Swelling and Lysis of Rat Liver Mitochondria Induced by Ferrous Ions*

F. EDMUND HUNTEE, JR., J. M. GEBICKI, P. E. HOFFSTEN, J. WEINSTEIN, AND A. SCOTT

From The Edward Mallinckrodt Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri

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The possibility that ascorbate- and reduced glutathione-induced swelling and lysis of mitochondria (1, 2) were dependent on some metal, such as iron, which was contaminating reagents, or even the nonheme iron in mitochondria led us to investigate the action of added iron. The ferrous ion was of special interest as a result of its role in several reactions catalyzed by ascorbate on some metal, such as iron, which was contaminating reagents, or even the nonheme iron in mitochondria led us to investigate the action of added iron. The ferrous ion was of special interest as a result of its role in several reactions catalyzed by ascorbate. The action of added iron. The ferrous ion was of special interest as a result of its role in several reactions catalyzed by ascorbate, or even the nonheme iron in mitochondria led us to investigate the action of added iron. The ferrous ion was of special interest as a result of its role in several reactions catalyzed by ascorbate.

EXPERIMENTAL PROCEDURE

The swelling of rat liver mitochondria was measured as the turbidity decrease of dilute suspensions at 520 μm (D20). Selected 8-ml test tubes give a light path of approximately 11 mm. Changes in light scattering are correlated with mitochondrial swelling (10, 11). The Bausch and Lomb Spectronic 20 shutter control and entrance slit were modified so that volumes as low as 1 ml could be read. The mitochondria were prepared as previously described (8), maintained at 0° in a concentrated stock suspension, and added in amounts giving an initial D20 of approximately 0.500 (protein = 100 to 150 μg per ml). In most experiments the basic medium was 0.175 M KCl-0.02 M Tris buffer, pH 7.4, equivalent in toxicity to our medium containing 0.33 M sucrose-0.02 M Tris (12). Earlier experiments had indicated that 0.25 M sucrose and its equivalents are probably hypotonic for rat liver mitochondria. Incubations were aerobic at 22-25°. Readings were taken or aliquots withdrawn at time intervals short enough to establish the character of the curves. This was every 2 or 3 minutes in the early time periods.

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All common chemicals were of analytical reagent grade. Ferrous and ferric ammonium sulfate were used as the source of metal ions. Fe++ ion stock solutions were prepared by dissolving the salt in cold unbuffered KCl solutions deoxygenated just before use. All concentrations of Fe++ refer to the initial value in the experiment. Distilled water was redistilled in a two-step all-quartz still. Special chemicals were of the highest grade obtainable from Sigma Chemical Company, California Corporation for Biochemical Research, Nutritional Biochemical Corporation, and the Eastman Company. BSA1 was a crystalline product of the Armour Company. Antimycin A was obtained from the Wisconsin Alumni Research Foundation, and gramicidin from the Schering Corporation. The oligomycin was a gift from Dr. H. A. Lardy and Dr. F. M. Strong, part of the material prepared by the late Dr. W. H. Peterson. Octylguanidine was a gift of Dr. B. C. Pressman. The C-factor was a crude preparation made according to the method of Lehninger (13).

Lipid peroxide formation was followed by a modification of the thiobarbituric acid method (14, 15). A 1-ml aliquot was withdrawn from the incubation mixture and pipetted into an 8-ml Pyrex tube. After the D20 was read, 0.25 ml of 40% trichloroacetic acid and 0.125 ml of 5 M HCl were added. After mixing, 0.25 ml of 2% sodium 2-thiobarbiturate was added promptly. In many experiments the 3 reagents were combined before addition. The use of part HCl instead of all TCA essentially eliminates difficulties from adsorption of color on the protein precipitate. The tubes were topped with 18-cm air-cooled condensers and placed in boiling water for 10 minutes, cooled, centrifuged at 2500 r.p.m. for 10 minutes, and the color read at 532 μm. In plotting lipid peroxide formation in the graphs, the absorbancy reading has been graphed directly rather than by means of a fixed conversion to malonaldehyde equivalents.

RESULTS

Comparison of Media—The swelling of mitochondria produced by Fe++ is usually so slow in 0.33 M sucrose as to be negligible. It occurs more readily in mannitol medium, but KCl is the most satisfactory medium for studying this phenomenon (Fig. 1). For comparison it might be mentioned that with 0.3 mM ascorbate, the lag period before swelling was 30 minutes in sucrose and 20 minutes in KCl. Fe++ has no effect at pH 8.0, possibly

1 The abbreviations used are: BSA, crystalline bovine serum albumin; HSA, human serum albumin; TBA, 2-thiobarbituric acid; DNP, 2,4-dinitrophenol.
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because of rapid conversion to Fe+++ and precipitation of ferric hydroxide.

Characteristics \( D_{520} \) Changes with Fe++—The curves for swelling and lysis of mitochondria with Fe++ ion had a superficial resemblance to those with ascorbate, but detailed study revealed distinct differences. Between concentrations of 1 and 10 \( \mu M \), Fe++ produces a swelling that shows essentially no lag period and a rate that is determined by the concentration of Fe++ (Fig. 2). With 10 \( \mu M \) Fe++ there may or may not be a brief lag period. As the concentration of Fe++ is raised, lag periods increase and the swelling after the lag period starts with a fairly sharp break and proceeds at a rapid rate which is nearly identical for all concentrations of Fe++ between 20 and 100 \( \mu M \) (Fig. 3). Provided sufficient time is allowed, all of these concentrations of Fe++ produce final plateau \( D_{520} \) readings that are essentially the same. The final low \( D_{520} \) reading (0.090 to 0.130) approaches that seen with ascorbate and GSSG + GSH (0.040 to 0.060), but it is distinctly and characteristically higher.

The very low \( D_{520} \) values reached with Fe++ are suggestive of lysis, a term we have used in connection with the effects of ascorbate and GSSG + GSH. However, since the end point with Fe++ may be different from that with the other substances, we have conservatively retained the term swelling. As will be seen in later sections, both phenomena probably occur.

Both Fe++ and Fe+++ Ions Affect Swelling Process—Experiments like that in Fig. 3 suggest that higher Fe++ concentrations have a definite inhibitory effect on the initiation of swelling. This lag-producing effect may disappear as the Fe++ concentration is lowered to optimal levels by conversion of Fe++ to Fe+++.

On the other hand, the shortening of the lag might be caused by the Fe+++ ion formed. In a large number of experiments with 50 \( \mu M \) Fe++, the lag period ranged from 10 to 50 minutes. The longer lag periods were seen when Fe++ stock solutions were most carefully prepared to avoid oxidation.

When combinations of Fe++ and Fe+++ ions are tested, the lag periods and swelling curves are often similar to those that would be seen with the same amount of Fe++ alone. Fig. 4 illustrates fairly typical effects of 1–40 \( \mu M \) Fe++ in the presence of a fourfold excess of Fe+++.

High Fe++ concentrations still inhibit initiation of swelling even in the presence of some Fe+++ ion. In general the lower concentrations of Fe+++ (0 to 40 \( \mu M \)) produce little or no effect, but at high concentrations, it does shorten the lag periods with Fe++ (Fig. 5). There may be an optimal Fe+++ to Fe++ ratio for shortening lag periods, but its definition is difficult because some Fe++ is always oxidized.

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Fe++ or Fe++ + Fe+++-induced swelling of mitochondrial suspensions in 0.33 M sucrose-0.025 M Tris medium as compared to 0.175 M KCl-0.025 M Tris. The concentration of added ions is indicated with each curve. Details as described under "Experimental Procedure."

![Figure 2](http://www.jbc.org/)

**Fig. 2.** (A) Swelling of mitochondria with different Fe++ concentrations. (B) Formation of lipid peroxide in the mitochondrial suspensions. KCl-Tris medium. Each curve is labeled with the \( \mu M \) concentration of Fe++ added initially.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** (A) Swelling of mitochondria and (B) lipid peroxide formation with higher concentrations of Fe++. KCl-Tris medium. Each curve is labeled with the \( \mu M \) concentration of Fe++ added initially. Note that this experiment used only approximately one-half of the standard amount of mitochondria.
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The final \( D_{540} \) plateau or end point reached when ratios of Fe+++ to Fe++ are used is almost identical regardless of whether the lag period is 2 minutes or 40 minutes or the total concentration of iron is 5 or 150 \( \mu \)M. It is the same as that reached with Fe++ ion alone.

Formation of Lipid Peroxide (TBA Color Reaction)—During Fe++- or Fe+++ plus Fe++-induced swelling of mitochondria, there is formation of material which reacts with 2-thiobarbituric acid to give a colored product. The absorption spectrum coincides exactly with that for malonaldehyde (16). Lipid peroxides are believed to be the precursors of this material (15). Formation of TBA color material shows remarkable correlation with the swelling and lytic changes as judged by the \( D_{540} \) reading (Figs. 2-5). During most of any lag period from 3 to 70 minutes, there is no color or a very small rise in color, but usually a minute or two before \( D_{540} \) changes start, the TBA color begins a rapid rise. However, in many experiments the increase in TBA color formation coincides with rather than precedes the beginning of swelling. The increase in lipid peroxides is much more rapid with Fe++ and Fe+++ plus Fe++ than with ascorbate.

The amount of TBA color material formed is determined by the amount of Fe++ added. Between 1 and 10 \( \mu \)M Fe++, the TBA color is roughly proportional to the amount of iron. The rise of TBA color occurs in a few minutes and then stops, as though the Fe++ were exhausted (10). It then remains unchanged for long periods instead of continuing to rise as with ascorbate. A second addition of Fe++ will produce a second rise (Fig. 6). With very low concentrations of Fe++, the amount of lipid peroxide is similar to that seen with 0.3 mM ascorbate lysis. At the concentration of Fe++ is increased, the amount of TBA color increases, even though a long lag period may precede the rise. With the highest concentrations of Fe++, the yield of TBA color is limited by the amount of unsaturated lipid of the mitochondria (Fig. 3). Fe+++ ion seems to have little or no effect on the amount of lipid peroxide formed.

The inhibitory or delaying effects of high Fe++ concentrations on swelling appear to be always associated with inhibition of lipid peroxide formation. Another type of experiment demonstrating such inhibitory effects is shown in Fig. 6; 10 to 20 \( \mu \)M Fe++ added late after 4 \( \mu \)M Fe+++ at zero time gives a proportional rise in lipid peroxide and an accelerated swelling. At 50-100 \( \mu \)M there is only a small rise in lipid peroxide. This is accompanied by a brief acceleration of swelling followed by some inhibition.

Effects on Fe+++-Induced Swelling—Our earliest observations on Fe+++-induced swelling raised the question whether or not complete lysis occurred, for the plateau of \( D_{540} \) was always distinctly higher than with ascorbate. This could be due to retention of structure, but it might also result from aggregation due to Fe++.

Phase microscope examination of mitochondria in samples withdrawn at various times during swelling experiments revealed two difficulties which prevented completely satisfactory results. In the KCl medium there was some tendency for mitochondria to aggregate in groups of 3 to 6. This tendency appeared to be increased somewhat by Fe++. The second difficulty was that when suspensions of mitochondria were placed between the coverslip and microscope slide, an appreciable number of the mitochondria became fixed on the surface of the glass and underwent very little further change once this occurred.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** (A) Swelling of mitochondria and (B) lipid peroxide formation with the Fe+++ to Fe++ ratio constant at 4:1. The curves are labeled with the \( \mu \)M concentration of Fe+++ + Fe++.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** (A) Swelling of mitochondria and (B) lipid peroxide formation with 35 \( \mu \)M Fe++ without and with various concentrations of Fe+++ ion added. KCl-Tris medium. With less than 40 \( \mu \)M Fe++ the curves were identical with that for 35 \( \mu \)M Fe++ alone.

We are indebted to Miss Claire Hollingsworth for many phase microscope observations and for the tests on reversibility of swelling by ATP + Mg + BSA + C-factor.
However, despite the difficulties mentioned, some swelling of mitochondria under the influence of iron could be followed by observing both single mitochondria and some of the aggregates. The beginning and progression of swelling was well correlated with the DszO change in the suspension. Swelling was followed by a variety of changes. Some mitochondria seemed to lyse quite clearly and just disappear. Others left distinct membrane outline ghosts or small clumps of membrane material.

Reversal of Fe**+-induced Swelling—ATP + Mg++ + BSA, with which Lehninger (17) has demonstrated reversal of mitochondrial swelling induced by a variety of agents, was studied for possible reversal of Fe**+-induced swelling. When this combination is added after the DszO plateau is reached in the usual (pH 7.4) Fe**+ swelling experiment, no reversal whatever is seen. The addition of a crude C-factor preparation either initially or after swelling made no difference. In two experiments where the ATP mixture (5 mM ATP + 2 mM Mg++, 1 mg per ml of BSA) was added at DszO = 0.225, before the plateau was reached, a small contraction was seen. The partial reversal may represent the response of mitochondria that are not yet completely swollen. Because most contraction studies with C-factor addition were done at higher pH, we carried out experiments with swelling induced by Fe**+ at pH 7.4, followed by adjustment of the pH to 8.0. Still there was no reversal with the ATP mixture. The presence of added iron did not inhibit in any way the substantial contractions seen when ATP + Mg++ + BSA was added after swelling produced by other agents.

Acceleration of Lipid Peroxide Formation and Swelling Induced by Fe**+-Gulonolactone, α-ketoglutarate, and GSSG were studied because they reduce or eliminate the lag period and accelerate lysis with ascorbate. L-Gulonolactone, 5 and 10 mM, produced only a slight shortening of the lag period with 50 μM Fe**, but 10 mM α-ketoglutarate induced a sharp rise in TBA color and virtually eliminated the lag before swelling. GSSG at 1 mM, which greatly shortens lag periods with ascorbate, did not produce swelling or lipid peroxide with 50 μM Fe**+

FIG. 6. (A) Swelling of mitochondria and (B) lipid peroxide formation with 4 μM Fe**+ added at zero time and various concentrations of Fe**+ (0, 10, 20, 50, and 100 μM) added at 11 minutes. By 11 minutes lipid peroxide formation induced by the 4 μM initial Fe**+ had ceased.

FIG. 7. The effect of GSSG alone and in combination with Fe**+ on swelling of mitochondria and lipid peroxide formation. The 100 μM Fe**+ used had a lag period of over 60 minutes in this experiment. The numbers on the right hand ordinate after each curve are the TBA color A323 readings for lipid peroxide determinations made at 60 minutes.

Within a 90-minute period. However, higher concentrations of GSSG reduce the lag period to 1 minute as the concentration is raised to 20 mM (Fig. 7). Such large amounts of GSSG alone result in some TBA color and a slow swelling. However, the primary determinant of the amount of TBA color is still the amount of Fe**+

Inhibitors of Lipid Peroxide Formation and Swelling Induced by Fe**+ It was of interest to test several types of inhibitors for the following reasons: (a) Electron transport chain inhibitors block phosphate- and thyroxine-induced swelling. (b) Complete uncoupling of phosphorylation blocks phosphate- and thyroxine-induced swelling. (c) Substances that destroy H2O2 or act as anti-oxidants might inhibit swelling associated with lipid peroxidation. (d) Swelling per se might render mitochondrial lipids available for rapid peroxidation.

Electron Transport Chain Inhibitors—NaCN, 1 mM, which blocks phosphate-induced swelling, ascorbate induced lysis, and GSSG plus GSH-induced lysis, and which has been reported to inhibit lipid peroxidation (18), produces only partial inhibition (10 to 50%) of Fe**+-induced swelling. Antimycin A blocks phosphate-induced swelling at 0.1 μM, but 3 to 4 μM are required to prevent ascorbate induced lysis. Fig. 8 shows that with Fe**+ even the higher concentration of antimycin A only lengthens the lag period and slows the rate of swelling. The formation of lipid peroxide is delayed, slowed, and the total yield is lowered. Results are similar with Fe**+ + Fe**+. The mitochondria were exposed to antimycin A 3 or 4 minutes before the addition of the iron. SN 5949, 3 μM, and 3 mM amytal produced no inhibition of Fe**+ plus Fe**+-induced swelling.

Phosphorylation Inhibitors—DNP in concentrations as high as 0.3 mM had no detectable effect on the swelling or lipid peroxidation produced by Fe**+.

Gramicidin at 1 μg per ml delayed
Fe\textsuperscript{2+}-Induced Swelling of Mitochondria

When a crude C-factor preparation was added to give 1 or 2 mg of protein per ml, the lag period was often unchanged, but the rate of swelling was usually greatly slowed, a fact that agrees with a slower rise of lipid peroxides. Such effects might result from nonspecific effects of the large amount of protein added, but it is of interest that crystalline BSA and HSA at similar concentrations of protein did not produce this effect.

**Surface Active Agents**—Fairly rapid swelling or lysis is caused by 0.1 mM Na oleate, 0.1 mM Na linoleate, 0.3 mM Na laurel sulfate, 0.05% saponin, and 1 mM gramicidin, but no TBA color material is formed during this swelling. When lipid peroxides have been previously formed through the action of Fe\textsuperscript{2+}, these substances do not decrease the yield of color in the TBA method. However, when these substances are present and have produced swelling, the formation of lipid peroxides upon the subsequent addition of Fe\textsuperscript{2+} is reduced in the case of saponin and gramicidin, slight in the case of laurel sulfate, and completely absent with the fatty acids. Undoubtedly the physical state of the mitochondrial lipids is altered drastically so that peroxidation may be inhibited or not lead to malonaldehyde. Formation of undissociated or insoluble iron salts may explain a number of these observations.

**Reduced Glutathione**—Because GSSG shortened the lag period with Fe\textsuperscript{2+}, and because of similarities between the action of GSH and Fe\textsuperscript{2+} on mitochondria, a range of GSH concentrations in combination with iron was tested. Between 1 and 3 mM GSH usually does not have any marked effect on the lag period or rate of swelling with 50 mM Fe\textsuperscript{2+} or Fe\textsuperscript{3+} plus Fe\textsuperscript{2+}, but the end point or final \(D_{500}\) plateau is the low one characteristic for GSSG + GSH instead of that with Fe\textsuperscript{2+} (Fig. 9). With these

![Fig. 8. The effect of antimycin A on Fe\textsuperscript{2+}-induced swelling of mitochondria (A) and lipid peroxide formation (B). The circumstances of this experiment were such that part of the added 50 \(\mu\)M Fe\textsuperscript{2+} was oxidized before the mitochondria were added.](http://www.jbc.org/)

lipid peroxide formation but caused swelling. Octyl guanidine at 100 to 200 \(\mu\)M is without effect against Fe\textsuperscript{2+}. Oligomycin (1 to 5 \(\mu\)g per ml) did not consistently reproduce the inhibition reported earlier (1).

**Phosphate and Substrates**—Inorganic phosphate, 5 mM, with or without substrates, seems to prevent the action of Fe\textsuperscript{2+}, yielding a mitochondrial swelling curve that is nearly identical with that for phosphate + substrate (\(\beta\)-hydroxybutyrate). Possibly there is a specific effect of phosphate on lipid peroxidation in mitochondria, but simple removal of Fe\textsuperscript{2+} as insoluble salts must be considered. \(\beta\)-Hydroxybutyrate, succinate, pyruvate, and oxaloacetate had essentially no effect at 2 mM concentration.

**Antioxidants**—\(\alpha\)-Tocopherol at 10 \(\mu\)M produced nearly complete inhibition of lipid peroxidation and swelling caused by 50 \(\mu\)M Fe\textsuperscript{2+}. Vitamin A, not ordinarily considered an antioxidant, showed similar effects at 10 to 100 \(\mu\)M. Vitamin K\textsubscript{1} was completely without effect. Fifty \(\mu\)M Mn\textsuperscript{3+}, a powerful inhibitor of lipid peroxidation (1, 19), produced nearly complete inhibition of lipid peroxidation and mitochondrial swelling induced by 50 \(\mu\)M Fe\textsuperscript{2+}. Serotonin (5-hydroxytryptamine), 100 \(\mu\)M, also an inhibitor of lipid peroxidation (20), greatly slowed and reduced both lipid peroxide formation and swelling with Fe\textsuperscript{2+}.

**Catalase, C-Factor, and BSA**—When crystalline catalase was added in concentrations between 50 and 115 \(\mu\)g per ml, there was usually some delay in the appearance of swelling with either Fe\textsuperscript{2+} or Fe\textsuperscript{3+} to Fe\textsuperscript{2+} mixtures. The rate was not slowed very much. The appearance of lipid peroxide is delayed a corresponding amount and the total yield was somewhat lower.

![Fig. 9. The effect of GSH on mitochondrial swelling (A) and lipid peroxide formation (B) with 25 \(\mu\)M Fe\textsuperscript{3+} + 25 \(\mu\)M Fe\textsuperscript{2+}.](http://www.jbc.org/)
low concentrations of GSH, the lipid peroxide continues to increase to values several times that seen with Fe++ alone. Higher concentrations of GSH (5 to 10 mM) markedly increase the lag period and slow the rate of swelling. During the long lag there is only a slight rise in lipid peroxide, but a rapid rise is associated with the rapid phase of swelling. When 10 mM GSH is added after the action of iron is complete (Fig. 9), there is an immediate resumption of lipid peroxide formation and the

**DISCUSSION**

Extreme degrees of swelling with Fe++ are seen only in KCl medium and not in sucrose. This would explain why such effects have not been seen in many mitochondrial studies. In KCl, lipid peroxide is formed and more extensive swelling occurs. Possibly sucrose complexes with Fe+++ or Fe++ ions to a degree which prevents catalysis of lipid peroxidation. However, a more likely possibility is that sucrose is directly protective to the mitochondria. In sucrose, the early structural and permeability changes seen with KCl may not occur. The existence of such differences is clearly indicated in the much greater tendency for mitochondria to show spontaneous swelling in KCl. These changes might permit Fe++ to penetrate to the site of certain unsaturated lipids. Sucrose may actually combine with lipoprotein in a way to prevent peroxidation of the lipids. The lytic action of gramicidin on mitochondria is seen in KCl but not in sucrose. Sucrose inhibition of lipoprotein enzymes of energy transfer or transphosphorylation systems in mitochondria has been reported (21). Sucrose does not have any such marked inhibitory effect on ascorbate or GSSG-to-GSH induced swelling, but there are indications that these agents affect structures or lipids not altered by Fe++.

The question of whether Fe+++ induced swelling is identical with ascorbate and with GSSG + GSH-induced swelling (22) is a fundamental one. All are associated with lipid peroxide formation and are more extensive than electron transport chain-supported swelling. Moreover, all are accelerated by GSSG and inhibited by similar concentrations of several inhibitors. A metal may be involved in ascorbate swelling (2), but whether or not it is Fe+++ ion in the medium is unknown. Heme or nonheme iron in the mitochondrial structure must be considered seriously, for ascorbate and GSH swelling, in contrast to that with added Fe++, readily occur in sucrose and at pH 8.0.

Fe+++ induced swelling shows one distinct difference from the others. The final D$_{20}$ plateau is characteristically higher, suggesting the possibility of less complete disintegration. The higher plateau with Fe+++ is not determined by the amount of Fe++ added or lipid peroxide formed. The fact that all concentrations of Fe++ from 5 to 100 μM produce the same final D$_{20}$ makes it doubtful that aggregation of residual material or precipitation of iron hydroxides can be the explanation for this difference from ascorbate and GSSG + GSH. Since a further fall of the D$_{20}$ to the lower plateau is seen when GSH is added after the higher plateau is reached with iron, it is possible that GSSG + GSH and ascorbate cause rupture of structural links or peroxidation of lipids at sites not reached or affected by Fe++.

Fe++ plays a direct role in the structural alteration of mitochondria, for the damage is directly related to the amount of Fe++ added. Lipid peroxidation is a likely mechanism (15, 19). The exact role of Fe++ in the mechanisms of such reactions may still be uncertain, but several very recent studies increase our knowledge in this area (23–20). In the mitochondrial system the reaction appears to stop when Fe++ becomes depleted. At present some stoichiometric reaction appears likely, but function as an oxidation-reduction catalyst may not be entirely ruled out.

Under certain conditions, lag periods are largely removed when Fe++ ion is added with the Fe++. There may be an optimal ratio of 5 or 10 to 1 for Fe++ to Fe++. This is consistent with the hypothesis of function as an oxidation-reduction couple and the earlier demonstration that GSSG and GSH are both required for GSH swelling (22). No requirement for dehydroascorbate has been demonstrated with ascorbate lysis, but this might be because monodehydroascorbate is the member of the oxidation-reduction couple. If a ratio of Fe+++ to Fe++ is important, it probably determines the length of the lag period rather than the rate of swelling. An alternative interpretation is that high concentrations of Fe+++, GSSG, and α-ketoglutarate shorten the lag period by producing changes which must precede the action of Fe++ ion.

Peroxide formation in unsaturated lipids was demonstrated as a likely cause of Fe++ -induced swelling by the close correlation between swelling or lysis and the thiobarbituric color reaction. However, a large production of lipid peroxide does not always precede swelling. Many times it occurs simultaneously. Therefore, the fundamental question is whether lipid peroxidation is the cause or the result of swelling. Our data suggest that it is the cause of a permeability or structural change. Possibly the lipid peroxidation and the structural change are simultaneous and synonymous. Addition of mitochondrial material that has undergone peroxidation to fresh mitochondria does not produce swelling. Most inhibitors prevent lipid peroxidation to exactly the same degree that they inhibit swelling. Any apparent discrepancies between lipid peroxide formation and swelling could be due to several factors: (a) Unsatisfactory methods for detecting early formation of lipid peroxides or early swelling changes. (b) Some swelling supported by endogenous substrate may occur during lag periods with Fe++. (c) Various reactions may destroy part of the lipid peroxides or the malonaldehyde being formed. Several workers (15, 10, 24, 27, 28) have observed lipid peroxide formation in mitochondria under various conditions. These changes are accompanied by alterations in electron transport and phosphorylation enzymes, which are known to represent organized systems in the mitochondrial membranes.

The amount of lipid peroxide formation required to cause permeability changes in the mitochondrial membrane is not large. With low concentrations of Fe++ there is a small amount of thiobarbituric color material formed in the first few minutes and then the reaction stops. Swelling begins at the same time, and the rate of swelling is related to the amount of lipid peroxide which was formed. Interestingly, the swelling (D$_{20}$ change) continues almost as a straight line until the final plateau is reached. No further lipid peroxidation occurs, yet swelling progresses over 30 to 60 minutes. This suggests that the swelling is the result of initial damage and permeability change when the lipid peroxide is formed. It would appear that all of
The mitochondria in the suspension may have their permeability altered to approximately the same degree during the initial action of the Fe²⁺. The amounts of lipid peroxide formation that give moderate rates of swelling with Fe²⁺ are of the same order as those seen with ascorbate and GSH for similar rates of swelling.

The TBA color method unfortunately does not measure lipid peroxides per se, but only those which decompose to release malonaldehyde. However, the correlation between the initiation and the rates of swelling and formation of TBA color material is excellent, although there is no evidence that malonaldehyde per se causes swelling. Making certain assumptions, it is possible to calculate the approximate number of lipid molecules affected for any given rate of swelling. If one assumes a yield of only one malonaldehyde per molecule of lipid, this means that one molecule of lipid in 600 has undergone peroxidation with the amounts of TBA color corresponding to slower rates of swelling, such as seen with 3 to 5 μM Fe²⁺. It should be noted that 10 μmoles of Fe²⁺ yield approximately 1 μmole of malonaldehyde.

The fatty acids known to be present in rat liver mitochondria and most likely to undergo peroxidation with the release of malonaldehyde are arachidonic and docosahexaenoic, 22:6 (25, 29, 30). Other substances like sialic acid (31), heme compounds, and pyrimidines are present and can give colored compounds with TBA, but only when the test is run with oxidizing agents present (32).

Swelling per se does not result in lipid peroxide formation, for swelling induced by phosphate, fatty acid, detergents, and aging in KCl is not associated with lipid peroxide formation. A small amount of substrate-induced swelling is not necessary for Fe²⁺ to penetrate and produce its action, as the effect of Fe²⁺ is almost identical in fresh- and in substrate-depleted mitochondria.

Although catalase at higher concentrations produces some delaying effect on lipid peroxide appearance and on swelling, this does not prove that H₂O₂ is a precursor of lipid peroxide. Presumably catalase would remain outside mitochondrial membranes, so it is interesting that added H₂O₂ and H₂O₂ generated with glucose oxidase produces no effect like that of Fe²⁺ (2, 15, 19). Catalase might act as a peroxidase and remove lipid peroxides until becoming inactivated (33). Very recently Neubert, Wojtczak, and Lehninger (34) have reported that C-factor is GSH peroxidase and catalase.

The mechanism by which high concentrations of Fe²⁺ inhibit or delay the appearance of TBA color material is unknown. It is noteworthy that similar delays are seen with high ascorbate, GSH, and some other reducing agents. Such substances can react with malonaldehyde, but it is more likely that they react with intermediates in the formation of lipid peroxides, as has been suggested for antioxidant (35). Whatever early steps of lipid peroxidation take place before such reactions, they do not suffice for permeability changes, since swelling is always delayed until TBA color (malonaldehyde) begins to appear.

Although cyanide and antimycin A show inhibitory effects on lipid peroxide formation with Fe²⁺, it is unlikely that electron transport is involved in this process. Electron transport would be completely inhibited at one-tenth of the concentrations which affect lipid peroxidation. These inhibitors might exert their effect by (a) complexing trace metal ions, (b) reacting with nonheme iron in the mitochondrial structure (30), (c) reacting with heme proteins (33), (d) reacting with mitochondrial lipoproteins, (e) serving as anti-oxidants, or (f) keeping the mitochondria impermeable to ascorbate, GSSG + GSH, and Fe²⁺. Antimycin A at 4 μM could not complex all of the iron added in these experiments.

The experiments described here demonstrate a clear similarity between Fe²⁺-induced swelling of mitochondria and that seen with GSSG + GSH or ascorbate, although the details of their action are not identical. On certain points the effect of Fe²⁺ can be studied more quantitatively. The peroxidation of lipids in the mitochondrial membranes occurs rapidly at temperatures below body temperature, especially when GSSG and certain other substances are present. Lipid peroxide changes may be the key to GSH swelling and the requirement of C-factor for contraction (1, 34). The possible relation of these changes to the control of mitochondrial swelling and contraction by electron transport and phosphorylation enzymes must be determined by further studies. Phosphate-induced electron transport supported swelling shows different characteristics, and no peroxidation of lipids has been detected under our conditions. However, Corwin (37) has recently shown that lipid peroxide formation may result from electron transport chain activity if the tissue is deficient in vitamin E. Thus, the possible role of lipid peroxide intermediates in electron transport, phosphorylation, and membrane permeability control is a subject for investigation.

**SUMMARY**

1. In KCl medium, Fe²⁺ ion induces a swelling and lysis of mitochondria which is quite different from electron transport-supported swelling. The light scattering of suspensions falls to a low level, approaching that seen with ascorbate and GSSG + GSH.

2. With 1 to 10 μM Fe²⁺ there is no lag period before swelling. As the Fe²⁺ concentration is increased to 50 μM, the beginning of swelling is delayed as long as 40 to 60 minutes. Fe³⁺ ion, GSSG, and α-ketoglutarate greatly shorten this lag period.

3. Although cyanide and antimycin A partially inhibit Fe²⁺-induced swelling, the concentrations required are higher than those necessary for respiratory chain inhibition. Uncouplers of oxidative phosphorylation, such as DNP, have no effect.

4. Lipid peroxide formation is very well correlated with swelling and lysis. Accelerators and inhibitors of this type of swelling affect lipid peroxide formation in an exactly parallel fashion.

5. Formation of lipid peroxide, possibly in some lipoproteins associated with electron transport and energy transfer or phosphorylation, causes first permeability increase and swelling. Extensive lipid peroxidation leads to lysis and disintegration.

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