

Mitochondrial Swelling Induced by Glutathione

By ALBERT L. LEHNINGER, Ph.D., and MARION SCHNEIDER*

(From the Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore)

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ABSTRACT

Reduced glutathione, in concentrations approximating those occurring in intact rat liver, causes swelling of rat liver mitochondria *in vitro* which is different in kinetics and extent from that yielded by L-thyroxine. The effect is also given by cysteine, which is more active, and reduced coenzyme A, but not by L-ascorbate, cystine, or oxidized glutathione. The optimum pH is 6.5, whereas thyroxine-induced swelling is optimal at pH 7.5.

The GSH-induced swelling is not inhibited by DNP or dicumarol, nor by high concentrations of sucrose, serum albumin, or polyvinylpyrrolidone, in contrast to thyroxine-induced swelling. ATP inhibits the GSH swelling, but ADP and AMP are ineffective. Mn^{++} is a very potent inhibitor, but Mg^{++} is ineffective. Ethylenediaminetetraacetate is also an effective inhibitor of GSH-induced swelling. The respiratory inhibitors amytal and antimycin A do not inhibit the swelling action of GSH, but cyanide does; these findings are consistent with the view that the oxidation-reduction state of the respiratory chain between cytochrome *c* and oxygen is a determinant of GSH-induced swelling.

Reversal of GSH-induced swelling by osmotic means or by ATP in KCl media could not be observed. Large losses of nucleotides and protein occur during the swelling by GSH, suggesting that the action is irreversible. The characteristically drastic swelling action of GSH could be prevented if L-thyroxine was also present in the medium.

A number of agents cause swelling of isolated rat liver mitochondria suspended in buffered sucrose solutions. These include thyroxine and other thyroactive compounds (1-3), inorganic phosphate (4, 5), Ca^{++} (1, 4, 6), heavy metal ions and certain of their derivatives, such as Ag^+ and *p*-chloromercuribenzoate (1, 7), phloridzin (8), and some detergents (9, 10). Of these agents, thyroxine, Ca^{++} and phosphate occur physiologically in concentrations capable of affecting mitochondrial swelling, and presumably contribute to the balance of intracellular factors affecting the morphological and physiological state of the mitochondria (3).

This paper reports the finding that reduced glutathione (GSH), and also cysteine, cause very pronounced and characteristic mitochondrial swell-

ing *in vitro*. This effect is noteworthy because concentrations of reduced glutathione equivalent to those found in fresh intact liver readily produce maximal swelling effects, suggesting that GSH may also be an intracellular factor concerned in maintaining the dynamic balance between swelling of mitochondria and the "active" contraction. Another significant feature of GSH-induced swelling is that it differs very strikingly from that induced by thyroxine with respect to kinetics, extent, inhibition, and reversal, suggesting that the two different kinds of mitochondrial swelling originate from rather different initial biochemical events and result in different types of morphological change.

Experimental

Rat liver mitochondria were prepared exactly as described in the preceding communication on thyroxine-induced swelling (3); they were used within 2 hours of preparation. The standard test system was 0.3 M sucrose-0.02 M tris (hydroxymethyl)aminomethane-

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(tris)-HCl, pH 7.4, unless otherwise designated. Solutions of sulfhydryl compounds were prepared and carefully brought to pH 7.4 at 0° just before use in order to minimize autoxidation by atmospheric oxygen.

Swelling Action of GSH and Cysteine.—GSH and cysteine cause a type of swelling of rat liver mitochondria which differs strikingly in extent and kinetics from spontaneous or thyroxine-induced swelling, which the preceding study demonstrated to be essentially similar (3). In Fig. 1 is shown a comparison of the action of 0.01 M GSH and 1×10^{-5} M thyroxine in a typical experiment. As shown before, thyroxine-induced swelling is characterized by a relatively short lag period, usually absent above 20–25°, followed by rapid swelling which approaches asymptotically a terminal optical absorbancy at 520 m μ of about 30 per cent of the initial absorbancy under the optical and geometrical conditions of measurement used (3). GSH, in contrast, shows a substantially longer lag period followed by a rather precipitous drop in absorbancy to an end value which is asymptotic to an optically clear solution. This characteristic picture is given by GSH concentrations of 0.005 M and above with freshly prepared mitochondria. At lower concentrations of GSH the lag period is longer. After aging of the mitochondria at 0° they become more sensitive to low concentrations of GSH and the lag period is shorter. For comparison, the concentration of GSH in fresh rat tissues ranges from 0.003 M to

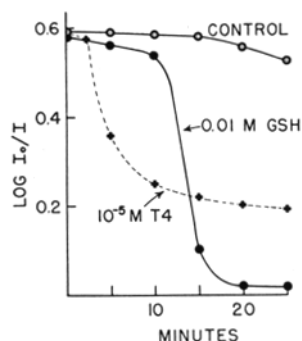


FIG. 1. Kinetics and extent of GSH- and thyroxine-induced swelling. The medium was 0.3 M sucrose–0.02 M tris pH 7.4 at 20.0°C.; final concentrations of 0.01 M GSH and 1×10^{-5} M L-thyroxine present at zero time as shown. Aliquots of rat liver mitochondrial suspension containing 50 mg. tissue equivalent of mitochondria added at zero time. Optical absorbancy measured at 520 m μ .

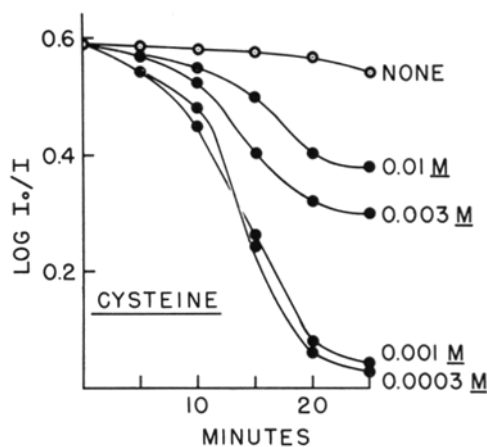


FIG. 2. Effect of cysteine concentration on swelling. Details as in Fig. 1; freshly neutralized cysteine added in concentrations shown.

0.02 M; in rat liver the GSH concentration is about 0.005–0.008 M (11, 12). These tests were carried out at pH 7.4; experiments described below show that mitochondria are considerably more susceptible to GSH-induced swelling at pH 6.5–7.0.

Cysteine is a far more potent swelling agent than GSH; data in Fig. 2 show that concentrations as low as 0.003 M produce about the same effect as 0.01 M GSH. However at higher concentrations (0.01 M) the action of cysteine is much less drastic; the data show a biphasic swelling response to cysteine concentration.

Substantial swelling is also given by the reduced form of coenzyme A at 0.001 M, but little or no swelling occurred with 0.005 M thioethanol, 0.001 M BAL (2,3-propanedithiol), 0.001 M reduced DL-lipoic acid, or 0.01 M methionine. Significantly, L-ascorbate at 0.01 M does not cause mitochondrial swelling.

Neither oxidized glutathione nor cystine causes swelling of rat liver mitochondria, nor do they alter the response to GSH or cysteine. However 0.001 M oxidized lipoic acid did increase the swelling rate.

In a medium of 0.15 M KCl–0.02 M tris pH 7.4, GSH also is effective in promoting the swelling. However, the terminal optical absorbancy is substantially higher than in sucrose, suggesting that in KCl GSH-induced swelling is much less drastic.

Factors Affecting the Swelling Action of GSH.—Swelling of mitochondria by thyroxine is inhibited by a variety of agents (1–3). In the following ex-

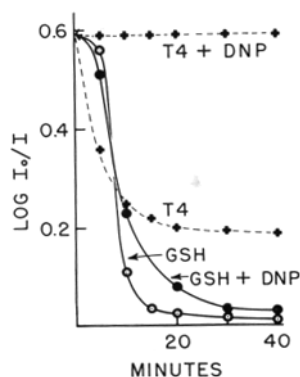


FIG. 3. Effect of 2,4-dinitrophenol (DNP). General conditions as in Fig. 1. L-Thyroxine was added at 1×10^{-5} M, GSH at 0.01 M, and DNP at 5×10^{-5} M.

periments, the action of these agents against GSH-induced swelling was surveyed, with the finding of quite characteristic differences between GSH- and thyroxine-induced swelling.

(a) *Action of 2,4-dinitrophenol.* This agent inhibits spontaneous swelling of mitochondria and completely blocks the swelling action of thyroxine (1, 2). Data summarized in Fig. 3 show that 5×10^{-5} M DNP has no inhibitory effect on mitochondrial swelling induced by 0.01 M GSH, although this concentration of DNP completely blocks the action of 1×10^{-5} M thyroxine. It is evident that the mechanism of action of GSH in inducing swelling must differ from that of thyroxine; the lack of response to DNP also suggests that GSH acts on some receptor site in the mitochondrion which may be less directly associated with the coupling of phosphorylation to respiration. In similar experiments it was found that 5×10^{-5} M dicumarol failed to inhibit GSH-induced swelling, but inhibited thyroxine-induced swelling completely.

(b) *Action of Mg^{++} and Mn^{++} .* Both Mg^{++} and Mn^{++} inhibit the swelling induced by thyroxine (1). However the typical experiments shown in Fig. 4 demonstrate that 0.005 M Mg^{++} has virtually no ability to inhibit swelling induced by 0.01 M GSH; this concentration of Mg^{++} suffices for virtually complete inhibition of thyroxine-induced swelling (1). On the other hand, 0.005 M Mn^{++} completely blocks swelling induced by GSH. Although Mn^{++} is known to catalyze autoxidation of cysteine, oxidative removal of the GSH in the test system during the test period was relatively slow and insufficient to account for the inhibitory action of Mn^{++} on the GSH swelling.

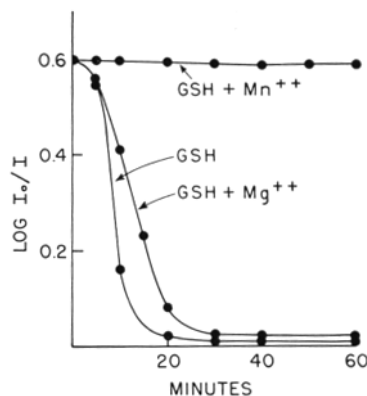


FIG. 4. Effect of Mg^{++} and Mn^{++} on GSH-induced swelling. Conditions arranged as in Fig. 1; the concentration of the $MgCl_2$ and $MnCl_2$ added was 0.005 M.

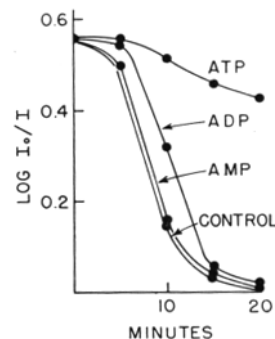


FIG. 5. Action of adenine nucleotides. Conditions as in Fig. 1. The nucleotides were added to yield final concentration of 0.003 M.

The striking difference between Mn^{++} and Mg^{++} suggests that the GSH-induced swelling may provide an approach for identification of the locus of the specific action of Mn^{++} claimed by Lindberg and Ernster (13) in reactivating oxidative phosphorylation in aged and swollen mitochondria and for the extensive accumulation and binding of Mn^{++} (7).

(c) *Action of adenine nucleotides.* The data collected in Fig. 5 show that ATP can very largely prevent the swelling action of GSH, but that equivalent concentrations of ADP and AMP are inactive, as is inorganic pyrophosphate. By contrast, in fresh mitochondria tested under the same conditions both ADP and pyrophosphate as well as ATP protect against thyroxine-induced swelling (1, 4). In similar tests it was found that GTP and ITP were as effective as ATP in protecting against GSH-induced swelling, but UTP afforded no

protection whatsoever. However, when the stock mitochondrial suspension in 0.25 M sucrose was aged at 0° for longer than 2 hours, then none of the nucleoside 5'-triphosphates protected against GSH-induced swelling.

There is now evidence that ATP can exert both specific and non-specific action against mitochondrial swelling (15, 16, 3). ATP, but not ADP, can reverse swelling, presumably because of a specific capacity to act as a phosphate donor. However, it may also act as a chelating agent for divalent metal ions such as Ca^{++} (14); ADP, inorganic pyrophosphate, and other nucleoside 5'-triphosphates have similar action and can also inhibit swelling. Presumably ATP has some specific action in the inhibition of GSH-induced swelling, since ADP and pyrophosphate are not effective. On the other hand, inhibition of GSH-induced swelling by concentrations of ethylenediaminetetraacetate (EDTA) as low as 1×10^{-4} M was observed. EDTA also inhibits thyroxine-induced swelling (1), but appears to be less potent.

(d) *Effect of sucrose.* In a medium containing 0.75 M sucrose, the swelling action of thyroxine is completely abolished (3), but similar experiments showed that this concentration of sucrose does not significantly inhibit the swelling induced by GSH. The inhibitory effect of high concentrations of sucrose on thyroxine-induced and spontaneous swelling appears to be caused by inhibition of an intermediate enzymic reaction in the coupling of phosphorylation to electron transport, a process which controls the contractility of the mitochondrial membrane (3). The lack of effect of sucrose

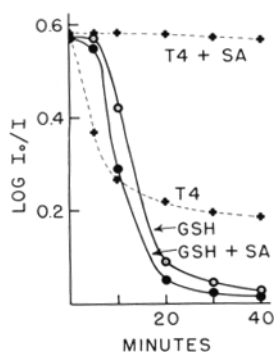


FIG. 6. Effect of serum albumin. Conditions as in Fig. 1, with 1.0×10^{-5} M thyroxine or 0.005 M GSH added as shown. Bovine serum albumin (crystallized, Armour) was added as shown in concentration of 10 mg. per ml. test medium.

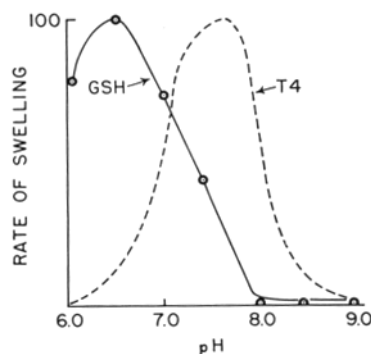


FIG. 7. Effect of pH. The rate of swelling in the presence of 0.01 M GSH was tested in a medium of 0.3 M sucrose buffered with tris-histidine mixtures as shown. The optimum pH curve of thyroxine-induced swelling is shown in the dotted line. The rates were arbitrarily measured as the reciprocal of the half-times to reach the limiting optical absorbancy.

on GSH-induced swelling indicates that GSH acts on some other susceptible site in the mitochondrial structure less directly associated with coupled phosphorylation.

(e) *Effect of high particle weight solutes.* Both bovine serum albumin (SA) and polyvinylpyrrolidone (PVP) (particle weight *ca.* 60,000) inhibit spontaneous and thyroxine-induced swelling, and this action appears to depend on the inability of these agents to pass through the mitochondrial membrane(s) (3), so that these solutes can provide a "colloid" osmotic pressure difference between extra- and intramitochondrial phases. Experiments such as shown in Fig. 6 demonstrate that bovine serum albumin (between 1.0 and 100 mg. per ml. medium) does not prevent the swelling induced by GSH, but does completely block swelling induced by thyroxine. Similarly it was found that 7.7 per cent PVP is also ineffective in preventing swelling induced by GSH. From these findings it may be suggested that swelling induced by GSH causes a change in permeability of the mitochondrial membrane(s) so that high molecular weight solutes such as PVP and serum albumin penetrate the membrane, permitting no substantially large osmotic pressure gradient between intra- and extramitochondrial phases to exist when such solutes are added. This interpretation is also supported by the finding described below that considerable protein is lost from the mitochondria during GSH-induced swelling.

Effect of pH.—The results of experiments testing

the effect of pH on the rate of GSH-induced swelling are shown in Fig. 7. It is seen that the optimum is at pH 6.5 or lower, and that the rate is far from maximum at pH 7.5. Most of the experiments on GSH-induced swelling in this paper were carried out at pH 7.4 and are thus not maximum in rate. On the other hand, both spontaneous and thyroxine-induced swelling are maximum at pH 7.5 (3) and occur only very slowly, if at all, at pH 6.5. These findings furnish additional evidence that GSH-induced swelling is different in nature from thyroxine-induced swelling.

Action of Respiratory Inhibitors.—It has been demonstrated that thyroxine-induced swelling can be completely prevented by the three characteristic respiratory chain inhibitors cyanide, antimycin A, and sodium amytal in the presence of O_2 and the respiratory chain reductant β -hydroxybutyrate (3, 17). These findings suggested that swelling induced by thyroxine does not occur when the DPN of the mitochondria is entirely in the reduced state, regardless of the oxidation-reduction state of the rest of the carriers.

The effect of varying the oxidation-reduction state of segments of the respiratory chain on the swelling action of GSH was studied, with the results shown in Fig. 8. It is seen that maintenance of all the carriers in the reduced state with cyanide caused complete inhibition of the swelling action of GSH. However, it is seen that GSH swelling was not prevented by amytal (DPN reduced, all other carriers oxidized) or by antimycin A (DPN,

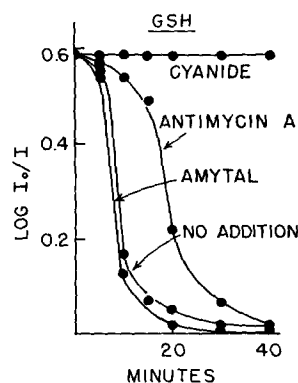


FIG. 8. Effect of respiratory inhibitors. Test systems arranged exactly as in preceding communication (3); cyanide was 0.001 M; antimycin A, 0.12 micrograms per ml.; and sodium amytal, 0.0018 M. Inhibitor tubes contained also 0.001 M DL- β -hydroxybutyrate as respiratory chain reductant.

flavoprotein, and cytochrome *b* reduced; cytochromes *c*, *a*, and a_3 oxidized). It is quite clear that GSH- and thyroxine-induced swelling differ very strikingly in their dependence on the state of the respiratory carriers. Whereas the swelling action of thyroxine appears to be dependent on the oxidation-reduction state of DPN, the action of GSH appears not to correlate with the oxidation-reduction state of DPN, flavoprotein, and cytochrome *b*, but is apparently dependent on the oxidation state of the segment of the respiratory chain between cytochromes *c* and a_3 , giving swelling when these are oxidized, but no swelling when these are in the reduced state. Alternative explanations (*cf.* reference 17) will be considered below.

Antagonism between Thyroxine and GSH in the Swelling Reaction.—In Fig. 9 is shown the interaction of thyroxine and GSH. When tested separately, they showed their characteristically different kinetics and extent of the swelling. When both agents were present together, swelling still ensued. However it is quite clear that the GSH and thyroxine appear to have some antagonistic action, at least on the terminal phase of swelling, since the characteristic approach to complete loss of optical absorbancy ordinarily produced by GSH does not occur when thyroxine is also present. The course of the swelling induced by the combination of GSH and thyroxine is thus not greatly different from that given by thyroxine alone. These findings suggest that there may be a common intermediate stage in the two modes of swelling in which thyroxine may prevent the much more drastic swelling action of GSH. The significance of these findings is discussed below.

Reversal of GSH-Induced Swelling.—Thyroxine-induced or spontaneous swelling can be reversed "passively," by addition of impermeant solutes such as PVP or serum albumin to the test medium, the mitochondria then presumably behaving as "passive" osmometers; or swelling can be reversed "actively," *i.e.* through enzymatic action, by adding ATP in saline media (3). Similar experiments revealed however that GSH-induced swelling cannot be either prevented or "passively" reversed by PVP or serum albumin, suggesting an irreversible action of GSH on the permeability of the mitochondrial membrane to these high molecular weight solutes.

Tests were, therefore, made for the ability of ATP, and combinations of ATP, Mn^{++} , and oxidizable substrates to reverse GSH-induced swell-

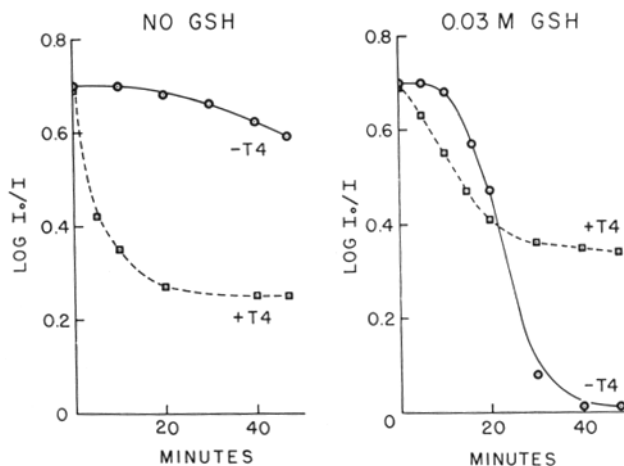


FIG. 9. Antagonism of GSH and thyroxine in swelling of mitochondria. Conditions as in Fig. 1; L-thyroxine was 1.0×10^{-5} M and GSH, 0.005 M.

TABLE I
*Loss of Nucleotides and Protein Following
GSH-Induced Swelling*

Basic test systems contained 5.0 ml. 0.30 M sucrose-0.02 M tris buffer pH 7.4, with 0.01 M GSH or 1×10^{-5} M L-thyroxine as shown. A standard aliquot of mitochondria, equivalent to the yield from 0.15 gm. fresh rat liver, was added to each tube. The tubes were incubated 30 minutes at 20°, during which the GSH and thyroxine had induced characteristic and maximal swelling. The tubes were centrifuged and the absorbancy of the clear supernatant fluids determined at 280 m μ and 260 m μ . Readings of a zero time tube were subtracted in each case.

	Protein in medium	Total nucleotides in medium
	mg. per ml.	micromoles per ml.
Control	0.013	0.013
GSH-swollen	0.265	0.083
Thyroxine-swollen	0.000	0.023

ing, in both sucrose and KCl media. However, although these agents can reverse spontaneous and thyroxine-induced swelling (3, 17) and can prevent GSH-induced swelling, they have shown no ability whatsoever to reverse GSH-induced swelling once it has reached a point where the suspension has achieved almost complete optical transparency. However when ATP and Mn⁺⁺ were added at points during the characteristic precipitous drop in optical absorbancy (in a KCl medium) the further decrease in absorbancy

could often be prevented but never reversed. It is possible that in its early stages the GSH-induced swelling may still be enzymically reversible.

Loss of Intramitochondrial Material during GSH-Induced Swelling.—The data in Table I show that considerable material absorbing at 260 m μ and also at 280 m μ is lost from mitochondria to the suspending medium after GSH-induced swelling has taken place, indicating the loss of both protein and nucleotides. On the other hand, much smaller losses of 260 m μ and 280 m μ -absorbing material occurred during thyroxine-induced swelling. These findings are in agreement with the relatively drastic and irreversible type of swelling produced by GSH and the milder and reversible swelling induced by thyroxine.

It appears very unlikely, however, that GSH produces frank lysis or rupture of the mitochondria, since centrifugal recovery of GSH-swollen mitochondria yields very voluminous pellets from the nearly clear suspensions, which contain considerably more water than pellets of thyroxine-swollen mitochondria recovered under the same conditions.

DISCUSSION

The results of this investigation indicate that reduced glutathione and other naturally occurring thiols such as cysteine and coenzyme A must be added to the relatively limited list of intracellular substances capable of greatly increasing the rate of mitochondrial swelling, which includes inorganic phosphate, Ca⁺⁺, and thyroid-active com-

pounds. Of these thiols, reduced glutathione occurs in highest concentrations in most tissues, sufficiently high to give nearly maximal mitochondrial swelling rates when added *in vitro*.

Because these thiols promote a type of swelling of mitochondria which differs in kinetics, extent, and other characteristics from thyroxine-induced swelling, it is probable that the thiols act on specific chemical receptors in the mitochondria and cause changes in the structure and physical properties of the mitochondria which are characteristic and different from those induced by thyroxine. The complex structure of the mitochondrion, which has at least two kinds of membrane and also inner compartmentation, obviously permits more than one mode of structural change which could lead to swelling. Possibly other physiologically occurring swelling agents may yet be found which will yield still other types of response.

Although GSH reacts with or involves receptor sites in the mitochondria which are obviously different from those affected by thyroxine or phosphate (presumably a bound form of mitochondrial DPN (3, 5, 19)), the GSH swelling also seems to involve the respiratory chain and the coupling mechanism. Thus, ATP and Mn^{++} prevent the GSH swelling. Furthermore, inhibition of the respiratory chain with cyanide, but not with amytal or antimycin, leads to inhibition of GSH-induced swelling. In addition, the presence of thyroxine prevents or antagonizes the normal action of GSH in producing the final, drastically swollen end-state.

The experiments on the action of respiratory inhibitors on GSH-induced swelling are of some interest, since they have a pattern quite different from that observed with thyroxine-induced swelling. They suggest that the action of GSH is connected with the function of that portion of the respiratory chain between cytochrome *c* or *c*₁ and oxygen, in such a manner that the swelling action of GSH occurs only when this segment of the respiratory chain is in the oxidized state. Reduction of the rest of the chain does not seem to affect the GSH-induced swelling. Chappell and Greville have suggested an alternative explanation for the inhibition of swelling by agents capable of blocking the respiratory chain, namely that respiration and phosphorylation are necessary for swelling to occur and that any respiratory inhibitor will inhibit swelling (18). Although experiments in this and the preceding paper (3)

also agree that swelling is dependent on the existence of some kind of "high-energy" intermediate generated by respiration or ATP, the oxidation-reduction state of the carriers is believed to represent an additional factor of significance, in view of the striking effects of the oxidation states of the carriers on ATPase and the ATP- P_i^{32} exchange in mitochondrial fragments (20, 21).

Cysteine and glutathione are oxidized *via* the cytochrome system in mitochondria. However, this process requires addition of external cytochrome *c*, and no phosphorylation is observed coupled to this oxidation (22). Some tests with P^{32} -labelled phosphate indicated that no significant uptake of P^{32} into the intramitochondrial nucleotides takes place during the course of GSH-induced swelling in the presence of antimycin A. L-Ascorbate, also a reducing agent capable of reacting with the cytochrome system but with high yields of coupled phosphorylation (23), does not cause mitochondrial swelling.

Recent studies by Lester *et al.* (24) suggest that a substituted quinone, namely "Q₂₇₅" or ubiquinone (25), may be an essential electron carrier preceding cytochrome *c*. Such quinones could be expected to interact with thiols to produce either respiratory chain inhibition or interference in the coupling of phosphorylation, depending on the precise nature of their function in respiration. BAL, cysteine, and other thiols do in fact inhibit respiration under certain conditions (26). GSH and cysteine, although readily oxidized by mitochondria, do not ordinarily yield coupled phosphorylation, although ascorbic acid, which similarly is capable of reducing cytochrome *c*, is oxidized with a high efficiency of phosphate coupling (23), suggesting that the thiol group has an inhibitory action as well as the ability to reduce cytochrome *c*. Furthermore, cysteine and, under some conditions, also glutathione uncouple phosphorylation associated with oxidation of β -hydroxybutyrate and other substrates by mitochondria (22, 23, 27). These considerations suggest a possible point of attack of thiols on the respiratory chain and also on the relationship between the action of the chain and the membrane properties. Interference with the function of mitochondrial lipoic acid or coenzyme A also appear to be possible mechanisms for this action of GSH.

The antagonistic action of thyroxine on the GSH-induced swelling is of some interest in connection with the finding of Staehelin (28) that

thyroxine prevents the inhibitory action of cysteine on respiration of mitochondria, and also with the finding of Park *et al.* (29) that GSH is capable of preventing the uncoupling of phosphorylation brought about by thyroxine in hamster liver mitochondria. Possibly GSH-induced swelling and thyroxine-induced swelling share some common chemical or morphological stage, permitting such an antagonism to occur.

It can be predicted from the inhibitory action of ATP and Mn^{++} toward GSH-induced swelling *in vitro* that in the intracellular environment the drastic swelling action of GSH would be largely opposed and prevented by these agents, as would also appear to be the case for the swelling action of thyroxine, which is successfully opposed by ATP, Mg^{++} , and intracellular soluble protein (3). GSH could thus be visualized as participating in a dynamic balance of factors regulating the size, shape, and function of the mitochondrion in its cytoplasmic environment. Since GSH can lead to irreversible swelling, it is conceivable that under certain circumstances it may participate in the intracellular degradation or lysis of mitochondria which has often been postulated to be a normal event in a turnover cycle of biogenesis and decay of mitochondria.

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