphenic acid; this is reduced to cystine by cysteine. Dithioformamidine has no effect on cystine.

Substituted thiocarbamides and related substances, such as ergothioneine, are catalytically active and a theory for the mechanism of the action has been put forward.

Glutathione behaves throughout in a manner similar to cysteine.

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