THE PRESENCE OF S°-CONTAINING IMPURITIES IN COMMERCIAL SAMPLES OF OXIDIZED GLUTATHIONE AND THEIR CATALYTIC EFFECT ON THE REDUCTION OF CYTOCHROME c*

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SUMMARY: The stimulatory effect of GSSG on the reduction of cytochrome c by GSH has been shown to be due to S°-containing impurities; GSSG itself has no stimulatory effect. Methods are described for the production of such impurities in high yield. Similar effects are shown by cystine trisulfide. The stimulation of the cytochrome c reduction rate has been found to be catalytic; one molecule of cystine trisulfide will induce the rapid reduction of at least 25 molecules of cytochrome c.

Recently Froede and Hunter[1] described the results of a study which showed that oxidized glutathione (GSSG) substantially enhanced the rate of reduction of cytochrome c by reduced glutathione (GSH). The phenomenon was ascribed to a molecular complex of GSSG and GS^- which could react by sequential one electron transfers generating $GS^\cdot$ and GS^+ species. In a later series of papers Painter and Hunter[2-5] claimed that this system could lead to oxidative phosphorylation. In view of the possible significance of these findings we have investigated the GSSG-enhanced reduction of cytochrome c. Evidence is presented that this phenomenon is not due to GSSG, but to impurities found in many commercial samples of GSSG. Our studies indicate that the chief impurity is probably glutathione trisulfide (GSSSG) and that the active species involved in rapid reduction of cytochrome c is probably the persulfide, GSS^-.

MATERIALS AND METHODS

Cytochrome c reduction rate was measured as described in Figure 1, i.e. at a GSH concentration of 3.33×10^{-4} M. The initial rate of cytochrome c reduction was found to be directly proportional to the product of the concentration of both GSH and stimulating compound, i.e. doubling the

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concentration of both resulted in a four fold increase in reduction rate. In all instances where the reduction rate is given in absolute terms it was measured at a GSH concentration of 3.33×10^{-4} M.

S°-content was measured essentially according to Fletcher and Robson[6]. The sample to be analyzed was mixed in a total volume of 2.25 ml with 0.2 ml 1 M NH_4OH and 0.25 ml 0.1 M KCN. After reaction at room temperature for 45 min 0.25 ml Sorbo's reagent[7] was added; the $Fe(SCN)_3$ formed was measured by its absorbance at 460 mμ. Under these conditions 1 μmole of NH_4SCN gave an A_{460} value of 1.32.

GSSG was assayed by the disappearance of TPNH (measured by absorbancy at 340 mμ) on mixing with yeast glutathione reductase[8]. GSH was estimated by reaction with $K_3Fe(CN)_6$ and measured by the decrease in absorbancy at 420 mμ when mixed with excess (6.7×10^{-4} M) $K_3Fe(CN)_6$ in 0.1 M phosphate, pH 7.6, (10^{-5} M $CuSO_4$ was added to catalyze the reaction). Pyruvate was estimated with beef heart lactate dehydrogenase by the method of Shifrin et al.[9].

Amino acid analyses were carried out using a Beckman Model B amino acid analyzer equipped with long path-length cells according to the accelerated methods of Spakman[10]. The resin used was Aminex-SB Blend Q-60.

RESULTS AND DISCUSSION

Figure 1 shows the effect of various commercial samples of GSSG, all at the same concentration, on the rate of reduction of cytochrome c by GS^-. It can be seen that the samples from Schwarz and Boehringer had very little effect, while four different batches from Sigma had very pronounced, though different, effects. Samples from Calbiochem, I.C.N. and P.L. Laboratories had intermediate effects. These results suggest that the rate stimulation is due to some impurity in the GSSG samples. The following possible contaminants were tested and found to have no significant effect; pyrrolidone carboxylic acid, acetone, D- and L-methionine, methionine sulfoxide.

While it did not prove possible to obtain information about the manufacturing process, it seemed feasible to us that trace metal catalysis of air oxidation of GSH was a likely process to be employed commercially in the preparation of GSSG. Accordingly we oxidized GSH with O_2 at slightly alkaline pH, employing Fe^{+++} and Cu^{++} in catalytic amounts. Very small positive effects were found at times when the GSH had just been completely oxidized. However it was found that stimulatory activity increased on standing for extended periods. Similar effects were found by incubating commercial GSSG with catalytic amounts of Fe^{+++} or Cu^{++}. The production of the compound(s) with stimulatory effect was markedly affected by temperature. Several hours incubation

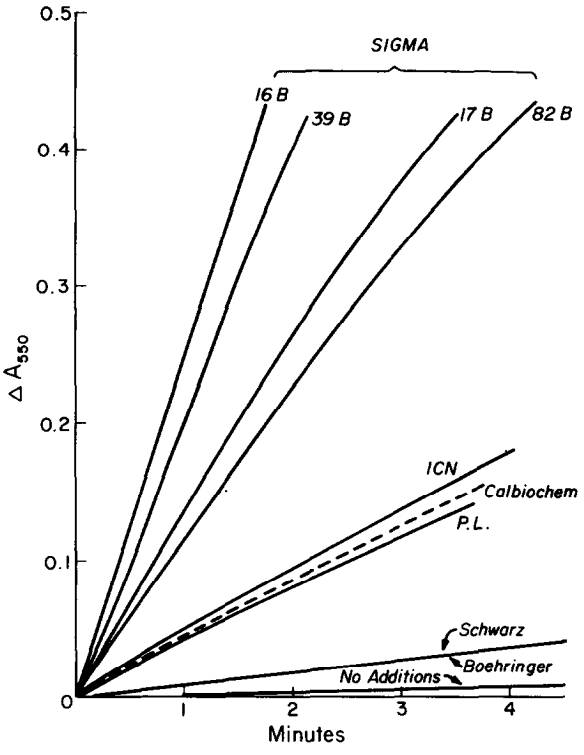

Fig. 1. Effect of different commercial samples of GSSG on the reduction of cytochrome c by GSH. Conditions: $3{\cdot}33 \times 10^{-5}$ M cytochrome c (Sigma, grade III), $3{\cdot}33 \times 10^{-4}$ M GSH, 0.1 M phosphate buffer pH 7.6, 3×10^{-4} M EDTA, 25°, final volume 3 ml. Cytochrome c reduction was recorded spectrophotometrically at 550 mμ, and was initiated by addition of GSH followed by GSSG, at a concentration of 10^{-3} M.

of GSSG at 90°, in the presence of catalytic amounts of Fe^{+++} gave rise to stimulatory activity an order of magnitude greater than that found in the most "active" GSSG samples of Figure 1. It was also found, over a wide variety of conditions, that the increase in cytochrome c stimulatory activity was correlated with the formation of compounds having sulfur in the zero valent state, as judged by the ability to form thiocyanate on incubation with cyanide under the assay conditions[6]. Compounds known to yield thiocyanate under these conditions are polysulfides, persulfides and elemental sulfur.

Figure 2 shows the results of a typical alkaline heating experiment. Also shown in this graph are the relative amounts of four new ninhydrin positive compounds formed by heating at 90°, determined with an automatic amino acid analyser. The first of these compounds elutes in a broad peak at the position of glutamic acid to proline and the remaining ones in a well-resolved cluster beginning at the buffer change. Two of these compounds (the first and

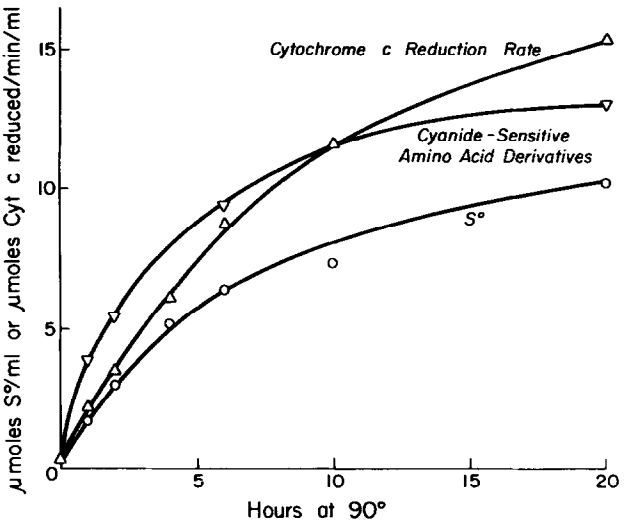

Fig. 2. Development of cytochrome c stimulatory activity by incubation of GSSG at 90°. Sigma GSSG, disodium salt (lot 16B), was dissolved in H_2O to a concentration of 0.05 M and 6.2 x 10^{-5} M $FeCl_3$ added. This solution was incubated with stirring in a stoppered flask at 90°, and samples withdrawn at intervals for analysis. The content of S°-containing material is expressed as μmoles/ml, the cytochrome c reduction rate (measured on 3-10 μl aliquots) as μmoles cytochrome c reduced per min per ml. The relative content of cyanide-sensitive compounds is given as amino acid analyser peak heights. The pH of the reaction mixture (measured at 20°) was 9.1 before heating and 6.9 after 20 hr heating.

one of the cluster) are also present in small but significant amounts in samples of Sigma GSSG and are just discernible in Boehringer GSSG. In all cases incubation at room temperature with 0.01 M KCN for several hours results in disappearance of these compounds (as judged by the amino acid analyser) and complete loss of stimulatory activity of cytochrome c reduction.

It was found also that considerable cytochrome c stimulating activity could be obtained by refluxing GSSG under acid conditions in the absence of added metal ions, for example at pH 4 in the presence of EDTA. Under these conditions pyruvate was also produced in considerable yield. Production of the latter appears to be optimal at a pH of approximately 1.2. Figure 3 shows the results under these conditions of analyses of GSSG, compounds containing sulfur in the zero valent state (S°), cytochrome c stimulating activity, pyruvate production and concentrations of five ninhydrin positive compounds as measured with the amino acid analyser, and which disappear on incubation with cyanide. These compounds eluted at the following positions: 10 ml after glutamic acid, 10 ml before glycine, 25 ml after the buffer change, 40 ml after the buffer change, and 73 ml after the buffer change. Under conditions of reflux at pH 1.2 the glutathione was 80% hydrolyzed in 14 hrs as judged by

the appearance of glutamic acid, glycine and cystine. The glutamic acid and glycine appeared at the same rate.

While there is a clear correlation between the ability to stimulate the reduction of cytochrome c by GS^- and the content of S°-compounds, the results of Figures 2 and 3 indicate that there are a number of S° compounds capable of causing this stimulation. Under the conditions of Figure 2 the rate of cytochrome c reduction is 1.12 - 1.6 moles cytochrome c reduced per minute per mole S°, with no systematic variation over the 20 hour heating period. However, under the conditions of Figure 3 the rate varies from 0.44 - 2.4 moles cytochrome c reduced per minute per mole S°, increasing steadily during the course of the incubation.

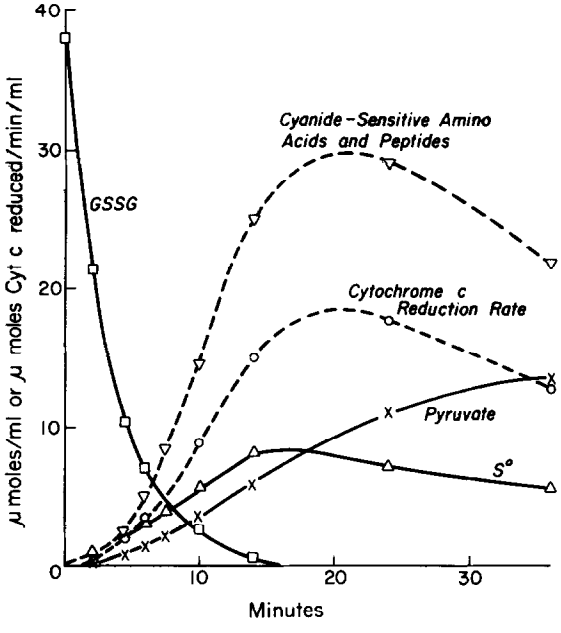

Fig. 3. Effect of refluxing 38 mM Boehringer GSSG with 0.3 N HCl. Samples were withdrawn at intervals and analysed. The concentrations of GSSG, pyruvate and S°-compounds are expressed as μmoles/ml and cytochrome c stimulatory activity as μmoles cytochrome c reduced per min per ml. The relative content of cyanide-sensitive ninhydrin-positive compounds is given as integrated amino acid analyser peak areas.

That compounds containing S° are indeed capable of catalysing the reduction of cytochrome c by GS^- was shown with cystine trisulfide (bis-(2-amino-2-carboxyethyl)trisulfide; the generous gift of Dr. J. C. Fletcher, obtained from wool[6]), and with elemental S. Figure 4 shows the effect of cystine trisulfide at several concentrations. The rate is 3.5 moles cytochrome c reduced per minute per mole of cystine trisulfide. From comparison of the progress

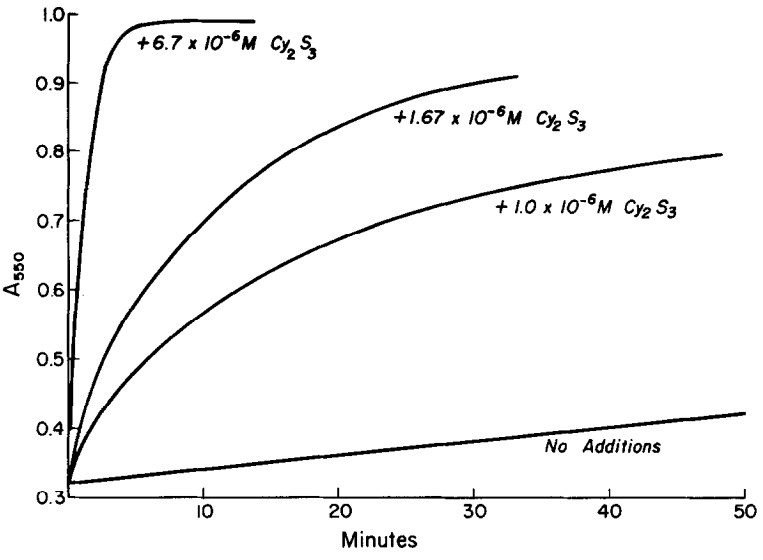

Fig. 4. Catalytic stimulation of GSH-reduction of cytochrome c by cystine trisulfide. The conditions were the same as those of Fig. 1, except that cystine trisulfide was used instead of GSSG.

curves in its presence and absence it may be estimated that each molecule of cystine trisulfide can induce the reduction of at least 20-25 molecules of cytochrome c by GS^-, i.e., the stimulatory effect is a catalytic one. Similar results are obtained with elemental S, which gives a rate of 1.15 moles cytochrome c reduced per minute per mole S°. However we consider that this is an unlikely contaminant in commercial sources of GSSG. Elemental S may be extracted readily from aqueous solution with benzene; such extraction has no effect on the stimulation of cytochrome c reduction given by commercial samples of GSSG.

An important clue to the mechanism of the rate stimulation came from the observation that when GS^- and Sigma GSSG are incubated together before the addition of cytochrome c, the stimulatory effect is lost rapidly. This process is accompanied by the liberation of H_2S, readily detected by its smell. The time course of the reaction is shown in Figure 5. When the reaction is carried out under anaerobic conditions there is less than 1% loss of GSH from the mixture in the time taken for almost complete loss of the stimulatory activity. This finding indicates that the impurity responsible for induction of cytochrome c reduction by GS^- is only present in trace amounts. This conclusion is borne out by analyses of S° content of various commercial samples of GSSG, listed in Table I. It is seen that the contaminant is present to the extent of less than 1%, even in the most "active" Sigma samples. It is also clear from this table that the rate stimulation per mole of S°

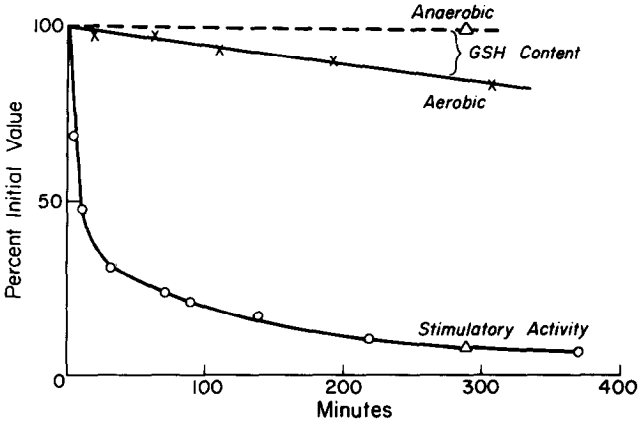

Fig. 5. Loss of cytochrome c - stimulatory activity of Sigma GSSG by preincubation with GSH. Sigma GSSG (lot 16B) and Sigma GSH were neutralized and made up in 0.1 M phosphate pH 7.6 containing 3 x 10^{-4} M EDTA. They were mixed and incubated in ice, each at a concentration of 0.05 M, and aliquots analysed at intervals for cytochrome c stimulatory activity and for GSH. The graph also shows the results obtained when the incubation was performed anaerobically(Δ).

TABLE I

S°-Contamination of GSSG and Cytochrome c Stimulatory Activity

The ability to stimulate the rate of reduction of cytochrome c by GS^- was measured under the standard conditions described in the Materials and Methods Section, as was the S°-content.

Source of GSSG	S°-content moles/100 moles GSSG	Cytochrome c reduction rate moles/min/mole S°
Boehringer	0.032	1.23
Schwarz	0.045	1.05
Pfalz & Bauer	0.029	1.02
P.L.	0.029	6.5
ICN	0.20	1.08
Calbiochem	0.028	7.2
Sigma 23B	0.154	1.96
Sigma 82B	0.35	1.55
Sigma 17B	0.63	1.0
Sigma 16B	0.73	0.90
Sigma 39B	0.79	1.45

varies somewhat from sample to sample; presumably differences in manufacturing conditions can lead to variations in the composition of the S°- compounds. In keeping with the hypothesis that the stimulatory effect of GSSG is due to contaminating S° species it is found that on preincubation of GSSG with KCN this stimulatory activity is lost rapidly (e.g. the stimulatory activity of the Sigma samples is lost completely on incubation with 0.05 M KCN at 25° for 10

min; the half life for the reaction being approximately 2 min.).

While it is clear that not one single S°-containing compound is responsible for the observed effects, the phenomena described above can be conveniently explained in terms of reactions of the most likely impurity, glutathione trisulfide (GSSSG). Thus the inactivation by preincubation with GS^- and the liberation of H_2S, can be accounted for by reactions 1 and 2, and the cyanide inactivation by reaction 3.

1) $GSSSG + GS^- \rightleftharpoons GSS^- + GSSG$

2) $GSS^- + GS^- \rightarrow GSSG + S^=$

3) $GSSG + CN^- \rightarrow GSSG + SCN^-$

The catalytic effect on reduction of cytochrome c by GS^- can be explained by reactions 1 and 4-6.

4) $GSS^- + \text{cyt } c^{+++} \rightarrow GSS^{\cdot} + \text{cyt } c^{++}$

5) $2GSS^{\cdot} \rightleftharpoons GSSSSG$

6) $GSSSSG + GS^- \rightleftharpoons GSSSG + GSS^-$

Net; reaction 1, 4-6: $2GS^- + 2 \text{ cyt } c^{+++} \rightarrow GSSG + 2 \text{ cyt } c^{++}$

These steps provide a chain mechanism for the oxidation of GS^- at the expense of reduction of cytochrome c; the catalytic efficiency presumably being determined by terminating reactions such as reaction 2. It should be emphasized that the impurities present in GSSG, or the S°-containing compounds produced from GSSG, do not by themselves reduce cytochrome c. Reaction of these compounds with GS^- (or other thiols - cf. ref. 1) is required to generate the reactive species. The overall stoichiometry of cytochrome c reduction is also determined by the GS^-; experimentally we find that 1 mole of cytochrome c is reduced per mole GSH added.

While we have not identified unequivocally the products of the alkaline-Fe^{+++} and acid modifications of GSSG, the production of such large concentrations of pyruvate after acid hydrolysis suggests strongly that dehydroalanine is an intermediate[11]. This could be formed by a β-elimination mechanism, which is known to be base-catalysed.

$GSSG + OH^- \rightarrow GSS^- +$ γ-glutamyldehydroalanylglycine

The GSS^- species, by oxidation would yield GSSSSG, or by reaction with GSSG, would yield GSSSG and GS^-. Alternatively, under acid conditions, the attacking species might be the sulfonium ion, GS^+ ($GSSG \rightleftharpoons GS^- + GS^+$). Attack by this species on GSSG would yield directly GSSSG and the protonated form of γ-glutamyldehydroalanylglycine. By analogy with known dehydroalanyl peptides[11] the γ-glutamyldehydroalanylglycine would be expected to hydrolyse readily at pH 1, yielding glutamic acid, glycine and pyruvate. All three

compounds have been measured in good yield (cf. Figure 3) by amino acid analysis and enzymic assay.

The pronounced reactivity of S°-compounds raises interesting possibilities concerning the role of such groups in biological processes. A protein-bound persulfide has been shown recently to be an essential constituent of xanthine oxidase[12]. Coupled with the observations of Hunter and his colleagues[1-5], the present work would suggest the possibility that a persulfide or related structure may be involved in the process of oxidative phosphorylation.

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