Studies on the Mechanism of Swelling, Lysis, and Distintegration of Isolated Liver Mitochondria Exposed to Mixtures of Oxidized and Reduced Glutathione*

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Lehninger and Schneider (1) studied the swelling of isolated liver mitochondria induced by reduced glutathione (GSH). One outstanding difference from thyroxine-induced swelling was that GSH-induced swelling was not reversible. These studies have been extended by Lehninger and Gotterer (2, 3) with the discovery that GSH-induced swelling is reversed by adenosine triphosphate + Mg++ + bovine serum albumin if a specific protein, C factor, is added. Recently Neubert, Wojtczak, and Lehninger (4) identified this protein as being a mixture of GSH peroxidase and catalase. Neubert and Lehninger (5–7) have also reported that oxidized glutathione (GSSG) has a marked stimulating effect on the rate of swelling with GSH. In fact, swelling with GSH may not occur at all if there is no GSSG present.

In our own work with GSH (8), the extremely low D250 readings reached led us to suspect that GSH-induced swelling was more closely related to ascorbate-induced lysis (9, 10) than to phosphate + substrate-induced swelling. Resolution of the question concerning the degree to which GSH-induced swelling is related to (a) substrate and electron transport-dependent swelling, (b) specific changes induced through membrane thiol and disulfide groups, or (c) lipid peroxide formation leading to swelling, lysis, and disintegration is very important. The mechanism of action of glutathione on mitochondrial membranes is of special interest because of evidence suggesting that disulfide interchange reactions in membrane structures may control permeability (11, 12).

This paper reports investigations on GSH- and GSSG + GSH-induced swelling. In earlier work, GSH-induced swelling usually reduced the D250 of dilute suspensions to values considerably below those reached with phosphate + β-hydroxybutyrate, but such was not always the case when as high as 20 mM GSH was used. Our initial working hypothesis was that a substrate role for GSH might be manifest at very high concentrations because the lytic effect of lower concentrations was suppressed. Such an inhibition of lysis is seen with high concentrations of ascorbate (9, 10). When Neubert and Lehninger (5–7) reported that GSSG greatly accelerated mitochondrial swelling induced by GSH, it was apparent that the amount of GSSG in GSH solutions might also determine the type of effect which predominates. We have examined carefully the possible relation of GSH- and GSSG + GSH-induced swelling to electron transport, coupled phosphorylation, and lipid peroxide formation (13). There is an excellent correlation between lipid peroxide formation and swelling and lysis.

**EXPERIMENTAL PROCEDURE**

Rat liver mitochondria were prepared as previously described (8, 10, 14), except that 0.1 mM EDTA was present in the homogenizing medium. When lipid peroxides were to be determined, the second washing and the final suspension were made with 0.175 M KCl + 0.025 M Tris pH 7.4 (10, 14). Fresh mitochondria were not needed for each experiment, since the results with GSSG + GSH are the same in freshly prepared and 24-hour-old preparations. The basic medium was either 0.33 M sucrose or 0.175 M KCl with 0.025 M Tris buffer, pH 7.4. Incubations were aerobic at 22–25°C.

Swelling and lysis were measured as the decrease in turbidity of dilute suspensions at 520 μm (D250) in a Bausch and Lomb Spectronic 20 spectrophotometer. Mitochondria were added in amounts giving an initial D250 of about 0.500 (protein, 100 to 150 μg per ml). Oxygen consumption was measured with a Beckman Instrument Company micro-Clark oxygen electrode. Lipid peroxide was measured by the thiobarbituric acid color reaction as previously described (10, 14). The absorbance readings at 532 μm have been graphed directly. Under the experimental conditions used (14), a reading of 0.090 for absorbance at 532 μm is equivalent to 1.0 mmole of malonaldehyde per ml of mitochondrial suspension.

All chemicals were of the highest purity available, obtained from the sources previously indicated (10). 5,5'-Dithiobis-(2-nitrobenzoic acid) was a gift from Dr. J. Newton. The GSSG and GSH were from the California Corporation for Biochemical Research or from Sigma Chemical Company. We detected no differences between materials from the two sources. Throughout this paper the expression GSSG + GSH will refer to 5 mM GSSG + 1 mM GSH except where other concentrations are specified.
RESULTS

Characteristics of Swelling (D_{520} Turbidity Changes) with Glutathione

Different Concentrations of GSH

In a detailed investigation over a wide range of GSH concentrations, we found that 0.1 to 2 mM GSH often produced no swelling or very slight swelling. At 5 to 10 mM, swelling and lysis were sometimes observed after a lag period which varied from 15 to 90 minutes, but in other preparations there was only slight swelling (Fig. 1). These observations did not completely agree with our earlier results, which showed a more rapid lysis (8, 9). The discrepancy probably can be explained by the presence of more GSSG in our earlier samples of GSH (6, 7). GSH alone may show shorter lag periods in sucrose-Tris than in KCl-Tris medium, but the results are similar. After the greatly different lag periods, there is a rapid D_{520} fall which leads to essentially the same very low readings with different amounts of GSH. Thus in many circumstances readings taken at any fixed time interval may be misleading.

GSSG

All experiments have confirmed our earlier findings (9) and those of Neubert and Lehninger (7). Up to 1 mM GSSG produces no swelling; 5 to 10 mM GSSG may produce a slow swelling (Fig. 1).

GSSG + GSH

Neubert and Lehninger (7) demonstrated that GSSG can greatly increase the rate of GSH-induced swelling. We reexamined this question in detail because in our experiments there was always some lag period and it appeared that rates of swelling and lag periods might be influenced separately. GSSG shortens the lag period according to the concentration added. The optimal ratio may be 5 to 10 GSSG:1 GSH. At the 10 mM concentration of glutathione frequently used by Neubert and Lehninger (7), the lag period is only 2 to 3 minutes. At lower glutathione concentrations, the lag may be 5, 10, or even 20 minutes (Fig. 2), so that the true characteristics of the phenomenon are seen only by following the whole curve.

Effect of GSSG on Other Types of Swelling

The accelerating effect of 1 mM GSSG on swelling and lysis induced by low concentrations of ascorbate, dihydroxyfumarate, dihydroxymalate, and dehydroascorbate (10) is not due to formation of small amounts of GSH, since added GSSG + GSH in similar concentrations produces only slow swelling. At 20 mM, ascorbate does not produce lysis for long periods of time, yet, the addition of GSSG does not hasten the process in this case.

GSSG has little effect on electron transport-supported swelling. GSSG at 1 mM does not replace phosphate, potentiate low levels of phosphate, shorten lag periods, or inhibit the swelling induced by phosphate + β-hydroxybutyrate. At 5 mM, GSSG slightly inhibits such swelling. Everything indicates that the reactions influenced by GSSG and responsible for ascorbate- or GSSG + GSH-induced swelling and lysis are probably not involved in phosphate-induced swelling (15).

Oxygen Consumption during Glutathione-induced Swelling

Oxygen is required for GSH-induced swelling. Since this requirement might be solely for conversion of GSH to GSSG, it was important to establish whether oxygen is also required when GSSG + GSH is used, especially in view of the high concentrations of GSSG found to be optimal by Neubert and Lehninger (6, 7). Oxygen is required, and Fig. 3 illustrates the fact that the rate of oxygen uptake increases just before the appearance of rapid swelling leading to lysis. A detailed study of the oxygen consumption is presented in Fig. 4. In medium alone, neither 1 mM GSH nor 5 mM GSSG causes much oxygen consumption. When the two are present together, the oxidation of GSH is increased several fold. This increased rate is inhibited very little by 0.1 mM EDTA. EDTA at 1 mM produces greater inhibition, but the rate is still twice as fast as with GSH alone. With mitochondria, GSH alone is not rapidly oxidized. When GSSG and GSH are both added to mitochondria, there is a higher
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Carefully prepared solutions of 1 to 20 mM GSH produce little swelling of mitochondria for long periods of time. The amount of TBA color material is barely more than in the control (Fig. 1). In cases when swelling does start after a long lag period, appearance of TBA color is associated with the phenomenon.

**GSSG**

At 1 to 5 mM, GSSG usually induces very little swelling and correspondingly little lipid peroxide formation (Fig. 1). Occasionally slow swelling occurs, and this is accompanied by slow formation of lipid peroxide.

**GSSG + GSH**

The more rapid swelling observed with various combinations of GSSG + GSH is always closely associated with an earlier appearance and more rapid increase of lipid peroxide (Figs. 1 and 2). In general, the rate of formation and the amount of lipid peroxide increase as the concentration of GSSG + GSH is increased. When the lag period for the D290 change is only 4 to 5 minutes, formation of lipid peroxide appears to start from zero time. When the lag periods before swelling are longer, the lipid peroxide does not appear until later, usually a little before swelling starts. In some experiments, the appearance of TBA color material lagged behind the D290 changes when high GSH:GSSG ratios were used. This merits further investigation, but it may result from destruction of the first small amounts of lipid peroxide in the presence of high GSH concentrations.

**Effect of Inhibitors on Glutathione-induced Swelling**

In studying the possible similarity between GSSG + GSH-induced swelling and ascorbate-induced lysis, we have examined the effects of many inhibitors on swelling and lipid peroxidation induced by GSH and GSSG + GSH.

**Metal Ions and Metal-complexing Agents**

**Metal Ions**—At 0.1 mM, Mg++ only slightly slows GSH- or GSSG + GSH-induced swelling, but it potentiates the effects of ATP. With 5 mM Mg++, marked inhibition occurs. Be-
tween 10 and 100 µM, Mn++ blocks all changes with 5 mM GSH or 5 mM GSSG + 1 mM GSH.

**EDTA**—EDTA at 10 µM prevents GSSG + GSH-induced swelling-lys as well as that with GSH, so that it does not function by inhibiting conversion of GSH to GSSG. Since EDTA also blocks swelling induced by phosphate, thyroxine, and nearly every other swelling-inducing agent (8), its action tells us nothing specific about glutathione-induced changes.

**8-Hydroxyquinoline**—With GSSG + GSH, 20 µM 8-hydroxyquinoline produces a 25-minute delay before swelling-lys, and 100 µM inhibits completely (Fig. 5). With 0.1 to 3 mM 8-hydroxyquinoline, GSSG + GSH-induced swelling-lys is blocked, but a slow swelling corresponding to that with 8-hydroxyquinoline alone is seen. Unlike EDTA, 8-hydroxyquinoline does not inhibit phosphate + substrate-induced swelling.

**Inorganic Triphosphate and Pyrophosphate**—These substances can prevent GSSG + GSH-induced swelling in concentrations as low as 10 µM (Fig. 5).

**Citrate**—Citrate at 10 µM can delay and slow GSSG + GSH-induced swelling, and 100 µM may block it completely (Figs. 5 and 9). Lipid peroxidation is also prevented. Such concentrations do not affect phosphate-induced swelling, although 5 to 10 mM can inhibit. The very low concentrations of citrate may act by direct antioxidant action rather than by formation of metal complexes.

**Electron Transport Inhibitors**

The effects of these substances on GSSG + GSH-induced lipid peroxidation and swelling are very similar to those described for ascorbate (10).

**Cyanide**—A careful study with a range of NaCN concentrations revealed that less CN− is required to block phosphate-induced swelling (dependent on electron transport) than to block GSSG + GSH-induced swelling (Fig. 6). When GSSG + GSH is used, 60 to 100 µM CN− increases the lag period, but 1 mM is needed to prevent swelling for 50 to 70 minutes. Similar effects are seen on lipid peroxidation.

**Antimycin A**—At 0.1 µM under our conditions, antimycin A blocks phosphate-induced swelling completely. It considerably increases the lag period for lipid peroxidation and swelling with GSH and GSSG + GSH, but does not prevent swelling (Fig. 7). At 1 to 4 µM, antimycin A blocks the action of GSSG + GSH for much longer periods of time.

**Phosphorylation Inhibitors**

Even high concentrations of uncoupling agents, such as 1 mM 2,4-dinitrophenol, 10 mM azide, and 5 µg of gramicidin per ml,
do not inhibit the action of GSSG + GSH (Fig. 8). Pre-exposure to 5 μM carbonyl cyanide phenylhydrazone uncouplers is also without effect. The energy transfer inhibitors oligomycin and octylguanidine have only slight delaying action.

**ATP and Substrates**

**ATP**—At 5 mM, ATP produces marked inhibitory effects, and ATP + Mg++ + BSA blocks completely with GSSG + GSH. Since both ATP and BSA have metal-binding capacities and very significant functions in contraction of mitochondria (3, 11), it is difficult to say which is responsible for preventing the action of GSSG + GSH. Lehninger and Schneider (1) reported that ATP inhibits GSH-induced swelling in fresh mitochondria, but Neubert, Rose, and Lehninger observed a marked acceleration (18). Emmelot (19) presented evidence that a delaying action of succinate on GSH-induced swelling may be mediated through ATP generation.

**α-Ketoglutarate**—This substrate always produces delays or inhibition of GSH- or GSSG + GSH-induced swelling and lysis, a sharp contrast to its accelerating effect with ascorbate (9, 10).

**Succinate**—With GSSG + GSH, we find that succinate produces some delay, just as Emmelot (19) reported for GSH.

**Oxaloacetate and Pyruvate**—These substances inhibit ascorbate-induced lysis (10), with 1 mM oxaloacetate sometimes producing complete inhibition. A similar action is also observed with GSSG + GSH. Lower concentrations are effective against GSH alone. Antioxidant and metal-complexing effects must be given serious consideration, but reaction with some group in the membrane is not ruled out.

**Antioxidants**

These substances all prevent GSSG + GSH-induced swelling in the same concentrations that inhibit lipid peroxidation. The effects of some are illustrated in Fig. 9. α-Tocopherol can prevent the action of GSSG | GSH, but the commercial food antioxidants butylated hydroxytoluene, butylated hydroxyanisole, and n-propyl gallate are effective in much lower concentrations. Phenothiazine derivatives such as promethazine and chlorpromazine prevent the effect of GSSG + GSH at 25 to 50 μM, presumably via their antioxidant action. High concentrations of ascorbate also counteract GSH and GSSG + GSH (10). At minimal concentrations for full antioxidant effects, all of these substances have essentially no effect on phosphate + P-hydroxybutyrate-induced swelling. At higher concentrations, some of them, 10 μM butylated hydroxytoluene for example, do have some effect on electron transport-supported swelling. This is probably due to uncoupling action of such phenols and phenothiazines.

**Miscellaneous Inhibitors**

Crystalline catalase at 50 μg per ml has relatively little effect (Fig. 10). At higher concentrations, such as 140 μg per ml, delay or slowing is sometimes observed. At very high concentrations (800 μg per ml), very long lag periods are produced. Such effects may be due to addition of protein rather than enzyme activity. BSA at 100 μg per ml slows the action of GSSG | GSH in KCl medium. At 2 mg per ml, BSA can virtually stop swelling-lysis in KCl medium and markedly slow it in sucrose medium.

**Action of Disulfides Other than Oxidized Glutathione**

Neubert and Lehninger (7) reported that several but not all disulfides have an action qualitatively like GSSG when combined
with GSH. We have tested the aromatic disulfide, dithiobis-(2-nitrobenzoic acid), used by Newton (20) in studies on disulfide cleavage of chromatophores of *Rhodospirillum rubrum*. Dithiobis-(2-nitrobenzoic acid) in concentrations ranging from 30 μM to 1 mM does cause some shortening of the lag period and acceleration of GSH-induced swelling-lysis. However, the effect was variable and quite small with some mitochondrial preparations.

**Effect of Glutathione on Pure Lipids**

With a suspension of pure methyl arachidonate, neither GSSG nor GSH alone leads to much peroxidation. When GSSG and GSH are both present in the same concentrations and exactly the same medium used in mitochondrial experiments, a rapid peroxidation occurs (Fig. 11). Similar results were obtained with ethyl linolenate, but there was much less TBA color material formed (21).

**DISCUSSION**

GSSG + GSH-induced swelling of isolated mitochondria is very closely correlated with the formation of lipid peroxides. The first appearance of the TBA color material slightly precedes swelling under nearly all circumstances. The rate of lipid peroxide formation and the rate of swelling and lysis show a direct quantitative relationship (Figs. 1, 2, and 10). The total lipid peroxide formed is greater than with ascorbate (20), and apparently represents the limit of peroxidizable material in the mitochondria. The O₂ consumption is considerably accelerated during the active phase of swelling, as would be expected from lipid peroxidation. No conditions have been found which result in swelling without lipid peroxide formation. All antioxidants prevent GSSG + GSH-induced swelling, so that there is little doubt that formation of lipid peroxides is intimately connected with the permeability increase which leads to swelling. A number of substances not ordinarily considered as antioxidants also probably inhibit GSSG + GSH-induced swelling by this mechanism.

Although some classical electron transport chain inhibitors can prevent GSSG + GSH-induced swelling, there is no indication that electron transport from substrate is involved. No endogenous or added substrate is needed. GSH itself does not serve as substrate. When GSSG is also present, oxidation of GSH is markedly increased, but the same effect is seen in medium without mitochondria. The rate of oxygen consumption with mitochondria during the lag period before detectable lipid peroxidation is similar to that in medium alone.

Not all electron transport inhibitors prevent GSSG + GSH-induced swelling. Malonate, Amytal, and rotenone are without effect. These substances might act at points too low in the electron transport chain to prevent entry of electrons from GSH. However, azide, which blocks in the upper part of the electron transport chain, also does not inhibit the swelling. In the case of the four electron transport chain inhibitors which do block the action of GSSG + GSH—cyanide, antimycin A, SN 5949, and 2-nonyl-4-hydroxyquinoline N-oxide—the concentrations required are 10- to 20-fold higher than those needed to block electron transport, and an antioxidant action is suggested. Lower concentrations do inhibit electron transport in a normal fashion with 5 mM GSSG + 1 mM GSH present, but it has not been possible to test for this during active lipid peroxidation (15). Reaction of these inhibitors with lipid peroxide intermediates in an antioxidant function might result in inactivation of the inhibitor.

Although electron transport from substrates to O₂ appears not to be involved in GSSG + GSH-induced changes, components of the electron transport chain might be involved. Participation in reactions induced by GSSG + GSH could be related to the functional properties or the structural position of the electron carriers. It is well known (22, 23) that cytochromes can catalyze lipid peroxidation. In mitochondria they are closely associated with lipid materials. Nonheme iron components that are closely associated with or part of the electron transport chain might also function as catalysts for lipid peroxidation and be the site of inhibition by higher concentrations of CN⁻, antimycin A, SN 5949, and 2-nonyl-4-hydroxyquinoline N-oxide. Added Fe⁺⁺ ion is known to induce lipid peroxidation (14). Many agents that form metal complexes prevent GSSG + GSH-induced swelling, but it is not known whether this results from interaction with metals in the mitochondria or with a trace metal in solution.

The question whether initiation of lipid peroxidation by GSSG + GSH involves specific sites or is a generalized nonspecific effect is a very complicated one. The early permeability changes probably involve very small amounts of lipid peroxidation, but the oxygen consumption and the TBA color reaction progress to amounts which represent generalized lipid peroxidation. Results with pure lipids indicate that nonspecific lipid peroxidation is quite possible. However, this does not tell us whether or not the initial reactions occurred at specific sites. There is some evidence that much of the early lipid peroxidation occurs in the area occupied by cytochrome c (24). The remainder may be at the site of other carriers, or it may represent a generalized attack on membrane lipids by GSSG + GSH.

There are numerous reasons for asking whether an initial selective action of GSSG + GSH involves lipids at sites where the formation of energy-rich intermediates of phosphorylation is coupled to the electron transport chain. Some of them are as follows. (a) The concentrations of antimycin A, SN 5949, and 2-nonyl-4-hydroxyquinoline N-oxide which do prevent the appearance of lipid peroxide are exactly the same as those which block swelling supported by any of the three phosphorylation-coupled segments of the electron transport chain, even the two segments in which electron transfer is not inhibited (25). (b) High concentrations of these inhibitors have effects on ATP₃₄PO₄ exchange reactions in mitochondria. (c) Phosphate and arsenate interrupt GSSG + GSH-induced swelling (15). (d) GSSG + GSH appears to cause immediate release from respiratory control in eliminating the lag often seen with phosphate + β-hydroxybutyrate-induced swelling. (e) The GSSG/GSH ratio determines the release of C factor, which has proven to be GSH peroxidase and is reported to be involved both in contraction mechanisms and in phosphorylation (3, 4, 7, 11). (f) ATP, and especially ATP + Mg⁺⁺ + BSA, prevents GSSG + GSH-induced changes. (g) The delaying action of dithiother in GSH-induced swelling is eliminated by dinitrophenol (19). (h) The action of dinitrophenol in a number of ATPase systems seems related to thiol groups. (i) Fluharty and Sanadi (26) have implicated dithiol groups in oxidative phosphorylation. (j) Jensen (27) has pointed out the reversible electron flow possi-
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Glutathione-induced swelling and lysis result from lipid peroxidation and are not dependent on electron transport and generation of high energy intermediates, as is the case for phosphate-induced swelling.  

7. GSSG + GSH-induced swelling consists of an initial swelling accompanied by a large decrease in turbidity. This is followed fairly quickly by disintegration of the membrane structures.  

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