Effects of Phosphate, Arsenate, and other Substances on Swelling and Lipid Peroxide Formation when Mitochondria Are Treated with Oxidized and Reduced Glutathione*

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As we have previously reported (1, 2) and as Neubert, Wojtczak, and Lehninger have confirmed (3), large amounts of lipid peroxide, as measured by the thiobarbituric acid color reaction (4), are formed during swelling and lysis of dilute suspensions of liver mitochondria in the presence of oxidized and reduced glutathione (GSSG + GSH). This swelling has quite different characteristics from that produced by phosphate (2). Many substances inhibit swelling produced by GSSG + GSH (2, 5-7), but of special interest are effects seen with phosphate, arsenate, and thyroxine. When phosphate or arsenate in rather low concentration is added with mixtures of GSSG and GSH, the phosphate-induced type of swelling seems to take precedence over glutathione-induced lysis, and the formation of lipid peroxide that is ordinarily induced by glutathione does not occur. This report deals with the detailed interrelationships of these phenomena. Such effects of phosphate and arsenate are of interest because several workers, including Calvin (8), Fluharty and Sanadi (9), and Newton (10), have suggested that disulfide cleavage might play a role in oxidative phosphorylation. In the preceding paper (2), we have pointed out that lipid peroxide might arise from some intermediate of oxidative phosphorylation under certain conditions.

The terms “swelling-lysis” or “lysis” are used here to describe the changes produced in liver mitochondria by GSSG + GSH in order to relate this work to literature on GSH-induced “swelling” of mitochondria. Actually, at least three different kinds of “swelling” occur with isolated liver mitochondria (2): (a) swelling, with very little loss of matrix protein, as seen with phosphate or thyroxine + substrate; (b) lysis, with loss of 50 to 60% of the protein in soluble form but with retention of membrane structures which may be “ghosts,” a change seen with low Fe** ion; and (c) disintegration, which is lysis with disintegration of the membrane into very small particles and soluble proteins. As indicated in the preceding paper (2), the action of GSSG + GSH probably involves all three.

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EXPERIMENTAL PROCEDURE

Rat liver mitochondria were prepared as previously described with 0.35 mM sucrose or 0.175 mM KCl + 0.025 mM Tris (2). Whenever mitochondria were not used within the first 2 hours, the conditions of the aging are specified. The test medium and conditions of incubation have been described previously (2, 11). Swelling and lysis were measured as the decrease in turbidity of dilute suspensions at 520 rnp (&) in a Bausch and Lomb Spectronic 20 spectrophotometer (12). Oxygen consumption was measured with a Beckman Instrument Company micro-Clark oxygen electrode.

Lipid peroxide was measured by a modification of the thiobarbituric acid color reaction (2, 12). The absorbance readings at 532 mnp have been graphed directly. An A532 reading of 0.090 is equivalent to formation of 1.0 mumole of malonaldehyde per mg of mitochondrial suspension.

Aging of mitochondria, removal of cytochrome c, and other treatments are described with the specific experiments discussed below. Rats were obtained from the Holtzman Company, Madison, Wisconsin. Distilled water was redistilled in a two-step all-quartz still. All chemicals were obtained from the sources previously indicated (2, 12, 13). Throughout this paper the expression GSSG + GSH will refer to 5 mM GSSG + 1 mM GSH except where other concentrations are specified.

RESULTS

Effect of Phosphate on Glutathione-induced Changes in Mitochondria

Swelling and Lysis—Fig. 1 shows a curve demonstrating the typical swelling and lysis seen when fresh rat liver mitochondria are exposed to a mixture of GSSG + GSH (1, 2, 6). When 5 mM orthophosphate is present along with the glutathione mixture, the $D_{532}$ curve is shifted to a form resembling that seen with phosphate alone or phosphate + ß-hydroxybutyrate. In the case of phosphate + the glutathione mixture, the swelling may proceed farther than with phosphate + substrate, but it always shows a plateau or end point that is unmistakably higher than the extremely low $D_{532}$ values reached when the glutathione mixture produces lysis and disintegration of the mitochondria. The effect of phosphate with GSSG + GSH is very similar...
whether the suspending medium is 0.33 M sucrose or 0.175 M KCl + 0.025 M Tris buffer.

Phosphate concentrations as low as 1 mM are quite effective with mitochondrial preparations in KCl-Tris medium. Partial effects are easily detectable with 0.2 mM phosphate. When phosphate is combined with the glutathione mixture, any lag period seen with phosphate alone is either eliminated (KCl-Tris medium) or greatly reduced (sucrose-Tris medium). These observations raised the question whether changes occurring during phosphate-induced swelling always preclude further stages involved in glutathione-induced swelling and lysis. A somewhat similar phenomenon was observed with thyroxine and GSH by Lehninger and Schneider (5).

Formation of Lipid Peroxide—Since appearance of TBA1 color material is so closely associated with GSSG + GSH-induced changes in mitochondria (1, 2), it was of great interest to see whether this still occurred in the presence of phosphate. In Fig. 1 it may be seen that amounts of phosphate which completely eliminate the GSSG + GSH type of swelling prevent the appearance of lipid peroxides. Smaller amounts of phosphate greatly reduce the rate of formation of lipid peroxides. Phosphate does not interfere in the TBA method for preformed lipid peroxide material.

Oxygen Consumption during Glutathione-induced Lysis—Since the TBA color method measures only the malonaldehyde breakdown product from certain lipid peroxides (14), the possibility existed that phosphate was preventing only the final stages of the breakdown of the fatty acid molecules. To check on this point, we studied the effect of phosphate on the oxygen consumption during GSSG + GSH-induced swelling and lipid peroxide formation. Earlier experiments (2) had demonstrated extra oxygen consumption during the lipid peroxidation.

When phosphate is added to mitochondria along with glutathione mixtures, the oxygen consumption parallels the formation of malonaldehyde measured by the TBA method. KCl-Tris medium. Swelling with phosphate alone and phosphate + β-hydroxybutyrate (β-OH-B) are shown for comparison.

Arsenate has effects on oxygen consumption very similar to those seen with phosphate. Arsenate at 1 to 5 mM, EDTA at 0.1 mM, citrate at 1 mM, and butylated hydroxytoluene at 1 μM do not significantly affect the spontaneous oxygen consumption with GSSG + GSH mixtures in medium alone, but they completely eliminate the oxygen uptake resulting from the action of such mixtures on mitochondria.

Phosphate Added after Glutathione—The addition of 1 mM phosphate after glutathione-induced swelling and lipid peroxidation have begun (or progressed fairly far) virtually terminates the formation of lipid peroxide and reduces the degree of lysis that is seen in the final Dmss plateau (15). The characteristic rapid initial fall in Dmss with phosphate in the presence of GSSG + GSH in KCl medium is also seen in these experiments. No preformed lipid peroxide disappears when phosphate is added. As was expected on the basis of these experiments, GSSG + GSH added after phosphate-induced swelling has reached its characteristic plateau has essentially no effect.

Phosphate-induced Swelling is Not Essential for Phosphate to Prevent Glutathione-induced Lysis—The action of GSSG + GSH is prevented by phosphate even when no swelling is produced. Experiments in which aging of the mitochondria or action of inhibitors prevents phosphate-induced swelling will be detailed in a later section.

1 The abbreviations used are: TBA, 2-thiobarbituric acid; SN 5049, 2-hydroxy-3-(2-methyloctyl)-1,4-naphthoquinone; butylated hydroxytoluene, 2,6-di-tert-butyl-p-cresol; butylated hydroxyanisole, a mixture of 2-tert-butyl-4-methoxyphenol and 3-tert-butyl-4-methoxyphenol.
Effect of Phosphate on GSSG + GSH-induced Mitochondrial Swelling

There are many similarities between GSSG + GSH- and ascorbate-induced lysis of mitochondria (2, 11, 13). The amount of lipid peroxide appearing with 0.3 mM ascorbate is less, but there are many reasons for believing that the mechanism is similar. Therefore it is important to record here certain differences when phosphate is added.

Effect of Phosphate on Ascorbate-induced Lysis of Mitochondria

Action of Phosphate with Ascorbate in Sucrose Medium—As previously reported (11, 13), ascorbate-induced lysis occurs in the presence of phosphate just as in its absence when the medium is 0.33 mM sucrose + 0.025 mM Tris buffer. The light scattering changes due to phosphate and to ascorbate seem essentially additive. Additional experiments have confirmed this point and shown that only with an occasional mitochondrial preparation does addition of phosphate hinder ascorbate-induced lysis in sucrose medium (13).

Action of Phosphate with Ascorbate in KCl Medium—When phosphate + ascorbate was studied in 0.175 mM KCl-0.025 mM Tris, it was discovered that phosphate affects ascorbate-induced lysis in a manner qualitatively similar to that observed with glutathione mixtures in both KCl and sucrose media. Low concentrations of phosphate, such as 1 mM, are much less effective against ascorbate than they are against glutathione. However, higher concentrations of phosphate (5 and 10 mM) produce a shift to the phosphate type of curve and delay lipid peroxidation for 20 to 30 minutes (Fig. 3). In some experiments, the inhibitory action of phosphate is only partial, so that the light scattering gradually decreases toward the lower plateau characteristic for ascorbate. Although 5 mM phosphate can prevent lysis due to GSSG + GSH or due to ascorbate in KCl medium, it produces only a 3- to 5-minute lag period with 5 mM GSSG + 0.3 mM ascorbate, a combination which produces extremely rapid lipid peroxidation and lysis (1).

Thus, it appears that with ascorbate-induced lysis, phosphate can partially prevent the reactions in KCl medium but not at all in sucrose medium. These observations contrast with those for glutathione mixtures, which lead to considerably more lipid peroxide formation but are almost completely inhibited by phosphate in both sucrose and KCl media.

Effect of Other Anions on Glutathione-induced Lysis and Lipid Peroxide Formation

Arsenate—Fig. 4 shows an experiment in which a range of arsenate concentrations has been tested against swelling-lysis due to GSSG + GSH. In KCl medium, arsenate produces the same effect as phosphate in similar concentrations. Arsenate at 1 mM produces nearly complete inhibition of GSSG + GSH-induced lysis and lipid peroxide formation, and easily detectable effects are seen at 0.1 mM. Arsenate, like phosphate, is less effective in preventing the action of ascorbate. Arsenate is also less effective against lysis with GSSG + GSH in sucrose medium. Sometimes 5 and 10 mM are quite effective, but in other cases the D50 plateaus first reached may be brief, indistinct, and followed by a slow further fall to the low light scattering characteristic after the action of GSSG + GSH.

Thyroxine—Thyroxine causes swelling similar to that seen with phosphate, and Lehninger and Schneider (5) noted that its action seemed to take precedence over that of GSH. In our experiments, 1 mM thyroxine produced very little swelling itself but did slow swelling-lysis and lipid peroxidation with glutathione (Fig. 5). Thyroxine at 10 mM, which produces swelling in fresh mitochondrial preparations but not in aged preparations, completely blocks lipid peroxide formation with GSSG + GSH in both fresh and aged preparations. Since thyroxine can prevent GSSG + GSH-induced effects without producing swelling itself, thyroxine must inhibit lipid peroxidation and glutathione-induced swelling-lysis rather than taking precedence over it.

Anions without Effect—Sulfate, nitrate, nitrite, acetate, bi-
carbonate, iodide, chloride, fluoride, and thiocyanate at 1, 5, and 10 mM do not produce any effect whatever on the GSSG + GSH-induced swelling-lysis curve.

**Anions that Prevent Effect of Glutathione but Do Not Produce Swelling Themselves**—As reported in the preceding paper (2), there are many substances which inhibit the swelling-lysis produced by GSSG + GSH. Whether their mechanism of action is similar to that of phosphate, arsenate, and thyroxine is unknown. Their inhibition of lipid peroxide formation correlates well with their inhibition of swelling-lysis.

**Effect of Electron Transport Inhibitors on Phosphate-induced Swelling in Presence of Glutathione**

All inhibitors of the electron transport chain block phosphate-induced swelling of mitochondria (7, 16). Somewhat higher concentrations of certain electron transport chain inhibitors can block glutathione- or ascorbate-induced swelling-lysis (1, 2, 5, 11, 13), but azide, Amytal, rotenone, and malonate are never able to prevent this type of swelling. It was of considerable interest to determine whether the phosphate-induced type of swelling seen when both phosphate and GSSG + GSH are present had the characteristic dependence on electron transport.

**Rotenone, Amytal, and Azure**—In concentrations which are known to block the electron transport chain, these inhibitors do not affect GSSG + GSH-induced swelling-lysis, but do prevent the phosphate-induced swelling which occurs in the presence of GSSG + GSH (Fig. 6). This is clear evidence that this is typical phosphate-induced swelling.

**Cyanide, Antimycin A, SN 5949, and 2-Nonyl-4-hydroxyquinoline N-Oxide**—Low concentrations of cyanide, antimycin A, SN 5949, and 2-nonyl-4-hydroxyquinoline N-oxide prevent phosphate-induced swelling in the presence of glutathione mixtures (Fig. 7). These concentrations are characteristic for selective electron transport inhibition and are not the higher concentrations which can directly inhibit the action of GSSG + GSH and ascorbate (2, 13).

**Effect of Uncoupling Agents on Phosphate-induced Swelling in Presence of Glutathione**

The effects observed with phosphate and arsenate made it of interest to look for any possible interrelationships between phosphorylation uncouplers and inhibitors and the glutathione-phosphate system.

**2,4-Dinitrophenol**—This uncoupling agent has no effect on GSSG + GSH-induced swelling-lysis and lipid peroxidation (2). When 10 to 100 μM dinitrophenol is added in addition to GSSG + GSH + phosphate, all swelling or lysis is completely prevented (Fig. 8). The phosphate prevents GSSG + GSH-induced lipid peroxidation, but phosphate-induced swelling occurs in a normal fashion. If an uncoupling agent is present also, the phosphate-induced swelling is eliminated (7, 16). The net result is no change at all.

**Gramicidin**—In sucrose, where gramicidin induces only uncoupling, its effects are similar to those of dinitrophenol. In KCl, the swelling or lytic action of gramicidin itself (17) makes similar tests on swelling impossible, but there is no indication that gramicidin affects the rate or amount of lipid peroxide formed.

**Carnbonyl Cyanide Phenylhydrazones**—These substances are powerful uncoupling agents (18). When mitochondria are pre-

![Fig. 5 (upper)](http://www.jbc.org/figure/5up.jpg)

**Fig. 5 (upper).** Effect of thyroxine on GSSG + GSH-induced swelling-lysis and lipid peroxidation in fresh mitochondria. KCl-Tris medium.

**Fig. 6 (lower).** Effect of Amytal, rotenone, and azide on phosphate swelling induced in the presence of GSSG + GSH. Fresh mitochondria, sucrose-Tris medium.

![Fig. 7](http://www.jbc.org/figure/7.jpg)

**Fig. 7.** Low concentrations of antimycin A and SN 5949 inhibit phosphate-induced swelling in the presence of GSSG + GSH. B0HB, β-hydroxybutyrate.
exposed to 1 to 5 μM carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone or carbonyl cyanide m-chlorophenylhydrazone before addition of GSSG + GSH, there is no effect on GSSG + GSH-induced swelling-lysis, but the swelling with GSSG + GSH + phosphate is completely eliminated. Pre-exposure of the mitochondria is essential; otherwise the uncoupler reacts with the GSH.

**Oligomycin and Octylguanidine**—These substances block reactions in the sequence coupling electron transport to phosphorylation, but are not uncouplers (19). In tightly coupled mitochondria, they can prevent phosphate-induced swelling. At 100 μM, octylguanidine markedly inhibits phosphate-induced swelling in the presence of GSSG + GSH, but oligomycin has little effect, probably because its action is more critically dependent on the maintenance of respiratory control in the mitochondria (20).

**Effect of Antioxidants on Swelling Induced by Glutathione and by Glutathione + Phosphate**

The preceding studies (2, 13) have shown that antioxidants prevent ascorbate- and GSSG + GSH-induced lysis of mitochondria. Since such observations raised the possibility that the effects of phosphate and arsenate were mediated by an antioxidant action, various inhibitors have been tested to determine whether their effects are independent or additive with phosphate.

**Agents that Form Metal Complexes**—EDTA at 10 μM will prevent ascorbate- and GSSG + GSH-induced lysis (2, 13) and the swelling seen with glutathione + phosphate. However, since EDTA also blocks swelling induced by phosphate, thyroxine, and nearly every other swelling-inducing agent, antioxidant effects cannot be distinguished from direct interaction with mitochondrial membrane structures. 8-Hydroxyquinoline is...
clearly more specific, since with glutathione mixtures + phosphate, 0.1 to 1 mM 8-hydroxyquinoline eliminates the GSSG + GSH contribution but leaves the phosphate portion essentially unchanged. Inorganic pyrophosphate and triphosphate as low as 10 μM can completely prevent GSSG + GSH-induced swelling-lysis, but have little effect on the swelling produced by phosphate in the presence of glutathione mixtures. If 100 μM is used, the triphosphate, possibly because it has some action like EDTA, markedly inhibits phosphate-induced swelling.

**Metal Ions**—Mn^{2+} is known to be a powerful antioxidant (4), and so its ability to prevent ascorbate- and glutathione-induced lysis (1, 2, 5, 11, 13) at 10 to 50 μM is not surprising. Such concentrations have no effect on swelling produced by phosphate in the presence of GSSG + GSH, although they produce partial inhibition with phosphate + β-hydroxybutyrate. At 100 μM or higher, Mn^{2+} completely prevents all phosphate-induced swelling as well as GSSG + GSH-induced swelling-lysis. At equal concentrations, Mg^{2+} is without effect, but 5 mM is partially inhibitory.

**Antioxidants**—Although 50 μM α-tocopherol, 50 μM chlorpromazine, 20 μM promethazine, 10 μM butylated hydroxyanisole, 10 μM p- propyl gallate, 100 μM citrate, 1 mM ascorbic acid, and 1 mM pyruvate eliminate GSSG + GSH-induced swelling-lysis, they have essentially no effect on phosphate-induced swelling in the presence of GSSG + GSH or on phosphate + β-hydroxybutyrate-induced swelling. At 10 μM, butylated hydroxytoluene partially inhibits phosphate-induced swelling as well as eliminating the GSSG + GSH-induced lysis (Fig. 9). The mechanism by which butylated hydroxytoluene slows phosphate-induced swelling remains to be determined (2).

**Effect of Alteration of Mitochondria on Responses to Glutathione and to Phosphate**

Since it was unknown whether the rapid lipid peroxidation seen with GSSG + GSH or the interruption of this process by phosphate was independent from or intimately tied to mitochondrial membrane structure and the enzymes of electron transport and phosphorylation, we tested mitochondria altered in several ways.

**Aged Mitochondria**—When mitochondria are aged at 0° in sucrose medium for 24 to 48 hours, or at 25° for 30 to 60 minutes, swelling with phosphate alone is absent or slight, but added β-hydroxybutyrate restores the phosphate-induced swelling. The rate is somewhat slower, and the final plateau reached is lower than in the fresh preparation (Fig. 10). Swelling and lysis induced by 5:1 or 1:1 mixtures of GSSG and GSH are a little faster in the aged preparations. When phosphate is added with the glutathione mixture, the rapid swelling-lysis is replaced by a slow swelling over several hours (Fig. 10). Similar effects are observed after aging in KCl medium. However, complete elimination of endogenous substrates seems to be more difficult in KCl medium than in sucrose. Phosphate inhibition of GSSG + GSH-induced lysis and lipid peroxidation remains unchanged.

Aging for 15 minutes at 45° altered the mitochondrial preparations, but they still showed light scattering changes with GSSG + GSH. After heating to 45° for 30 minutes or to 60° for 15 minutes, mitochondrial suspensions showed no change in turbidity on subsequent addition of glutathione mixtures. Nevertheless, in all cases GSSG + GSH-induced lipid peroxidation occurred to about the same degree. This peroxidation was inhibited 60 to 75% by 1 mM phosphate, 90% by 0.1 mM citrate, and 100% by 1 mM citrate.

**Mitochondria Depleted of Pyridine Nucleotides**—Mitochondria can be depleted of pyridine nucleotides by being treated aerobically with 20 mM phosphate at 25° for 30 minutes, resolubilized by centrifugation, and washed once (21). GSSG + GSH produced typical swelling-lysis curves and lipid peroxide formation with such treated mitochondria (22). Phosphate at 5 mM also exerted its typical inhibitory action against GSSG + GSH-induced swelling-lysis.

**Mitochondria Depleted of Cytochrome c**—It is possible to remove essentially all of the cytochrome c from liver mitochondria by hypotonic and KCl treatments (23). Such mitochondria still oxidize substrate and show considerable phosphorylation when cytochrome c is added. Since hemep compounds like cytochrome c can catalyze lipid peroxidation (24, 25), we prepared mitochondria depleted of cytochrome c to see whether there was any significant change in their behavior when exposed to mixtures of GSSG + GSH.

In Fig. 11 it may be seen that removal of cytochrome c eliminates a large part of the GSSG + GSH-induced changes. The slow swelling seen may be dependent on traces of cytochrome c not removed, or on other cytochromes not removed at all by the procedure of Sanadi (23). Lipid peroxide formation is greatly reduced. Addition of 1 μM cytochrome c with the GSSG + GSH results in a swelling-disintegration curve and lipid peroxide formation almost identical with that seen in control preparations with GSSG + GSH alone. Thus there is complete restoration of the action of GSSG + GSH when 1 μM cytochrome c is added.

In relation to this, it is of some interest that addition of up to 10 μM cytochrome c to normal mitochondria having a full complement of endogenous cytochrome c does not significantly alter the swelling-lysis curve, although there may be some increase in lipid peroxides (22).

Phosphate and β-hydroxybutyrate are unable to induce swelling in mitochondria from which cytochrome c has been removed, as would be predicted from the fact that generation of high energy intermediates appears to be required for this type of swelling (20). When cytochrome c is added to restore electron transport, phosphate and substrate induce a normal electron transport-dependent swelling that is fully blocked by 20 μM dinitrophenol.

Addition of phosphate to cytochrome c-depleted mitochondria + GSSG + GSH + added cytochrome c inhibits lipid peroxidation and swelling-disintegration in the same manner as with normal mitochondria. Under these conditions, a phosphate type of swelling is seen even when evidence for substrate other than GSH is slight. Ordinarily GSH does not serve as a substrate for phosphate-induced swelling, particularly in sucrose medium, but it is known that added cytochrome c establishes the best conditions for GSH to feed electrons into the electron transport chain (5, 26, 27). However, further investigation is necessary, since there is no rapid oxygen uptake with phosphate and GSH or GSSG + GSH. Moreover, there is evidence that GSSG is essential as well as GSH to produce phosphate-induced swelling in the system consisting of cytochrome c-depleted mitochondria + phosphate + added cytochrome c.

*Full details of this work will be presented in a separate communication by F. E. Hunter, Jr., and A. Scott.*
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In this paper it has been shown that physiological levels of phosphate (or equal amounts of arsenate) largely prevent the lipid peroxide formation and the disintegration of mitochondrial structure seen with GSSG + GSH. These observations also raise the question whether the action of glutathione is related to oxidative phosphorylation mechanisms. It is easy to visualize the entry of phosphate or arsenate at the appropriate point in the oxidative phosphorylation sequence as being a reaction which might prevent activated intermediates from leading to or being converted to lipid peroxides per se, thereby preventing the complete cleavage or breakdown of the membrane structure.

However, in the preceding paper (2), it has been shown that GSSG + GSH-induced changes are probably not dependent on electron transport. This greatly weakens the argument that GSSG + GSH-induced lipid peroxide formation is connected with activity in the electron transport system, but it does not answer the question whether lipid peroxides can also arise during normal activity of the electron transport chain (32). The extensive swelling (but not disintegration) seen when both GSSG + GSH and phosphate are present with fresh mitochondria has been shown to be typical phosphate-induced swelling, dependent on electron transport and generation of high energy intermediates, as has been clearly established for phosphate + β-hydroxybutyrate (20).

It is important to know the mechanism by which phosphate and arsenate eliminate the GSSG + GSH-induced lipid peroxidation. If it represents diversion of oxidative phosphorylation intermediates away from a side reaction leading to lipid peroxides, then one is dealing with reactions between electron transport and formation of activated intermediates. However, if the effect is a nonspecific one, the action of phosphate and arsenate may not suggest a relation to oxidative phosphorylation at all.

Phosphate might inhibit the action of GSSG + GSH in any one of a number of ways, such as complex formation with GSH or with a contaminating metal ion, reaction with nonheme iron in the mitochondria, prevention of loss of C factor, reaction with key disulfide or thiol groups in the membrane, conversion of some factor to a phosphorylated intermediate, or action as a membrane stabilizer or as an antioxidant. There are arguments against most of these possibilities. Phosphate inhibits when GSH is in excess. Phosphate has only partial inhibitory effects on the spontaneous oxidation of glutathione mixtures. The presence of GSSG itself inhibits the loss of C factor. Even reaction of phosphate with a membrane thiol can be questioned, as treatment with p-chloromercuribenzoate or N-ethylmaleimide increases rather than decreases the yield of lipid peroxide with ascorbate (2, 13). Effects limited to specific sites, such as those for phosphorylation, seem somewhat unlikely, since phosphate inhibits all of the lipid peroxidation represented by a rather large O₂ consumption. However, we cannot be sure of the amount of lipid at such sites.

It seems clear that phosphate inhibition of GSSG + GSH-induced swelling-lipid and lipid peroxidation does not require mitochondrial structural integrity, electron transport, respiratory control, active phosphorylation enzymes, or endogenous ATP. Extensive investigation of many other substances has revealed that antioxidants in general and certain metal-complexing agents also prevent the action of GSSG + GSH (2). This raises the question whether the effect of phosphate could be entirely due to an antioxidant action. The finding that 5 mM phosphate almost completely inhibits peroxidation initiated by

**Effect of Phosphate on Glutathione-induced Peroxidation in Pure Lipid Materials**

GSSG + GSH mixtures catalyze peroxidation of methyl arachidonate at neutral pH and 25°C (2, 28). The rate is increased when 0.1 μM cytochrome c is added. Phosphate or arsenate at 5 mM is a powerful inhibitor of the peroxidation whenever GSSG + GSH is present (Fig. 12). Phosphate is a very poor inhibitor for peroxidation induced spontaneously at 60°C, or by ascorbate or by cytochrome c alone at 25°C. There are large amounts of arachidonic acid in mitochondria.

**DISCUSSION**

The mechanisms by which various substances affect GSSG + GSH-induced swelling, lysis, and disintegration of mitochondrial membrane structures are of considerable interest, since they may provide clues to the mechanism of action of glutathione and the possible relationship of effects in vitro to effects in vivo. Current speculations postulate roles for GSSG, GSH, and membrane protein disulfide, dithiol, and thiol groups in control of mitochondrial membrane permeability and in the mechanisms of oxidative phosphorylation. In fact, there are many suggestions that these may be essentially one and the same thing (7, 20, 29). Moreover, the discovery (1) that GSSG + GSH-induced swelling of mitochondria is intimately associated with lipid peroxide formation raised some interesting questions about the origin of these lipid peroxides. Since the action of glutathione appeared to be dependent on electron transport, and since lipoproteins had been postulated to be involved in the creation of energy-rich intermediates between electron transport and phosphorylation, it was important to consider the possibility that the initial intermediates of oxidative phosphorylation involved changes in polyunsaturated fatty acids that could lead to lipid peroxide formation or accumulation under certain conditions.

Such speculations had additional support from the fact that treatment with GSH leads to separation of a normal mitochondrial membrane component involved in ATP-induced contraction and possibly in oxidative phosphorylation (6, 30, 31). More recently this factor has been identified as GSH peroxidase (3). Removal of such an enzymatic factor might interrupt the oxidative phosphorylation mechanism sequence, leading to accumulation of lipid peroxide. However, it is not clear why GSSG would be so critical to lipid peroxide formation when it can in large measure prevent release of GSH peroxidase from the membrane structure by GSH (6).
GSSG + GSH with pure lipid materials demonstrates that its action in the mitochondrial system might be as a simple antioxidant. This antioxidant action is not necessarily due to metals that yield complexes (33, 34). Stuckey (33) has pointed out that phosphoric acid may belong in the electron-donating class of antioxidants. In the food industry, phosphoric acid is generally considered as an accessory rather than a primary antioxidant. In the mitochondrial system it might act in concert with any one of a number of substances, including α-tocopherol.

The antioxidant action of phosphate may have significant degrees of specificity, since in our preliminary experiments phosphate showed little or no antioxidant action on ascorbate-induced peroxidation of arachidonate, even when the rate of peroxidation was considerably less than with GSSG + GSH. Phosphate also had relatively little effect on peroxidation catalyzed by 0.1 μM cytochrome c or by 60°C temperature. These findings are completely consistent with the fact that phosphate has no effect on ascorbate-induced lysis of mitochondria in sucrose medium, and with the fact that studies on ascorbate- and cytochrome c-catalyzed lipid peroxidation have been carried out in phosphate buffer in some cases (35, 36). Possibly phosphate acts primarily on the mechanism for initiation of lipid peroxidation which is active with GSSG + GSH. It is well recognized that different antioxidants act by different mechanisms (33, 34).

The fact that phosphate can act as an antioxidant with pure lipids under certain conditions requires one to exercise caution in assigning possible physiological significance to its interaction with GSSG + GSH and mitochondria. However, this does not prove that its action on mitochondria is completely nonspecific and unphysiological. In fact, one must keep in mind that the fundamental property responsible for its antioxidant action might be the very basis for its entry into chemical reactions which are elevated to major physiological roles and channeled in appropriate directions by the enzyme catalysts of the mitochondria. Possibilities for reversible electron transfer in phosphorus and sulfur compounds have been discussed by others (37, 38). Controlled interactions of disulfides, dithiols, phosphate, polyunsaturated fatty acids, lipid peroxides, and GSH peroxidase offer many possibilities for physiological reactions. The role played by cytochrome c also requires further study. Although it does catalyze lipid peroxidation with pure lipid materials, in the mitochondrial system with GSSG + GSH, cytochrome c bound at its normal functional site in the mitochondria is more effective than much larger amounts of added cytochrome c.

Phosphate prevents the GSSG + GSH-induced disintegration of mitochondria, but this is not the only interrelationship between the two substances. The GSSG + GSH mixtures decrease or eliminate lag periods with phosphate-induced swelling dependent on electron transport from endogenous substrate. They also cause this swelling to be more extensive in nearly every case. GSSG or GSH alone does not have this action. Possibly such effects are due to immediate elimination of respiratory control. So far as can be detected, they are not associated with lipid peroxide formation, so that direct interchange with membrane disulfides or thiols may be involved.

There is very little evidence for any glutathione-induced swelling in preparations where phosphate is present and phosphate swelling is prevented by depleting endogenous substrates, inhibiting electron transport, or producing complete uncoupling of phosphorylation. Thus, it is questionable whether GSSG or GSH GSH alone, in the concentrations used, has any effect on permeability or structure when electron transport-supported swelling and reactions which lead to lipid peroxidation are eliminated. Neubert and Lehninger (6), Friedkin and Lehninger (26), and Maley and Lardy (27), using mitochondria prepared in 0.25 M sucrose and a different test medium (0.125 M KCl), have studied a mitochondrial swelling induced by high GSH concentrations and characterized by the loss of C factor. Further work is necessary to determine whether swelling with 10 to 20 mM GSH is dependent on endogenous substrate in addition to glutathione, α Neovert, Rose, and Lehninger (31) always used fresh mitochondria. This swelling has been reported to be inhibited by several electron transport inhibitors and dinitrophenol (30). This initial swelling with high concentrations of GSH alone is not seen with our preparations, and the reasons for the difference are not clear, as we have checked all known differences in experimental conditions. If GSH were an accelerator of swelling due to endogenous substrate, the difference might indicate less endogenous substrate in our preparations. Release of C factor might be a simultaneous event dependent on GSH.

**SUMMARY**

1. Phosphate or arsenate at 1 to 5 mM, and thyroxine at 10 μM, prevent mitochondrial swelling, lysis, and disintegration induced by mixtures of oxidized and reduced glutathione (GSSG + GSH). The extra oxygen consumption and lipid peroxidation associated with these changes is eliminated.

2. The effect appears to be direct, for phosphate- or thyroxine-induced swelling changes are not essential for modifying the action of GSSG + GSH. Conversely, phosphate-induced electron transport-dependent swelling occurs normally and independently in the presence of GSSG + GSH.

3. The lytic effect of GSSG + GSH and its inhibition by phosphate are seen in mitochondria depleted of substrate, with electron transport blocked, and after aging to inactivate phosphorylation enzyme systems.

4. Phosphate prevents the peroxidation of pure arachidonate induced by GSSG + GSH. This antioxidant effect of phosphate may have some specificity related to glutathione, as phosphate shows very much less or no effect on peroxidation of pure lipids catalyzed by ascorbate or cytochrome c.

5. Present evidence suggests that phosphate inhibits GSSG + GSH-induced changes in mitochondria by a direct antioxidant action, possibly in a nonspecific manner. The only evidence that could relate these effects to specific sites of electron transport or phosphorylation is that (a) removal of cytochrome c greatly reduces the effects of GSSG + GSH, and (b) inhibitors of the antimony A type block lipid peroxidation in the same concentration which blocks phosphate-induced swelling regardless of which segment of the electron transport chain is generating the essential high energy intermediates.

**REFERENCES**


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