Studies on the Mechanism by Which Anaerobiosis Prevents Swelling of Mitochondria in Vitro: Effect of Electron Transport Chain Inhibitors*

F. EDMUND HUNTER, JR., JEROME F. LEVY, JOAN FINK, BEVERLY SCHUTZ, FRANCISCO GUERRA, AND ARYEH HURWITZ

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

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Several years ago it was observed that strict anaerobiosis prevented the swelling of isolated liver mitochondria. This was true for spontaneous swelling and for swelling induced by phosphate, certain metal ions, and other substances (1–3). This observation created a sharp paradox with the generally accepted view that adenosine triphosphate or active phosphorylation was essential in maintaining mitochondrial structure and integrity (4, 5), for under anaerobic conditions one would expect the mitochondrial ATP level to fall to low levels. Subsequent studies (6–8) have indicated that anaerobiosis protects against virtually every agent known to induce mitochondrial swelling by a selective action. In addition, the unexpected observation that some uncoupling agents, like 2,4-dinitrophenol, can prevent mitochondrial swelling has been firmly established as fact (7, 9–11).

In our earlier experiments (1) we concluded that blocking the electron transport chain at cytochrome oxidase with NaCN did not result in protection against swelling, whereas anaerobiosis did. This resulted in the suggestion that the effect of anaerobiosis was to prevent some easily oxidizable group in the mitochondrial membrane from undergoing the oxidative change essential for the permeability change responsible for swelling. In reinvestigating this question, Lehninger and Ray (6) observed good protection with 10^{-3} M NaCN. They postulated (6, 7) that the action of anaerobiosis and NaCN resulted in keeping one or more components of the electron transport chain in the reduced form.

This paper reports additional studies which bear on the possible role of the state or the activity of the electron transport chain in controlling the permeability of the mitochondrial membrane and thereby determining mitochondrial swelling. Using dilute mitochondrial suspensions we have obtained effects with NaCN very similar to those reported by Lehninger and Ray (6, 7). The exact reason for the discrepancy with the earlier results with more concentrated suspensions has not been established. Studies with other blocking agents for the electron transport chain and with various substrates suggest that active electron transfer or some dynamic state is essential for swelling rather than just the oxidized state. Blocking the electron transport chain does block the effect of most agents which produce swelling, but differences in the kinetics and the extent of swelling with certain substances suggest that several fundamental changes may be involved. While this manuscript was being prepared, Lehninger and Schneider (8) reported that cyanide, antimycin A, and Amytal® block thyroxine- and phlorizin-induced swelling.

EXPERIMENTAL

Materials and Methods

Male rats, 100 gm. each, were obtained from the Holtzman Rat Company, Madison, Wisconsin. They were fed Purina laboratory chow ad libitum and usually weighed between 125 and 250 gm. when used. The liver mitochondria were isolated in 0.33 M sucrose by a slight modification (12) of the method of Schneider (13). They were washed twice and suspended in 0.33 M sucrose (mitochondria from 1 gm. of liver in 2 ml.). This stock suspension was kept at 0°C. Swelling experiments were carried out as promptly as possible, preferably within the 1st hour after the isolation was completed. After 2 hours or more the preparations showed signs of changes in sensitivity to agents which cause swelling.

Swelling was followed by changes in the optical density at 590 m\text{\textmu}L. Numerous earlier workers have used similar procedures. Tedeschi and Harris (14) and others (15, 16) have published studies on the correlation between optical density changes and mitochondrial volume changes. Anaerobic experiments were carried out in Thunberg tubes which could be read directly in the spectrophotometer. Mitochondria from 40 to 75 mg. of liver diluted to 3.5 ml. give an initial optical density reading of 0.500 to 0.700. In general, the initial readings have not been corrected for small variations caused by the fact that the tubes were not perfectly matched.

The basic medium was 0.33 M sucrose containing 0.025 M Tris, pH 7.4. The incubation was at room temperature, 22–24°C. In most cases all substances were placed in the tube, and the experiment was started by adding the mitochondria, mixing by inversion, and taking the first reading. This procedure proved to be more convenient and satisfactory than adding the swelling-producing agent last. However, it could not be used when it

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1 F. E. Hunter, Jr., unpublished experiments.
was essential to expose the mitochondria to a blocking agent 5 or 10 minutes before the addition of the swelling-inducing agent, as is the case with antimycin A and SN 5949. The suspension in the tube was remixed in the longer experiments, but no significant optical density change occurred on remixing. Experiments with NaCN were carried out with filled and capped tubes to minimize loss of HCN.

For anaerobic experiments the mitochondria were suspended in 0.3 ml. of 0.33 M sucrose and placed in the hollow stopper of Thunberg tubes, with all other constituents in the main tube. The entire evacuation and flushing with nitrogen was carried out at 3°. Repeated shaking of the contents of the tubes over a 20-minute period is essential to remove the dissolved oxygen. The tubes were then warmed to 24° in a water bath and the contents mixed.

Pyridine nucleotide determinations were carried out by the method of Lowry et al. (17). Because of the well known fact that metal ions can influence mitochondrial swelling (10, 18), the highest purity chemicals and water redistilled in Pyrex glass were used in all cases.

RESULTS

Effect of Electron Transfer Chain Inhibitors on Phosphate-induced Swelling

1. Cyanide—Figs. 1 and 2 illustrate experiments with NaCN. The swelling of mitochondria in the presence of phosphate was blocked by 2 mM NaCN. Because this relatively high concentration of NaCN might react with components other than cytochrome oxidase, lower concentrations of cyanide were investigated. At 0.1 mM NaCN was not always adequate to prevent completely spontaneous swelling or swelling induced by low concentrations of phosphate. As the concentration of phosphate was increased the amount of NaCN required to prevent swelling increased. One mM NaCN was necessary to block completely the effect of 10 mM phosphate. Although the general concentration ranges of NaCN permit the tentative conclusion that it acts by blocking electron transport, it is well to remember that 1 mM NaCN may also reduce disulfide bonds or regenerate sulfhydryl groups from their metal complexes (19). The amount of phosphate used affects the amount of cyanide required in a manner suggestive of a competitive effect.

2. Azide—Spontaneous swelling was largely but not always completely prevented by 2 mM sodium azide. This concentration decreased the rate of swelling due to 2 mM phosphate by about 50 per cent but was unable to block the action of 10 mM phosphate. Higher concentrations of azide, 10 mM, were able to block spontaneous swelling and swelling induced by 2 mM phosphate (Fig. 3), but were unable to block the effect of 20 mM phosphate.

3. Antimycin A—Antimycin A in concentrations which inhibit electron transport (1 to 2 μg per ml.) prevented swelling with all concentrations of phosphate (Fig. 4). As is the case with electron transport, the antimycin A inhibition of swelling is more effective if a little time is allowed for it to react with the tissue.

4. SN 5949—This agent, which is reported to block the electron transport chain between cytochromes b and c (Ball et al. (20)), like antimycin A, prevented swelling induced by a considerable range of phosphate concentrations (2 to 20 mM, Fig. 4).

5. Amytal—This inhibitor, which blocks the electron transfer chain between pyridine nucleotides and flavoprotein in intact mitochondria (21–23), results in pyridine nucleotides remaining reduced and the remainder of the electron transport chain becoming oxidized under aerobic conditions (21, 24). It may be seen from Figs. 5, 6 and 7 that Amytal, in the same concentrations (2 to 4 mM) which provide a fairly complete block of electron transfer, prevented spontaneous and phosphate-induced swelling of mitochondria.
I

\[ \alpha\text{-ketoglutarate} + \text{NH}_4 + \text{DPNH} \]

\[ + \text{H}^+ \rightarrow \text{glutamate} + \text{DPN}^+ \quad (1) \]

Acetoacetate + DPNH + H\(^+\) \rightarrow \beta\text{-hydroxybutyrate} + \text{DPN}^+ \quad (2)

The data in Table I represent one of several experiments which indicate that 80 to 100 per cent of the pyridine nucleotide (direct chemical determination) can be converted to the oxidized form, yet swelling does not occur if an Amytal block of the respiratory chain is present (Fig. 6). Therefore, reduced pyridine nucleotides do not seem to be essential for prevention of swelling.

**Effect of Electron Transfer in Presence of Amytal Block**

Since Amytal blocks the electron transport chain between pyridine nucleotides and flavoprotein but does not inhibit succinate oxidation, it is possible to feed electrons into the system above the point of the Amytal block by means of succinate (21, 23). These electrons enter the main electron transport chain at cytochrome b. Other substances which may reduce certain components of the respiratory chain are ascorbate, GSH, cysteine, sulfit., and others. The effect of these and related substances on the swelling of mitochondria in the absence and in the presence of Amytal, antimycin A, SN 5949, azide, and cyanide was investigated.

1. **Succinate**—The swelling-inducing effect of succinate was observed by Raafbaub (26, 27) and confirmed by Tapley (10). Raafbaub suggested that succinic oxidase might play a special role in the permeability of the mitochondrial membrane. The concentration of succinate which produces swelling (1 to 2 mM) is sufficiently low to indicate that impurities are not likely to be the explanation.

Although the swelling induced by phosphate, \(\beta\text{-hydroxybutyrate} \), or glutamate was blocked by 2 to 4 mM Amytal, the swelling induced by succinate was not blocked (Fig. 7). Agents which do block electron transport from succinate, such as antimycin A, SN 5949, azide, cyanide, and malonate, did prevent the swelling caused by succinate (Fig. 8). The blocking of the swelling-producing effect of succinate by malonate (Fig. 9) was first observed by Raafbaub (27) and was confirmed by Tapley (10). Malonate inhibition in the presence of Amytal emphasizes the fact that succinate oxidation must occur for swelling to occur under these conditions (Fig. 10).

Malonate had little effect on swelling produced by phosphate, malate, fumarate, and \(\beta\text{-hydroxybutyrate} \) (Figs. 11 and 12).
Sometimes malonate showed small inhibitions with sulfite and ascorbate. Possibly some of the electrons from these agents enter the electron transport system via the flavoprotein of succinic dehydrogenase. Malonate gave a partial block of spontaneous swelling, an effect which may be due to blocking the oxidation of some endogenous succinate. Since most of the spontaneous swelling appears to be due to oxidations sensitive to Amytal, malonate would not be expected to give a complete block in preparations with high rates of spontaneous swelling. When the spontaneous swelling is eliminated with Amytal, malonate produces a complete block of succinate-induced swelling (Fig. 10).

2. Ascorbate—In low concentrations (0.1 to 0.3 mM) ascorbate caused rapid swelling of mitochondria in the presence of an electron transfer block by Amytal (Fig. 13). Isoascorbate produced similar effects but dehydroascorbate was much less effective (Fig. 14). Characteristically there was a lag period before the onset of rapid swelling. The swelling produced by ascorbate was prevented completely by anaerobiosis, EDTA, 8-hydroxyquinoline, Mn++, ATP + Mg++ + bovine serum albumin, and higher concentrations of ascorbate (15 to 20 mM) (Figs. 13 and 14). Inhibitions by antimycin A, SN 5949, and NaCN ranged from slight to nearly complete in different preparations. More consistent inhibition by antimycin A and by SN 5949 was produced by somewhat higher concentrations (Fig. 15). Small inhibitory effects were sometimes seen with azide, Mg++, and dehydroascorbate. Amytal, dinitrophenol, and malonate usually had no effect.

3. Glutathione and Cysteine—Glutathione, 5 to 6 mM, and cysteine, 6 mM, produced swelling similar to that seen with low concentrations of ascorbate. Oxidized glutathione produced only a slow steady swelling without a sharp break. It may be partially reduced by the mitochondria. Glutathione-induced swelling differed somewhat from that produced by ascorbate in sensitivity to inhibitors. In general it was more sensitive to antimycin A, SN 5949, NaCN, and possibly Amytal, but less sensitive to 8-hydroxyquinoline. Glutathione may feed electrons into the electron transfer chain in the pyridine nucleotide-flavin region to a greater extent than does ascorbate. Azide,}

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Reoxidation of pyridine nucleotide in presence of Amytal

β-OH-Butyrate and Amytal were added at zero time, 5 mM α-ketoglutarate + 5 mM NH₄Cl at 3 minutes, and 5 mM phosphate at 13 minutes.

<table>
<thead>
<tr>
<th>Initial additions</th>
<th>Pyridine nucleotide*</th>
<th>Optical density 520 mµ</th>
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<tbody>
<tr>
<td></td>
<td>3 min. after addition of</td>
<td>Total PN⁺⁺</td>
</tr>
<tr>
<td></td>
<td>β-hydroxybutyrate</td>
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<tr>
<td>0.5 mM β-Hydroxybutyrate</td>
<td>4.1</td>
<td>1.9</td>
</tr>
<tr>
<td>0.5 mM β-Hydroxybutyrate + 4.6 mm Amytal</td>
<td>3.9</td>
<td>1.0</td>
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<tr>
<td>0.5 mM β-Hydroxybutyrate</td>
<td>α-Ketoglutarate + NH₄Cl added</td>
<td></td>
</tr>
<tr>
<td>0.5 mM β-Hydroxybutyrate + 4.6 mm Amytal</td>
<td>α-Ketoglutarate + NH₄Cl added</td>
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* µmoles X 10⁻⁴ per 29.5 µl of mitochondrial suspension.
† PN⁺⁺, oxidized pyridine nucleotide.

![Fig. 8. Effect of EDTA, antimycin A, Na₃, and malonate on swelling induced by succinate.](http://www.jbc.org/)

**Fig. 8.** Effect of EDTA, antimycin A, Na₃, and malonate on swelling induced by succinate.

![Fig. 9. Effect of malonate on swelling induced by succinate.](http://www.jbc.org/)

**Fig. 9.** Effect of malonate on swelling induced by succinate.

![Fig. 10. Effect of malonate on swelling induced by succinate in the presence of Amytal.](http://www.jbc.org/)

**Fig. 10.** Effect of malonate on swelling induced by succinate in the presence of Amytal.
Additional Substances which Produce Swelling

1. Substrates—According to our working hypothesis, spontaneous swelling and phosphate-induced swelling would be dependent on the presence of endogenous substrates. Addition of substrates which can be oxidized by mitochondria should promote swelling. Raafaub (27, 28) and Tapley (10) have reported that α-ketoglutarate, fumarate, malate, and glutamate increase the rate of swelling. These results have been confirmed and several additional substrates investigated.

Glutamate—At 1 to 3 mM, this substrate hastened the swelling of the mitochondria (Fig. 10). This effect was blocked by anaerobiosis and by those electron transport chain inhibitors which prevent pyridine nucleotide-dependent oxidations (antimycin A and Amytal). Ten mM malonate did not block glutamate-induced swelling.

β-Hydroxybutyrate—β-Hydroxybutyrate, 1 or 2 mM, caused rapid swelling resembling that seen with phosphate (Fig. 12). This swelling was prevented by anaerobiosis, cyanide, antimycin A, and Amytal. Malonate had essentially no effect. Electrons note should be taken of the fact that ascorbate, glutathione, and cysteine produced swelling that either was different in character or contained an additional component, for what appeared to be virtually complete lysis occurred, with the optical density approaching zero (Figs. 13, 14, and 15).

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10 mM did not block swelling caused by glutathione. There may be some direct interaction.

The results with succinate, ascorbate, and glutathione are completely consistent with the working hypothesis that restoration of electron transfer through the cytochromes (by reduction of cytochrome b or cytochrome c) permitted swelling to occur even in the presence of the Amytal block. However, special
from β-hydroxybutyrate enter the electron transfer chain via pyridine nucleotides. β-Hydroxybutyrate has the advantage that the oxidation product, acetoacetate, does not undergo further oxidation in liver mitochondria (9).

Fumarate-Malate—With 1 mM fumarate there was swelling which was completely blocked by Amytal (Fig. 11). This clearly indicates that succinate-induced swelling is not due to formation of fumarate. Since fumarate is in equilibrium with malate, electrons from this substrate enter the electron transfer chain via pyridine nucleotides. Maleic acid is not oxidized by mitochondria and did not induce swelling.

α-Ketoglutarate—in our experiments, 2 to 10 mM α-ketoglutarate either alone or with equimolar amounts of NH₄Cl induced swelling that was blocked by Amytal (Fig. 6).

Sulfite—This reducing agent was tested for comparison with ascorbate. In concentrations of 0.3 to 3 mM it caused rapid swelling (Fig. 17). It is of interest that the swelling curve with sulfite resembled the curve with phosphate and substrates more than that with ascorbate. It has been known for several years that sulfite is oxidized enzymatically by mitochondria, partially via cytochrome-sensitive electron transfer coupled to phosphorylation (29).

Like other substrates, sulfite appears to produce swelling through effects on electron transport. Its action was almost completely blocked by anerobiosis, cyanide, azide, and antimycin A, with partial inhibition by Amytal and malonate. These latter observations suggest that electrons from sulfite enter the electron transfer chain at several points, including some reduction of the flavoprotein of succinic dehydrogenase. Since cyanide completely blocks sulfite-induced swelling but only partially blocks sulfite oxidation (29), it appears that oxidation not involving electron transfer via cytochrome oxidase does not promote swelling.

Nitrite—in concentrations up to 8 mM, nitrite neither produced nor prevented swelling. Nitrite is not oxidized by mitochondria (30).

Metal Ions—Hunter et al. (1) showed that a number of metal ions which promote mitochondrial swelling aerobically do not do so under anaerobic conditions. Lehninger and Ray (6) found that the action of Ca⁺⁺ is blocked by cyanide. Further investigation is needed with other electron transport chain inhibitors.

Arsenate—the swelling induced by arsenite is known to be blocked by anerobiosis (1). The experiment in Fig. 18 illustrates that it is also blocked by antimycin A and Amytal. The swelling was delayed for considerable periods by 0.6 mM cyanide, 10 mM azide, and SN 5949. Arsenite is not ordinarily considered to be a substrate, but it can be oxidized to arsenate. If, as is more probable, its action is via reaction with thiol or dithiol groups, the reaction or the expression of it as seen in mitochondrial swelling is conditioned by the electron transport chain.

p-Chloromercuribenzoate—A number of workers have observed that p-chloromercuribenzoate causes rapid and pronounced swelling of mitochondria (2, 6, 7, 10, 11, 31). Iodoacetamide produces a similar effect (10). The action of p-chloromercuribenzoate is blocked by anerobiosis (see below) and by cyanide (7). However, in contrast to what was observed with arsenite, the effect of p-chloromercuribenzoate was not blocked by antimycin A or Amytal (Fig. 19). Malonate produced a slight slowing of the swelling.

Anaerobic Experiments

Anerobiosis prevented swelling in all cases with the materials used in this study. It is very important that the last traces of oxygen be removed by repeated and thorough evacuation and flushing. Traces of oxygen permit swelling and probably were responsible for the fact that Tapley (10) reported that N₂, CO, and so on did not prevent swelling with several agents now known.
to be blocked by strict anaerobiosis. Even after 1 hour of anaerobiosis, swelling occurred with ascorbate when air was admitted (Fig. 20). This indicates that the ascorbic acid was not converted to some other material anaerobically. Phosphate, \( \beta \)-hydroxybutyrate, succinate, and glutathione also still produce swelling after a period of anaerobiosis.

**Additional Blocking Agents**

It has been known for some years that a number of substances such as EDTA, \( \text{Mn}^{2+} \), \( \text{Mg}^{2+} \), citrate, and dinitrophenol could prevent swelling. Since these substances are not known inhibitors of electron transfer, it was of interest to test them against many agents which cause swelling.

1. **EDTA**—This chelating agent is well known to block mitochondrial swelling induced by many agents. EDTA, 0.1 to 1 mM, is very effective in blocking the additional substances studied in this paper, including ascorbate and glutathione. The universal effect of EDTA and the remarkable degree of stability it produces suggest that it stabilizes the mitochondrial membrane by forming complexes with some group in it (32).

2. **\( \text{Mn}^{2+} \) and \( \text{Mg}^{2+} \)**—It has been known (18) that these ions inhibit the type of swelling induced by phosphate. In the present investigation it was shown that 5 to 8 mM \( \text{Mg}^{2+} \) and 0.5 to 1 mM \( \text{Mn}^{2+} \) had considerable inhibitory effect on the swelling-inducing properties of succinate, sulfite, and arsenite. \( \text{Mn}^{2+} \)
could completely neutralize the effect of ascorbate. There is considerable reason to believe that these ions stabilize the mitochondrial membrane by forming complexes with some group in it (32).

3. **8-Hydroxyquinoline and \( \beta \)-Phenanthroline**—In comparison with EDTA these chelators had relatively small effect against most agents which cause swelling. They were more effective against ascorbate and GSH. 8-Hydroxyquinoline, 0.1 mM, completely blocked the swelling caused by ascorbate. This suggests that the somewhat different type of swelling caused by ascorbate is the result of the action of an ascorbate-metal complex + \( \text{O}_2 \).

4. **Inorganic Triphosphate**—This polyphosphate ion blocked swelling induced by phosphate. There is reason to believe that it decreases the permeability of the mitochondrial membrane (12). Like ATP, ADP, and pyrophosphate, which have been shown to inhibit swelling produced by several agents, triphosphate may act in a manner similar to EDTA.

5. **Bovine Serum Albumin**—Added alone bovine serum albumin had only small inhibitory effects. It was additive with ATP, and ATP + \( \text{Mg}^{2+} \) + bovine serum albumin were quite effective, even against swelling produced by ascorbate and glutathione.

6. **High Concentrations of Ascorbate**—Fifteen mM ascorbate, in contrast to 0.2 to 1 mM, did not cause swelling. Although a high concentration of ascorbate prevented the swelling seen with low concentrations of ascorbate and glutathione, it did not inhibit phosphate, \( \beta \)-hydroxybutyrate, succinate, or sulfite-induced swelling (Fig. 21).

7. **2,4-Dinitrophenol**—The striking blocking effect of dinitrophenol, first observed by Lehninger (9) with swelling produced by thyroxine and phosphate, applies also to swelling induced with \( \beta \)-hydroxybutyrate, succinate, sulfite, and arsenite. However, dinitrophenol is not able to prevent the type of swelling induced by ascorbate, glutathione (Fig. 22), or p-chloromercuribenzoate.

**DISCUSSION**

The fact that five electron transport chain inhibitors, like anaerobiosis, prevent phosphate-induced swelling of liver mitochondria suggests that the effect of anaerobiosis in preventing swelling is due to interruption of electron transport. With anaerobiosis all of the electron transport carriers would be in the reduced form (6, 21). However, reduction of cytochromes \( a, a', c, c', \) and \( b \) or of flavoprotein is not essential for prevention of
swelling, because Amytal blocks swelling. In the presence of Amytal all of the electron transfer chain except the pyridine nucleotides would be oxidized (Fig. 23). This suggests that keeping pyridine nucleotides in the reduced form prevents the permeability changes responsible for swelling (see also Lehninger and Schneider (8)).

If the reduced form of the pyridine nucleotides were the key to prevention of swelling, reoxidation of the pyridine nucleotides in the presence of an Amytal block should result in all of the carriers being in the oxidized form, and swelling should occur. However, even when the pyridine nucleotide was reoxidized experimentally, phosphate did not induce swelling. Another situation in which most of the pyridine nucleotide would be expected to be in the oxidized form (21) would be with dinitrophenol aerobically, yet swelling does not occur (9). Lester et al. (33) have reported that the pyridine nucleotides shift to the oxidized form after a period with EDTA, which prevents swelling.

These observations suggest that some factor other than the oxidation-reduction state of the pyridine nucleotide or any other electron carrier is involved. Perhaps active electron transfer with accompanying cyclic changes in the membrane creates a situation in which agents that induce swelling can produce their effect. In the presence of the electron transport blocking agents, carriers above the point of the block would be oxidized, and those below would be reduced, but there would be no active transport or electron flux and no swelling would occur.

If this hypothesis is correct, feeding electrons into the chain above a block should result in swelling, if electron flux in the upper part of the chain is a critical factor in swelling. Succinate feeds electrons into the chain above the point of the Amytal block (23), and succinate produces swelling in the presence as well as in the absence of Amytal. Antimycin A and all inhibitors acting higher in the chain block succinate oxidation and succinate-induced swelling. Very striking, then, is the fact that malonate inhibits succinate-induced swelling with relatively little effect on swelling induced by phosphate, \( \beta \)-hydroxybutyrate, fumarate, malate, glutamate, or ascorbate.

The fact that succinate induces swelling in the presence of Amytal eliminates the possibility that Amytal merely reacts with a key electron carrier (or other group) to produce a situation equivalent to reduction of that carrier or group. If this were the case Amytal should block succinate-induced swelling just as anaerobiosis, cyanide, azide, antimycin A and SN 5949 do.

On the basis of the working hypothesis just outlined, substances which could reduce cytochromes \( c_1 \), \( c \), \( a \), or \( a_1 \) should produce swelling even in the presence of antimycin A, because they would feed electrons into the transport chain above the antimycin A block. Ascorbate was tested as a likely possibility. It is oxidized at a low rate by mitochondria (34, 35) without added cytochrome \( c \), and coupled phosphorylation indicates that some electrons are entering the electron transport chain. Low concentrations of ascorbate produced swelling, as predicted, but the fact that antimycin A can completely block ascorbate-induced swelling in some preparations strongly suggests that electrons from ascorbate are passed to cytochrome \( b \), either directly or via a flavoprotein.

These experiments with electron flux in part of the electron transfer chain, with other sections blocked, point to the conclusion that the portion of the chain in which an electron flux may be essential for many agents to produce swelling includes cytochromes \( a_2 \), \( a \), \( c \), \( c_1 \), and \( b \).
substances with a fairly drastic direct action on the membrane structure. Substances like EDTA, which appears to have a direct stabilizing effect on the membrane, would block all swelling except that produced by organic solvents, detergents, lecithinases, lyssolecithin, and so on.

Various substrates should promote swelling. Most of them do, and their effect is nullified by blocking their oxidation. Secondary chelating or membrane-stabilizing effects may cause certain substrates to prevent swelling. Citrate certainly belongs in this class; oxaloacetate and pyruvate may belong here, since Tapley (10) has reported that they inhibit swelling.

It seems very likely that the spontaneous swelling frequently seen is dependent on the endogenous substrates in the mitochondria. The great variation in spontaneous swelling from one preparation to another may be due to different levels of endogenous substrates. Since isolated heart mitochondria exhibit much greater stability than liver mitochondria, it is interesting that Chance and Bunteheoffaly (36) find strong indications that the former contain much less endogenous substrate. Cooper and Tapley (37) have reported that mitochondria from livers of rats fasted overnight are less sensitive to the swelling-producing effects of thyroxine and digitoxin. In our experiments, mitochondria aged for 24 hours at 0° became quite resistant to the swelling-producing effects of phosphate. This may be due to loss of endogenous substrates or cofactors or of both. Small temperature differences undoubtedly also contribute to the variability in spontaneous swelling (7).

Phosphate appears to catalyze oxidative changes which occur in most preparations more slowly and spontaneously. Emmelot and Doe (38) have reached similar conclusions. Phosphate seems to increase the rate of swelling much more than the end point of swelling. The mechanism by which thyroxine causes swelling may be similar to that for phosphate-induced swelling. However, the action of phosphate and thyroxine is not identical. With the latter Tapley (10) has reported characteristic differences in the end point with each concentration.

Further work is necessary to define the nature of the action of phosphate and thyroxine. The most likely possibility appears to be stimulation of the rate of the membrane changes which occur secondary to electron transfer, either by catalysis (possibly as a metal complex) of the oxidation of some labile group, or by reaction with substances such as Mg++ or Mn++ in the membrane structure. Removal of such ions would render the membrane more labile. However, until investigated directly, stimulation of electron transfer from endogenous substrates cannot be ruled out. Lehninger (7) has pointed out that swelling produced by thyroxine is probably not related to uncoupling of phosphorylation, but Chance (39) favors the view that its action is related to shifts in concentrations of energy-rich intermediates within mitochondria. Earlier Brenner-Holzach and Raafflub (4, 27) concluded that ATP within the mitochondria fell to a low level before swelling began. Phosphate is known to release the pyridine nucleotide from mitochondria, but whether this is primary or secondary to swelling is not entirely settled. Conceivably, release of DPN from enzymes in the membrane could render the membrane more susceptible to oxidative change.

It is well known that a number of metal ions which catalyze the oxidation of sulfhydryl groups, and reagents which react with sulfhydryl groups induce swelling (9, 18, 31). Moreover, certain other metal ions such as Mn++, which may form complexes with sulfhydryl groups without catalyzing their oxidation (40), prevent swelling. Reagents which react with sulfhydryl groups tend to uncouple phosphorylation. By such an action they might promote electron transfer. Another possibility is that in the membrane structure, reaction of a sulfhydryl group with p-chloromercuribenzoate might be the equivalent of oxidation to a disulfide link as far as permeability is concerned. Arsenite and p-chloromercuribenzoate must act at somewhat different sites. The effect of arsenate is blocked by electron transport inhibitors. The action of p-chloromercuribenzoate is inhibited by cyanide (7) and EDTA, but not by antimycin A or Amytal.

EDTA, like anaerobiosis, protects against virtually every agent which produces swelling. It might act by chelating detrimental metal ions in the medium, thereby preventing metal-catalyzed oxidative changes in the membrane. However, other chelating agents are usually less effective. It is more probable that most of the effect of EDTA is due to complex formation directly in the mitochondrial membrane. This membrane stabilization could result from complex formation with metals such as Mn++ or from protection of a sulfhydryl group.

Mg++ and Mn++ might inhibit swelling by inhibiting electron transfer under the conditions of these experiments. Chance and Williams (21) observed inhibition of succinate oxidation by Mg++ when ADP was absent. However, there is a good possibility that the protective effects of EDTA, dinitrophenol, Mg++, and Mn++ are at sites one or more steps removed from electron transfer itself. Cyclic changes occurring during electron transfer may create the situation in which the physical-chemical shifts responsible for swelling can occur, but the actual shifts may be blocked by the direct action of certain substances without any effect on electron transfer.

Dinitrophenol blocks swelling produced by phosphate, sulfite, and several substrates. With the uncoupling by dinitrophenol there should be a rapid transfer of electrons from substrate to oxygen, and promotion of swelling would be predicted. Therefore, if the active electron transfer hypothesis is correct, dinitrophenol must have a protective effect which supersedes its uncoupling effect.

There are several possible mechanisms by which dinitrophenol might produce its protective effect. One would at first think that the uncoupling action of dinitrophenol or the shunt of electron carriers toward the completely oxidized state must be related to prevention of swelling. However, on this basis all potent uncoupling agents should produce a similar effect. This does not seem to be the case (7, 10). Actual inhibition of electron transfer under the conditions of these experiments is a possibility but seems unlikely. Dinitrophenol may interrupt the series of reactions responsible for the labile state of the membrane and keep the steady state concentrations of structures or groups in the labile form at such a low concentration that the oxidative changes in the membrane do not occur. Uncouplers like gramicidin may act at a somewhat different site in the reaction sequences and not produce the same result. The fact that dinitrophenol does not block swelling induced by ascorbate and GSH suggests that it does not act by the same direct membrane-stabilizing effect as EDTA.

Obviously there are two kinds of swelling. The work with ascorbate and GSH indicates either a completely different mechanism of swelling or a second mechanism superimposed on the first. Both types of swelling are dependent on the presence of oxygen. Similarity is indicated by the fact that anaerobiosis, EDTA, and antimycin A can block ascorbate-induced swelling. On the other hand, the shapes of the swelling curves are different,
and dinitrophenol and Amytal do not block swelling produced by ascorbate. The first type of swelling must be due to increased permeability, but it stops without osmotic rupture when the membrane becomes quite permeable. The second type of swelling (more accurately optical density change) might result from rupture of links holding subunits together in the mitochondrial membrane. If the action of antimycin A is specific for electron transfer, the second type of structural change is either dependent on electron transfer or cannot occur until the first type of change has preceded it. Since auto-oxidation of ascorbate may give rise to \( \text{H}_2\text{O}_2 \), this must not be overlooked as a possible cause of some of the effects.

The unexpected finding that high concentrations of ascorbate block swelling induced by low concentrations of ascorbate requires an explanation. This protective effect applies only to the type of swelling seen with ascorbate and glutathione. Therefore the general phenomena of inhibition of electron transport or direct stabilization of the membrane are not the explanation. The most likely explanation for the action of high concentrations of ascorbate is complex formation with some metal or group essential to make manifest the effect of low concentrations of ascorbate.

Substantial arguments favor the concept that electron transfer inhibitors block mitochondrial swelling by their inhibition of electron transport. However, although many of these substances are considered to be almost completely specific for their best known action, possible alternative explanations of their action should be examined. The almost universal protective effect of EDTA raises the question whether most or all of the substances which prevent swelling do so by virtue of chelation or complex formation.

Simple chelation of detrimental ions is probable for citrate, 8-hydroxyquinoline and o-phenanthroline. The latter two substances are especially effective in blocking the effect of ascorbate and GSH. Although some chelating potential is recognizable in cyanide, azide, antimycin A, SN 5949 and Amytal, it seems unlikely that they would all chelate free metals in the same degree and at the same concentrations at which they first achieve complete block of electron transport. It also seems unlikely that all these agents would form complexes with the same groups in the mitochondrial membrane. The fact that some of them interact with the metals in electron transport enzymes is well known, and in the proposed hypothesis this would be the very basis of their action. Furthermore, it is unlikely that malonate would form a protective complex when succinate was the substrate and not when \( \beta \)-hydroxybutyrate, glutamate, or phosphatase was causing the swelling. The converse is true for Amytal.

We do not know much about the action of dinitrophenol and Dicoumarol, but simple chelation of detrimental metals seems unlikely in view of the fact that dinitrophenol does not block the action of low concentrations of ascorbate and GSH. However, there is the possibility that marked differences in chelating activity by uncoupling agents would explain why some inhibit swelling and others do not.

The apparently intimate association of mitochondrial swelling with changes in the electron transfer chain and the interesting effect of dinitrophenol raise the question whether the mechanisms of swelling are not closely related to the mechanisms for oxidative phosphorylation coupled to electron transfer, dinitrophenol-activated ATPase, and the \( \text{H}_2\text{PO}_4^-\)-ATP exchange reactions. Lehninger (7) has also pointed out such possible relationships. Only recently has the study of the effect of electron transport inhibitors on some of these reactions been undertaken (7, 32, 41, 42).

The idea that either the state or the activity of the respiratory chain influences the permeability of the mitochondrial membrane is an intriguing one. More information about such effects should provide us with some knowledge about the structure of the mitochondrial membrane. Since liver mitochondria vary in their sensitivity to swelling-producing agents with various metabolic and disease states (43), changes in the membrane in different circumstances should be studied.

The hypothesis discussed in this paper brings into a plausible framework the action of many substances which cause swelling of isolated mitochondria. However, it is difficult to fit some observations into this framework. Much more information is needed. Many of the points raised can be investigated by direct experimentation.

**SUMMARY**

It is suggested that active electron transfer between substrates and oxygen is a prerequisite for swelling of isolated rat liver mitochondria suspended in 0.23 M sucrose + 0.025 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, at 22-24°. This suggestion is based on the following findings.

1. Swelling is promoted by the addition of many substances dependent on diphosphopyridine nucleotide-linked enzymes for their oxidation.

2. This swelling is prevented by inhibitors of electron transfer between pyridine nucleotides and oxygen (Amytal, antimycin A, SN 5949, NaN₃, and NaCN).

3. Succinate induces swelling in the presence of an Amytal block of the electron transport chain. It is well known that succinate feeds electrons into the electron transport chain above the point of Amytal inhibition. Malonate is fairly specific for blocking succinate-induced swelling.

4. Reoxidation of the pyridine nucleotides in the presence of an Amytal block does not result in swelling.

5. Swelling induced by inorganic phosphate and several other substances behaves as though it were dependent on oxidation of endogenous substrates.

Ascorbate, glutathione and cysteine produce a different type of swelling or disintegration of structure.

Possible mechanisms for relating the action of ethylenediaminetetraacetate, 2,4-dinitrophenol, \( \text{Mn}^{2+} \), and other substances to the proposed hypothesis are discussed.

**Addendum**—After this manuscript had been submitted, the letter of J. B. Chappell and G. D. Groville (Nature, 189, 813 (1958)) came to our attention. Using experiments of a slightly different design, they reached conclusions similar to a number of those presented here.

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F. Edmund Hunter, Jr., Jerome F. Levy, Joan Fink, Beverly Schutz, Francisco Guerra and Aryeh Hurwitz


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