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Studies on the Reduction of Cytochrome *c* by Thiols

A Preliminary Report

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The reduction of cytochrome *c* by reduced glutathione is catalyzed by an impurity or impurities present in oxidized glutathione and cystine. In contrast to the conclusions arrived at by V. Massey, C. H. Williams, and G. Palmer [*Biochem. Biophys. Res. Commun.* **42**, 730 (1971)], we conclude that this catalysis is not primarily caused by trisulfides. Heating of cystine or GSSG solutions results in a marked increase in the catalytic properties of the solutions. The formation of the catalytic compounds by heating is favored at alkaline pH, with an apparent p*K* value of about 9.0. The catalytic compounds appear to possess absorption spectra with maxima at 285 nm for GSSG solutions and 300 nm for cystine solutions. The compounds appear to be stable at pH values between 1.3 and 11. The identity of these compounds is presently being investigated.

INTRODUCTION

Several years ago Froede and Hunter (*1*) described a series of experiments in which they showed that the rate of reduction of cytochrome *c* by various thiols is significantly enhanced by oxidized glutathione. The catalytic action of the disulfide was ascribed to the formation of a complex of reduced glutathione (GS^-) and oxidized glutathione (GSSG), generating a ring of three sulfur atoms. The complex reduced two molecules of cytochrome *c* by sequential transfers of one electron, leading to the reformation of GSSG and the formation of a sulfonium ion (GS^+). The sulfonium ion reacts with another molecule of GS^- to form another molecule of GSSG. The hypothesis that a sulfonium ion may be formed during this reaction is of considerable interest, since evidence has been obtained in several laboratories suggesting that the formation of such a species may be a primary step in the mechanism of energy conservation (*2–5*).

The GSSG-catalyzed reduction of cytochrome *c* was further investigated by Massey *et al.* (*6*), who demonstrated that the catalysis was not caused by GSSG, but by one or more impurities present in commercial GSSG preparations. Several impurities in GSSG preparations were identified by these authors as polysulfides of glutathione (GSSSG, GSSSSG). Massey *et al* (*6*) concluded that the enhancement of the thiol-induced reduction of cytochrome *c* by GSSG was caused by the trisulfide and tetrasulfide present in the GSSG preparations. However, the data presented by these authors indicate that little or no correlation exists between the trisulfide content of commercial

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samples of GSSG and their ability to catalyze cytochrome *c* reduction (see Table I in ref. *6*), thus raising serious questions as to whether or not the trisulfide is the sole impurity in GSSG that is responsible for the catalytic effects.

We have therefore initiated some studies aimed at a closer examination of the kinetics of the catalytic effects exerted by GSSG preparations, as well as at obtaining more information regarding the physical properties of the compound(s) present in GSSG preparations that is (are) responsible for the caralytic effects. Some of the results that we have obtained thus far are described in this paper.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type III), reduced and oxidized glutathione, dithiothreitol, and β-mercaptoethanol were purchased from Sigma Corp. Cystine and cysteine were products of General Biochemical, and methyl trisulfide was obtained from Eastman–Kodak. Cysteine trisulfide was generously donated by Dr. J. C. Fletcher, Wira Company, Leeds, England. All other reagents were commercial products of the highest available grade.

The rate of cytochrome *c* reduction was generally followed spectrophotometrically at 550 nm using a Beckman Model 24 spectrophotometer. The total volume of the reaction mixture was always 3.0 ml. In all cases the reaction was initiated with the addition of the reducing agent. The rate obtained when 33.3 μ*M* cytochrome *c* is reduced with 333 μ*M* reduced glutathione in 0.1 *M* Tris–HCl buffer, pH 7.8, at 25°C was taken as the standard rate.

RESULTS

Reduction of Cytochrome c by Various Thiols

Figure 1 illustrates the rates at which cytochrome *c* is reduced by various sulfhydryl compounds. Reduction of the cytochrome with reduced glutathione is quite slow,

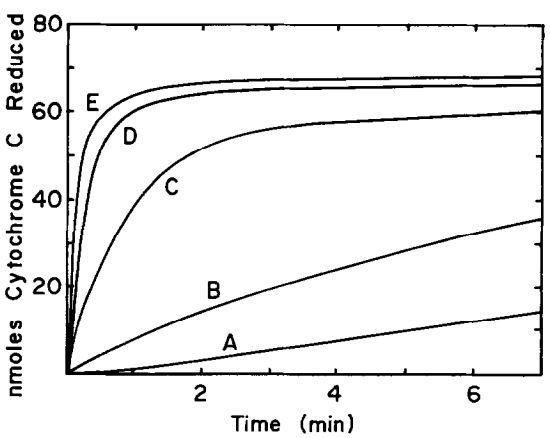

FIG. 1. Rate of reduction of cytochrome *c* by various thiols. Concentrations: 33.3 μ*M* cytochrome *c*; 333 μ*M* reduced thiol, 0.1 *M* Tris–HCl buffer, pH 7.3, 25°C. A, reduced glutathione; B, 2-mercaptoethanol; C, dithiothreitol; D, cysteine; E, ascorbate (used as a comparison).

amounting to 2.3 nmol of cytochrome reduced/min under the given conditions. Faster rates are obtained with β-mercaptoethanol (10.0 nmol/min), dithiothreitol (48 nmol/min), and cysteine (129 nmol/min). Ascorbate, which is normally used for the reduction of cytochrome *c* (*7*) yields a rate of 193 nmoles per min under our conditions.

These data suggest that the rate of reduction of the cytochrome may be affected by some of the structural properties of the thiol. These properties may include molecular size, p*K* of the thiol group, and the presence or absence of certain groups other than the thiol group. The fact that cytochrome *c* is reduced about 60 times faster by cysteine than by glutathione cannot solely be attributed to the smaller size of cysteine, since the rate of reduction by β-mercaptoethanol is considerably slower than that of cysteine. Although our data are insufficient to allow for any conclusions at the present time, more information may be obtained from a detailed kinetic analysis of the reactions. These experiments are presently in progress.

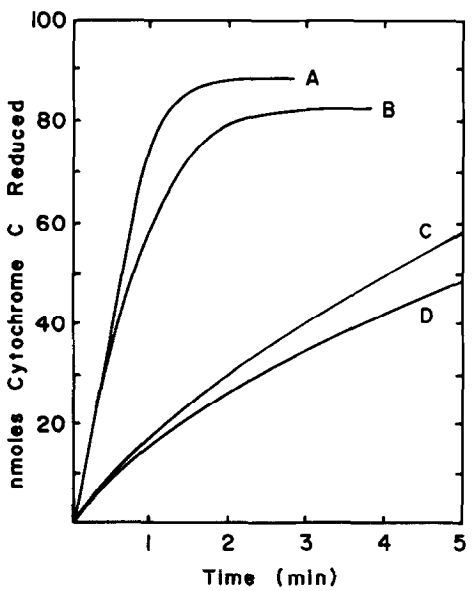

FIG. 2. Activation of the thiol-induced reduction of cytochrome *c* by disulfides. Disulfides were added to the standard reaction mixture to a final concentration of 500 μ*M*. A, cystine; B, oxidized glutathione; C, oxidized dithiothreitol; D, no disulfide added.

Enhancement in the Rate of Cytochrome Reduced by Added Disulfides

Froede and Hunter (*1*) observed that the addition of oxidized glutathione to a mixture of cytochrome *c* and reduced glutathione causes a significant increase in the rate of reduction of the cytochrome. This result was confirmed by Massey *et al* (*6*), who also showed that the addition of cystine causes an enhancement in the rate of cytochrome reduction by cysteine.

Figure 2 shows the enhancement obtained when 500 μ*M* of a disulfide is added to a reaction mixture containing cytochrome *c* and reduced glutathione. The addition of oxidized glutathione as well as of cystine caused a marked enhancement in the rate of cytochrome reduction, whereas the addition of oxidized dithiothreitol only had a small

effect. No reduction was observed with any of the oxidized compounds in the absence of reduced glutathione, which confirms the conclusion by Froede and Hunter (*1*) that the enhancement in rate is not due to the presence of free thiol in the disulfide solutions.

The oxidized dithiothreitol was prepared in our laboratory by bubbling air through a solution of dithiothreitol until no more sulfhydryl groups could be detected with *p*-hydroxymercuribenzoate. Oxidized glutathione is commercially prepared in a similar manner, except that small amounts of Fe^{2+} or Cu^{2+} ions are added as a catalyst (*8*).

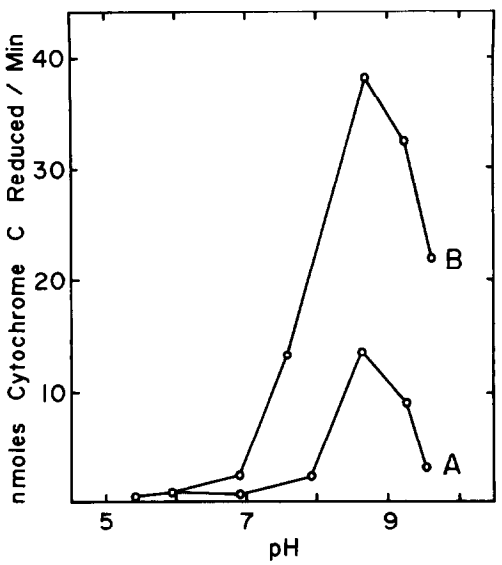

FIG. 3. Reduction rate of cytochrome *c* by GSH as a function of pH. Concentrations: 33.3 μM cytochrome *c*; 333 μM GSH, 25°C, acetate–phosphate–borate buffer, 0.1 *M* each. Curve A, no additions; curve B, in presence of 1 m*M* oxidized glutathione.

pH Dependence of the Cytochrome c Reduction and of Its Enhancement by Disulfides

The rate of reduction of cytochrome *c* by reduced glutathione is pH dependent, as shown in Fig. 3, curve A. The activity increases with increasing pH, until a maximum is attained at about pH 8.5. A further increase in pH causes a decrease in activity. A similar pH dependence is observed when the reduction is performed in the presence of oxidized glutathione (Fig. 2, curve B) or of oxidized cystine.

These data indicate that the rate of reduction of cytochrome *c* by reduced thiols is dependent on a group with a p*K* value in the alkaline pH region. This behavior could result from a group on the cytochrome molecule, but it appears more likely that the data point to a mechanism by which the cytochrome is reduced by the thiol anion ($R-S^-$) rather than by the unionized R—SH form. Such a mechanism would be consistent with other reducing systems involving thiols (*9*). Above pH 8.5 the rate of the reaction decreases, suggesting that above this pH value the reaction mechanism becomes more complex.

Of particular interest to us is the fact that the activation by the disulfides also shows a pH dependence and is more pronounced at alkaline pH values (Fig. 3, rate B/rate A).

Such a pH dependence renders it rather unlikely that the catalysis of the reaction is promoted by the disulfide group or by a trisulfide group as suggested by Massey *et al.* (*6*), since these compounds should show activation that is independent of pH. Our data suggest that the catalyst possesses an ionizable group with a p*K* in the alkaline region.

Catalysis by Trisulfides

Massey *et al.* (*6*) showed that the reduction of cytochrome *c* by thiols is strongly accelerated in the presence of cysteine trisulfide Cy–SSS–Cy), obtained from wool hydrolysates (*10*). We have repeated these experiments and obtained similar results. The rate of activation by cysteine trisulfide is about 3.5 mol of cytochrome *c* reduced/min/mol of trisulfide.

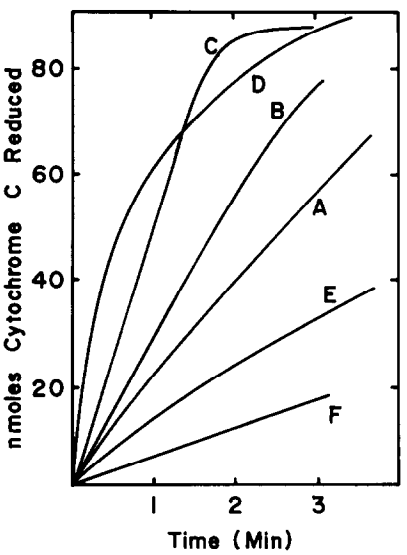

FIG. 4. Catalytic effect of trisulfides on the reduction of cytochrome *c*. The standard reaction mixture was used with the following additions: 6.7 μM methyl trisulfide (curve A), 13.4 μM methyl trisulfide (curve B), 26.8 μM methyl trisulfide (curve C), 6.7 μM cysteine trisulfide (curve D), 1.68 μM cysteine trisulfide (curve E), or no addition (curve F).

In order to ascertain whether the activation is solely a property of the polysulfide chain or if other parts of the cysteine molecule contribute to the process, we did a similar series of experiments using dimethyl trisulfide (CH_3–SSS–CH_3). The results, shown in Fig. 4, indicate that dimethyl trisulfide is also capable of catalyzing the reduction of cytochrome *c*, but to a much lesser extent than cysteine trisulfide. The rate increase obtained with dimethyl trisulfide is about 0.55 mol of cytochrome *c* reduced/min/mol of trisulfide.

It appears thus that the activation of the cytochrome *c* reduction obtained with trisulfides is much more pronounced with cysteine trisulfide than it is with the methyl derivative, suggesting that in some manner the groups attached to the trisulfide chain participate in the catalytic mechanism, either directly or indirectly.

Effect of Heating Disulfide Solutions on the Catalysis of Cytochrome c Reduction

Massey *et al.* (*6*) observed that a further increase in the catalytic effect may be obtained if a solution of oxidized glutathione is heated for several hours at 90°C in the presence of small amounts of Fe^{3+} ions. When we heated a 30 m*M* solution of oxidized glutathione in 0.1 *M* Tris–HCl buffer pH 7.8, for 5 hr at 95°C in the absence of added Fe^{3+}, we also obtained an increase in stimulation of the cytochrome reduction. The increase in the rate of reduction was 0.560 mol/min/mol of added oxidized glutathione after heating, whereas the increase obtained with unheated oxidized glutathione was 0.031 mol/min/mol of GSSG (see Fig. 5).

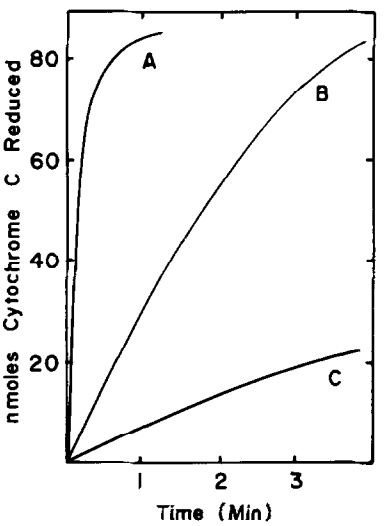

FIG. 5. Effect of heating disulfide solutions on catalytic activity. A 30 m*M* solution of oxidized glutathione was heated at 95°C for 5 hr in a sealed tube. An appropriate aliquot (final concentration, 250 μM) was dried and then added to the standard reaction mixture (curve A). Curve B was obtained in a similar manner, except that the glutathione solution was not heated. Curve C, no oxidized glutathione added.

A similar increase in activation was found when a 10 m*M* solution of cystine was heated under the same conditions, suggesting that a similar reaction may occur during heating of either disulfide. Our experiments show that the addition of Fe^{2+} ions is not a requisite for the formation of the catalyst. It should be pointed out, however, that Fe^{2+} or Cu^{2+} ions are used during the commercial preparation of oxidized glutathione (*8*), and hence the presence of trace amounts of metal ions in our oxidized glutathione cannot be ruled out.

Heating of a 10 m*M* cysteine trisulfide solution under the same conditions, however, led to a rapid destruction of the compound and complete disappearance of any activating properties.

These observations suggest that heating of the disulfide solutions leads to the formation of an additional amount of catalytic material. It appears less likely, however, that this material is a trisulfide in view of the instability of the trisulfides under the conditions used for the formation of the compound.

Time-dependence of the Formation of the Catalyst during Heating of Oxidized Glutathione Solutions

A 15-ml solution of 30 m*M* oxidized glutathione was prepared in an acetate–phosphate–borate buffer, 0.1 *M* each, pH 10.5, and heated in a water bath at 95°C. At regular time intervals 10-μl samples were removed from the solution and evaporated to dryness to remove the H_2S that formed during the reaction. The samples were then added to a reaction mixture, and their stimulating effect on the reduction of cytochrome *c* was determined. Furthermore, 10 μl of the incubation mixture was added to 3 ml of 0.1 *M* Tris–HCl buffer, pH 7.8, and the absorption spectrum of the incubation mixture was determined, using an equivalent amount of nonheated solution of GSSG as a reference.

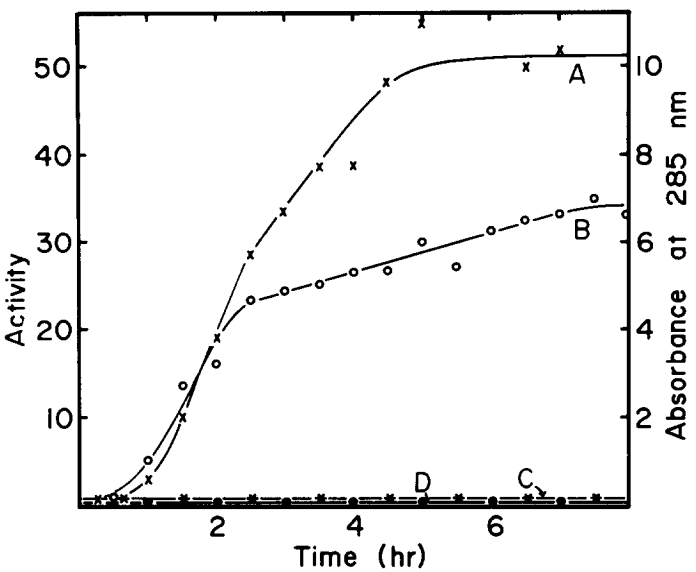

FIG. 6. Time dependence of the formation of the catalyst in heated GSSG solutions. Two 15-ml solutions of 30 m*M* oxidized gutathione in 0.1 *M* acetate–phosphate–borate buffer, one at pH 10.5 and the other at pH 5.7, were placed in an oven at 95°C in closed tubes. Samll aliquots (10 μl) were removed at the indicated times, and the absorption spectrum was determined after appropriate dilution, using an unheated sample as the reference. Another aliquot was removed, evaporated to dryness, and added to the standard reaction mixture (final concentration, 1 m*M* GSSG) for activity determinations. Curve A, activity of GSSG heated at pH 10.5; curve B, absorbance of same solution at 285 nm; Curve C, activity of GSSG heated at pH 5.7; curve D, absorbance of same solution at 285 nm.

The results of this experiment are shown in Fig. 6. The data show that a compound with an absorption maximum at 285 nm was slowly formed during the course of the incubation at 95°C, simultaneously with the appearance of the catalytic acitivty. When the experiment was repeated using the same buffer at pH 5.6, no increase in catalytic activity was obtained, and no increase in absorption at 285 nm was observed.

Figure 7 shows that a linear correlation exists between the catalytic activity of the samples at pH 10.5 and their absorption at 285 nm. This suggests that the catalytic compound may possess an absorption spectrum with a maximum at 285 nm.

A similar study with cystine as the disulfide yielded comparable results, except that the absorption maximum of the compound formed during incubation was at 300 nm instead of 285 nm. In both cases the absorption curve was very broad, extending into the visible region.

pH Dependence of the Formation of the Catalyst during Heating of Oxidized Glutathione Solutions

Two-milliliter aliquots of a 15 m*M* oxidized gutathione solution were incubated for 4 hr at 95°C in 0.1 *M* acetate–phosphate–borate buffer of various pH values. After incubation the absorption at 285 nm as well as the catalytic activity of the solutions were determined. The results, shown in Fig. 8, indicate that the formation of the catalyst as well as the formation of the material absorbing at 285 nm is promoted at alkaline pH values. Both curves show a p*K* value of about 9.0.

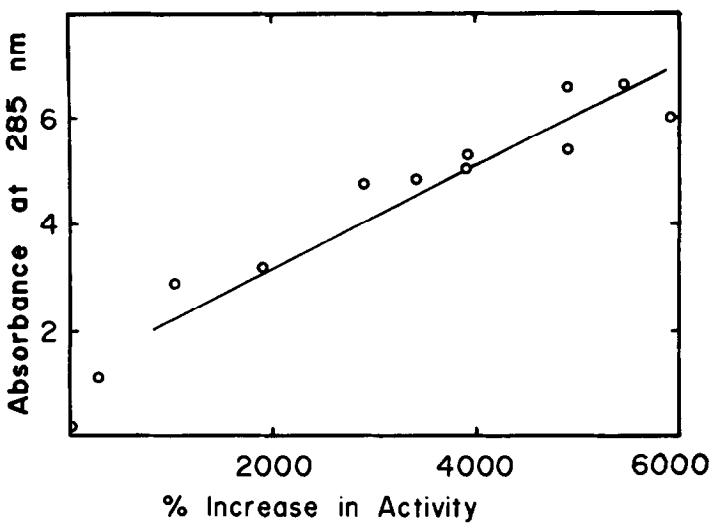

FIG. 7. Relationship between activity and absorbance of GSSG solution heated at pH 10.5. The data in curve A (Fig. 6) are plotted as a function of the data shown in curve B. The correlation coefficient for the least square fit is 0.970.

The correlation between the absorption at 285 nm and the catalytic activity is shown in Fig. 9 and is linear. These observations thus provide further support for the thesis that the material absorbing at 285 nm is the material that promotes the reduction of the cytochome.

A p*K* value of about 9.0 was also found when the experiment was done with cystine instead of oxidized glutathione.

Stability of the Catalytic Compound

In order to obtain further information about the physicochemical properties of the catalytic compound formed by heating disulfide solutions, we investigated its stability under various conditions. Experiments described earlier in this paper have already

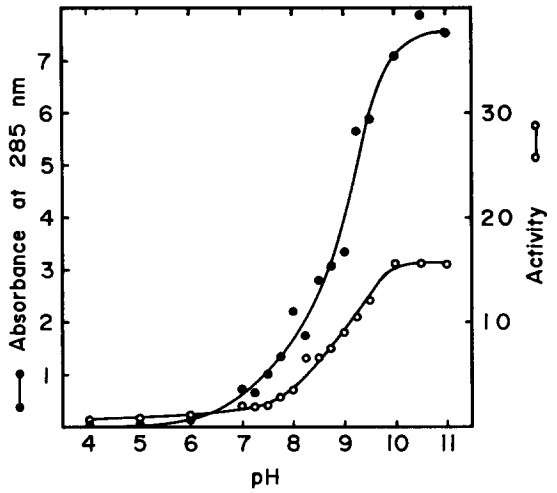

FIG. 8. pH dependence of the formation of the catalyst in heated GSSG solutions. Solutions of 15 m*M* oxidized glutathione (2 ml each) were made in 0.1 *M* acetate–phosphate–borate buffer of the indicated pH and were heated in an oven for 4 hr at 95°C. The solutions were then cooled, and nitrogen gas was bubbled through the solutions to remove H_2S. Subsequently their activity and absorbance at 285 nm were determined as described in Fig. 6.

shown that the catalyst is stable at alkaline pH values up to pH ~11, and at temperatures up to 95°C. When a solution of heated disulfide was acidified, no loss in activity occurred at pH values as low as 1.3. Furthermore, a heated solution of oxidized glutathione containing the catalytic compound may be stored at 5°C for several months without any loss in activity.

These data show that the compound is very stable under a variety of conditions, in contrast with cystine trisulfide which was shown to be quite unstable at neutral and alkaline pH values (*11*).

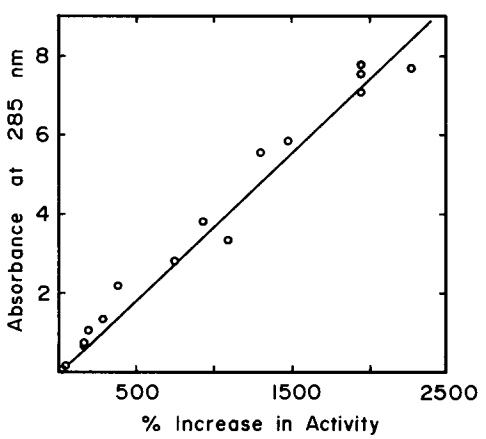

FIG. 9. Relationship between activity and absorbance of GSSG solutions heated at a various pH values. The activities of the solutions described in Fig. 8 are plotted as a function of their absorbance at 285 nm. The correlation coefficient for the least square fit is 0.984.

DISCUSSION

A study of the mechanism by which cytochrome *c* is reduced by thiols and especially a structural knowledge of the compounds that are capable of catalyzing this reduction are of considerable importance for several reasons:

1. Little is known about the various interacting species that facilitate the movement of electrons through the mitochondrial electron transport chain, or about the chemical groups that mediate the electron transport. The elucidation of the mechanism of a model system could help in elucidating the biological mechanism.

2. The mechanism by which a two-electron redox pathway changes to a one-electron pathway in the electron transport chain is still not known, and the mechanisms proposed thus far are subject to criticism. Our model could be of some value in a further understanding of this process.

3. The reduction of hemin by thiols has been associated with the transfer of high-energy phosphate from one molecule to another. Bäuerlein and Wieland have shown that the reduction of hemin by thioglycolate in the presence of ADP and P_i in a pyridine solution may lead to a conversion of as much as 15% of the ADP to ATP (*12*, *13*). Bäuerlein also showed (*14*) that the reduction of cytochrome *c* by reduced glutathione in aqueous solutions can lead to an adenylate kinase-type reaction in which a phosphate from a mole of ADP can be transferred to another mole of ADP, yielding AMP and ATP. These findings indicate that the system described in this paper may be of value in our understanding of the mechanism by which energy is conserved during electron transport.

The data presented in this paper show that a compound is present as an impurity in preparations of oxidized glutathione and cystine, and that it is capable of catalyzing the reduction of cytochrome *c* by thiols. This fact was previously established by Massey *et al.* (*6*), who concluded that the impurity was trisulfide as well as possibly some higher polysulfides. A number of inconsistencies appeared, however, when the contents of polysulfides in commercial glutathione samples were correlated with their ability to catalyze cytochrome *c* reduction (*6*). This suggested to us that another factor besides or instead of the polysulfides is responsible for the catalytic effects.

There appears to be no question about the fact that trisulfides are capable of catalyzing the reduction of cytochrome *c* by thiols, as stated by Massey *et al.* (*6*), although the nature of the trisulfide is of importance with respect to the degree of catalysis observed. Our data presented here as well as some of Massey's own data, however, strongly indicate that the catalytic effects obtained with oxidized glutathione are not solely due to trisulfide contaminants, but are at least in part due to another compound or other compounds. The possibility that these other compounds are trisulfide or polysulfide derivatives has, of course, not been ruled out.

Prolonged heating of a solution of oxidized glutathione at alkaline pH results in the formation of a catalytic compound with an absorption maximum at 285 nm, and a similar compound is formed when solutions of cystine are heated under the same conditions. The physical properties of this compound are quite different from those of the polysulfides. Fletcher and Robson (*10*) have shown that cysteine trisulfide is stable at acid pH values, but very labile at neutral and alkaline pH. The stability of the catalytic compounds is quite different from this; the compound is stable from pH 1.3 to

11, and in fact, it appears to be somewhat more stable in the alkaline pH region than it is under acidic conditions. Furthermore, the catalytic compound appears to be stable at temperatures where the trisulfide is rapidly destroyed. These data strongly suggest that the catalyst is not a trisulfide or higher polysulfide. We have also shown evidence suggesting that the catalytic compound has an absorption spectrum with a maximum in the 280- to 300-nm region. The absorption spectra of cysteine trisulfide and dimethyl trisulfide show no maximum in this region.

Several pieces of information have been obtained thus far that may be of value in the elucidation of the structure of the catalyst. First, it is formed by heating a solution of oxidized glutathione or of cystine. If an impurity is involved in the formation of the compound, this impurity is present in both preparations. It is clear, however, that the catalytic compound obtained from oxidized glutathione is not identical with the compound obtained from cystine, as shown by the difference in the absorption maxima. Although it is possible that the disulfides contain impurities that are closely related but not identical, it appears more likely that the disulfides themselves are involved in the formation of the catalyst and that the difference in the absorption maxima is due to a difference in the amino acid residues.

Second, the formation of the catalytic compound appears to involve a group with a pK around 9.0, possibly a sulfhydryl group, an α-amino group, or an unknown group, such as a metal complex. As mentioned earlier, the presence of traces of metal ions in the commercial preparations of the disulfides is not unexpected. In Massey's experiments (*6*) a considerable increase in polysulfide content was obtained as a result of heating oxidized glutathione solutions for up to 20 hr, which parallels an increase in catalytic activity. The mechanism by which the polysulfides are formed is not clear, but it appears that Fe^{3+} ions are required for the reaction. The fact that our catalytic compound is stable over a wide pH range as well as at elevated temperatures, however, makes it unlikely that this catalyst is a polysulfide, since polysulfides are not stable under these conditions.

It is as yet not known whether or not the catalytic compound that is formed by heating the disulfide solutions is identical to the catalytic material that is already present in the disulfide preparations. It is of interest, however, that cystine is commercially prepared by acid hydrolysis of feathers at elevated temperatures.

Further studies are presently being done in our laboratory which involve a detailed kinetic study on the mechanism of cytochrome *c* reduction and the mechanism of catalysis by trisulfides. In addition we intend to purify the catalyst formed by heating solutions of disulfides and identify its chemical nature.

ACKNOWLEDGMENT

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