THE OXIDATIVE METABOLISM OF MALATE PLUS PYRUVATE,
AND a-KETOGLUTARATE BY VITAMIN E-DEFICIENT
RAT LIVER MITOCHONDRIA

by

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ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td>An Effect of Vitamin E Deficiency on Rat Liver Homogenates</td>
<td>2</td>
</tr>
<tr>
<td>An Effect of Vitamin E Deficiency on Rat Mitochondria</td>
<td>3</td>
</tr>
<tr>
<td>Antioxidant Effect of Vitamin E</td>
<td>4</td>
</tr>
<tr>
<td>A Further Understanding of the Effect or Effects of Vitamin E Deficiency on Rat Liver Mitochondria</td>
<td>5</td>
</tr>
<tr>
<td>EXPERIMENTAL PROCEDURE</td>
<td>6</td>
</tr>
<tr>
<td>Subjects and Diet</td>
<td>6</td>
</tr>
<tr>
<td>Mitochondrial Preparation From Liver</td>
<td>6</td>
</tr>
<tr>
<td>Oxygen Electrode Determination of Mitochondrial Respiration</td>
<td>8</td>
</tr>
<tr>
<td>Mitochondrial Substrates and Vitamin E Used in Vitro</td>
<td>9</td>
</tr>
<tr>
<td>Protein Determination of Mitochondria</td>
<td>10</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>12</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>13</td>
</tr>
<tr>
<td>State 4 Respiration</td>
<td>13</td>
</tr>
<tr>
<td>State 3 Respiration</td>
<td>13</td>
</tr>
<tr>
<td>Vitamin E Added in Vitro</td>
<td>16</td>
</tr>
<tr>
<td>Respiratory Control Ratios</td>
<td>17</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>18</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>20</td>
</tr>
<tr>
<td>VITA</td>
<td>22</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>State 4 respiration of rat liver mitochondria from E-def and E-suff rats expressed as nA₀₂ per mg of protein per min.</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>State 3 respiration of rat liver mitochondria from E-def and E-suff rats expressed as nA₀₂ per mg of protein per min.</td>
<td>15</td>
</tr>
</tbody>
</table>
INTRODUCTION

For the past several years many claims have been made about the healthful benefits of vitamin E in the diet. Because of such claims, much recent research has been stimulated. Even though the role of vitamin E as an antioxidant in the body has been well established by experimentation (1) little is known about its metabolic function at the cellular level.

Previous work by Corwin et al (2) and Grove et al (3) demonstrated some negative relationships in rat liver mitochondria between the levels of vitamin E and the rate of respiratory decline. These findings opened up a new area of research possibilities where mitochondrial studies might be used to establish the level of vitamin E needed in the diet and to show evidence for one or more of vitamin E's functions in the body. To gain additional understanding of this area, the effect of dietary vitamin E deficiency on the oxidative metabolism of rat liver mitochondria was studied.
This review deals only with work involving the effect of vitamin E deficiency on rat liver homogenate and mitochondrial respiration of malate plus pyruvate, and $a$-ketoglutarate. These substrates were chosen because they are readily utilized by liver mitochondrial preparations. Oxygen uptake is rapidly increased in liver mitochondria when these substrates are available.

Effect of Vitamin E Deficiency on Rat Liver Homogenates:

The influence of vitamin E on the oxidative metabolism of $a$-ketoglutarate by rat liver homogenates was covered by Corwin et al (4). The rats used in the study were 50-75 g weanling male rats. A "torula yeast" diet deficient in vitamin E was fed to the experimental group. The controls received the same basal diet supplemented with 20-50 mg of dl-$a$-tocopherol acetate per 100 g of diet. Each rat consumed the diet for 4 weeks. All data on respiration by liver homogenates were obtained with the use of a Warburg respirometer. The homogenates were prepared in 0.25 M sucrose solution. The buffer used was a pH of 7.4 and included 40 $\mu$M Na$_3$P0$_4$, 300 $\mu$M NaCl, 12 $\mu$M KCl, and 4 $\mu$M MgSO$_4$·7H$_2$O. The incubation temperature was 30°C. The results obtained showed that liver homogenates deficient in vitamin E had significant respiratory decline as compared to controls.

A more extensive study was accomplished by Grove and researchers (5). These experiments were conducted under the same conditions as those by Corwin et al (4), with the exception of using two additional
substrates, pyruvate and malate. They also found that vitamin E deficiency caused liver homogenates to have an increased rate of respiratory decline.

The Effect of Vitamin E Deficiency on Rat Liver Mitochondria:

The next step was to see if the same results would be found in isolated rat liver mitochondrial respiration. Was vitamin E in some way controlling liver homogenate respiration through its control on the mitochondria? To answer such a question Corwin et al (2) prepared rat liver homogenates under the same method as before, and, then, isolated the mitochondria. The homogenate was centrifuged at 1000 rpm in a refrigerated centrifuge for 10 min to remove extraneous material, and then at 11,000 rpm for 10 min to remove the mitochondria. The substrates used were malate, malate plus pyruvate, and α-ketoglutarate. The incubation temperature was again 30°C in the Warburg respirometer. The buffer used at pH 7.4 was 50 μM KH2PO4, 40 μM tris (hydroxy methyl) amino methane, 4μM MgCl2, and 14μM NaF. The E-deficient mitochondria gave oxygen utilization rates similar to controls, while the respiratory decline was smaller for controls. Dietary supplements of vitamin E were beneficial in reducing the rate of respiratory decline.

Grove and co-workers (3) tried to confirm and extend this research. They duplicated the study for all but the preparation media (0.3 M mannitol and 0.1 M tris) and substrates used (α-ketoglutarate and malate only). These workers also showed that vitamin E supplements did not improve initial respiration rates, but did decrease the rate of respiratory decline.
Antioxidant Effect of Vitamin E:

According to the work of Scott (1) with chicks, vitamin E prevents deficiency diseases such as muscular dystrophy, encephalomalacia, and erythrocyte hemolysis, etc. primarily through its function as an antioxidant in the body. Could this be the role of vitamin E in rat liver mitochondria?

Both Corwin et al (2) and Grove et al (3) included in their work with rat liver mitochondria some studies to test the antioxidant theory. They included Factor 3 (selenium containing compound) in the diet of E-deficient rats. Selenium overcame any liver problems brought about by the lack of methionine or sulfur amino acids in the vitamin E-deficient "torula yeast" diet, and it slightly improved oxygen uptake rates of E-deficient rat liver mitochondria. The rates were not greatly improved over rates without selenium, indicating that vitamin E may act in a way other than just as an antioxidant. Both teams of researchers felt that the vitamin E effect seen in rat liver mitochondria was more than an antioxidant effect.

Contrary to this belief, Tappel (6) contended that impaired respiration of the mitochondria from livers of rats deficient in vitamin E was the result of peroxide damage to their sensitive membranes. Grove and fellow investigators (3) showed, through their mitochondrial experiments with the substrate α-ketoglutarate, that dietary vitamin E was ineffective in preventing peroxide damage when large concentrations of α-ketoglutarate were present during incubation in vitro. These results support the non-antioxidant theory of vitamin E action in mitochondria.
A Further Understanding of the Effect or Effects of Vitamin E Deficiency on Rat Liver Mitochondria:

Schwarz (7) observed that rat liver mitochondria with inadequate vitamin E became swollen or enlarged. This may indicate its function in membrane stability. Schwarz feels that vitamin E may control respiration in mitochondria directly by participating as a co-factor or catalyst, or indirectly by exerting a regulatory effect on enzyme levels. Vitamin E may be important in both the physical and physiological nature of the mitochondria.

Schwarz further states that the reason for conflicting oxygen uptake results of experimental and control mitochondria are due to the loss of "a protein like vitamin E activator" in isolating the mitochondria. He claims that when this "activator" is separated from the microsomal supernate in preparing the mitochondria and then added back to them, that actual rate changes can be seen between the deficient and sufficient mitochondrial respiration.

Further research emphasis is needed to provide information on whether or not vitamin E can improve rat liver mitochondrial oxidative metabolic rates with no added substrate (endogenous), with malate plus pyruvate as substrate, and with \( \alpha \)-ketoglutarate as substrate. Also it is important to see if vitamin E can improve vitamin E-deficient rat liver mitochondrial respiration when added in vitro. If this is possible, then both an optimal and toxic level of vitamin E might be determined in vitro.
EXPERIMENTAL PROCEDURE

Subjects and Diet:

The subjects in this study were twelve 50 g, Sprague-Dawley, weanling male rats. They were fed a vitamin E free diet for 4 weeks. One half of the rats were supplemented with 10 mg of dl-a-tocopherol acetate per day. The remainder of the rats were not supplemented with vitamin E. The two groups here after will be referred to as E-suff and E-def, respectively.

Mitochondrial Preparation From Liver:

Each rat, after the 4 week diet period, was killed by decapitation and the liver was extracted immediately. The liver mitochondria were isolated by the following procedure:

1. Decapitate rat
2. Extract Liver (MSE at 4°C)
3. Homogenize (MSE at 4°C)
4. Place homogenate in 3 (50 mL) polypropylene centrifuge tubes

1 Casein based diet obtained from Nutritional Biochemical Co., Cleveland, Ohio.


3 MSE (225 mM mannitol, 75 mM Sucrose, 0.1 mM Tris-EDTA. pH 6.8-6.9)
Centrifuge at 1000 rpm (121 x g) for 5 min at 4°C.

Remove the white membraneous liquid from the top of the supernatant with pasteur pipet and then pour the supernatant through cheesecloth (gauze) into 3 new tubes. Balance each with cold MSE to the same level. Throw out the pellets of the old tubes.

Recentrifuge at 7000 rpm (5900 x g) for 10 min at 4°C.

Again remove any white membraneous liquid and pour off the supernatant and keep the pellets. The pellets contain the mitochondria.

Resuspend (use a pasteur pipet and cold MSE) the mitochondrial pellet (avoid any blood spot that may be present in the middle of pellet) into new tubes and fill all tubes to the same level with MSE.

Recentrifuge at 7000 rpm for 10 min at 4°C.
Pour off the supernatant and resuspend the pellet with cold MSE into two tubes. Again fill the tubes to the same level with MSE.

Recentrifuge at 7000 rpm for 10 min at 4°C.

Pour off the supernatant and resuspend the mitochondria in ~3 ml volume with cold MSE.

End product is a liver mitochondrial homogenate from extracted liver which is ready for the respiration studies.

The Oxygen Electrode Determination of Mitochondrial Respiration:

The oxygen consumption of both E-def and E-suff mitochondria was accomplished with the use of a Yellow Springs Model 53 oxygen analyser with sensor electrode housed in an all glass reaction vessel, 1 ml capacity, (containing 0.1 ml mitochondria and 0.9 ml regular buffer,1 pH 7.2-7.3) in a circulating water jacket at 37°C.2 The respiration rates were measured with the use of a Fisher Recordall Series 5000,

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1Regular buffer (150 mM KCl, 20 mM Tris-HCL, and 5 mM KH₂PO₄ pH 7.2-7.3); Communications with Dr. A. B. Fisher, M.D., Dept. of Physiology, Univ. Pa., Phila., Pa.

2Incubation temperature was 37°C to simulate the body temperature of the rat. This was higher than the incubation temperature (30°C) referred to in the literature.
10 inch potentiometric recorder with 0-100 mv input. Calibration of the oxygen analyser and recorder was done by methods described by the manufacturer.3

Mitochondrial Substrates and Vitamin E Used in Vitro:

The mitochondria from both groups underwent treatment with 3 substrate conditions. The three substrate conditions were: no substrate added, 0.1 M malate plus 0.5 M pyruvate added, and 0.25 M a-ketoglutarate as substrate. All substrates were dissolved in distilled water and kept in an ice bath.

Vitamin E was also used in vitro for evaluation of its effect on both groups of mitochondria. This was done with the expectation that vitamin E added in vitro might have a greater effect on the E-def mitochondria than the E-suff as well as possible determining any toxic or beneficial effects of in vitro additions of the vitamin on respiration. After narrowing down the possible range of concentrations of vitamin E to be used, three levels were selected. They were: 0.05 mg/ml, 0.1 mg/ml, and 10 mg/ml of vitamin E dissolved in absolute ethanol.

The use of 0.25 M ADP4 (adenosine diphosphate) was undertaken so state 3 respiration (active state) as well as state 4 (resting state, 3This method gave direct respiration rates per second calculated in nA02/mg protein/min. This differed from the methods used in the literature where respiration rates were calculated per 30 min or longer using a Warburg respirometer.

4Communications with Dr. A. B. Fisher, M.D., Dept. of Physiology, Univ. Pa., Phila. Pa.
rate of mitochondrial respiration without ADP) could be investigated for substrate effects and vitamin E effects. ADP was further used so that respiratory control ratios could be computed (state 3/state 4) to determine mitochondrial integrity.

**Protein Determination of Mitochondria**

The protein determination of the amount of mitochondria being evaluated for oxygen uptake from each group of rats was accomplished to compensate for differing amounts of mitochondria that were obtained from each liver, so such differences could be taken into consideration in the calculations of the respiration rates. The following represents the procedure used:

0.1 ml mitochondrial homogenate (sample)  
↓  
Spin with vortex mixer.  
↓  
Add 4 ml of 5% PCA (perchloric acid) to the sample in graduated V-centrifuge tube.  
↓  
Centrifuge at 2000 rpm for 10 min.  
↓  
Pour off supernatant.  
↓  
Spin pellet with vortex mixer.

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5Communications with Dr. A. B. Fisher, M.D., Dept. of Physiology, Univ. Pa., Phila., Pa.
Add ~ 4 ml of 50% ethanol plus 2 drops of 40-50% PCA.

Spin pellet with vortex mixer.

Centrifuge at 2000 rpm for 10 min.

Pour off supernatant.

Spin pellet with vortex mixer.

Add ~ 4 ml 95% ethanol.

Spin pellet with vortex mixer.

Let stand for 30 min.

Centrifuge at 2000 rpm.

Pour off supernatant.

Spin pellet with vortex mixer.

Add 4 ml of biuret reagent plus 0.9 ml distilled H₂O (total volume 5 ml). Then after 30 min read on spectrophotometer at 550 nm in the visible range.

The protein concentration was determined by a biuret colorimetric method using lyophilized bovine serum albumen as a source of protein for the standard curve.
Statistical Analysis:

A paired t-test (8) was used to test the statistical significance of the data. The animals were paired based on time started on diet and time of assay. The paired t-test was selected to remove variation associated with these conditions.
RESULTS AND DISCUSSION

Final body weight averaged 173 ± 42 and 179 ± 40 for the E-def and E-suff animal groups, respectively. For these same two groups, food consumption averaged 392 ± 85 g and 416 ± 70 g, respectively. No significant difference was seen between the two groups for these two parameters.

State 4 Respiration:

In state 4 respiration, or the resting state, little difference in oxygen uptake was seen between the two groups in the endogenous state or when pyruvate plus malate was the substrate, however, when \( \alpha \)-ketoglutarate was added, the respiration rate was significantly faster in the E-def group (table 1). This may indicate that \( \alpha \)-ketoglutarate was able to penetrate at a faster rate the mitochondria from animals deficient in vitamin E. When all data in state 4 were considered together, respiration was significantly faster by the mitochondria from the E-def group. Much of the variation in the data was associated with between-pair variation. No logical explanation was found for this variation.

State 3 Respiration:

In state 3 or the active state, respiration was significantly faster in the E-def group in the endogenous state (table 2). The average respiration rate was faster in the E-def group when either \( \alpha \)-ketoglutarate or malate plus pyruvate were added as substrates, however, the differences were not statistically significant in either
TABLE 1

State 4 respiration of rat liver mitochondria from E-def and E-suff rats expressed as nA0₂ per mg of protein per min.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E-Def</th>
<th>E-Suff</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>22 ± 8¹</td>
<td>18 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Pyruvate² plus malate²</td>
<td>29 ± 16</td>
<td>27 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>α-Ketoglutarate³</td>
<td>48 ± 18</td>
<td>33 ± 16</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Combined Data</td>
<td>33 ± 16</td>
<td>26 ± 11</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

¹ SD, 6 rats per treatment each run in triplicate.

² 10 μl of 0.5 M pyruvate and 5 μl of 0.1 M malate were added per ml of reaction mixture.

³ 10 μl of 0.25 M α-ketoglutarate per ml of reaction mixture.
TABLE 2

State 3 respiration of rat liver mitochondria from E-def and E-suff rats expressed as nA0₂ per mg of protein per min.

<table>
<thead>
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<th>Substrate</th>
<th>E-Def</th>
<th>E-Suff</th>
<th>Significance</th>
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<tr>
<td>None</td>
<td>43 ± 3₁</td>
<td>33 ± 12</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Pyruvate plus malate²</td>
<td>74 ± 20</td>
<td>61 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>a-Ketoglutarate³</td>
<td>89 ± 18</td>
<td>71 ± 21</td>
<td>NS</td>
</tr>
<tr>
<td>Combined Data</td>
<td>67 ± 26</td>
<td>52 ± 23</td>
<td>P &lt; 0.05</td>
</tr>
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₁SD, 6 rats per treatment each run in triplicate.

²10 ul of 0.5 M pyruvate and 5 ul of 0.1 M malate were added per ml of reaction mixture.

³10 ul of 0.25 M a-ketoglutarate per ml of reaction mixture.
case. When all data in state 3 were considered together, respiration was significantly faster by the mitochondria from the E-def group. Again these findings may indicate that the substrate was getting into the mitochondria faster or the enzymes were being freed from the mitochondria. These data seem to be supportative of the concept that vitamin E functions to stabilize the integrity of the cell membrane, in this case the mitochondrial cell membrane (9). If this is true, the mitochondrial membranes from the rats deficient in vitamin E would have been more easily penetrable by the substrates, or else the membranes from the E-def group might have been disrupted to a greater extent than those from the E-suff group.

These results seem to further support the previous work of Schwarz et al (10) who showed with a Warburg respirometer that mitochondria deficient in vitamin E had faster respiration rates than mitochondria not deficient in the vitamin initially, but after 30 min or more the E-def mitochondria had slower respiration rates. This work may then additionally strengthen the theory that vitamin E is beneficial in mitochondrial respiration.

**Vitamin E Added in Vitro:**

The addition of vitamin E in vitro did not alter the respiration rates in either group under any condition of assay. The failure of vitamin E to have the same effect when added in vivo and in vitro supports the concept that it acts to stabilize the membrane by being incorporated into the membrane structure. Alternatively, it may be metabolized to a different active compound when provided in vivo.
Respiratory Control Ratios:

The respiratory control ratios computed in this experiment averaged between 2.0 and 3.0, which is lower than normal for rat liver mitochondria (11). At this time further research is being carried out to improve this situation and at the same time verify the existing data.
CONCLUSIONS AND SUMMARY

Rats were fed vitamin E-def or vitamin E-suff diets for 4 weeks. The mitochondria isolated from the livers were examined in three different ways: with no substrate added, with malate plus pyruvate as substrate, and with \( \alpha \)-ketoglutarate as substrate. The mitochondria from E-def and E-suff rats were also tested with three different levels of vitamin E added \textit{in vitro} in an attempt to find optimal and toxic levels of the vitamin with regard to their oxidative metabolism. No statistically significant differences were seen in respiration rates associated with vitamin E supplementation \textit{in vitro}.

Vitamin E-def mitochondria generally had greater oxygen consumption rates than the E-suff mitochondria. This may have resulted from an action of vitamin E on membrane stability and physiology, rather than any depressing effect of the vitamin. The results of this study are supportative of the findings of others which show that vitamin E influences membrane stability of cells (9, 12), affects mitochondrial shape and size (7, 10), and prevents cellular and subcellular degeneration (13). The above findings would explain why mitochondria, lacking sufficient vitamin E, could respire faster than mitochondria getting an abundant supply of the vitamin. If the membranes are swollen and/or broken as a result of insufficient levels of E in the diet, the damaged mitochondria could respire faster than normal because the substrates that are provided come into contact with the enzyme system of the mitochondria faster and thus are oxidized faster.
This study supports the belief that vitamin E plays one of its important roles in the body through its beneficial influence on mitochondrial membrane stability and physiology, and thus influences the rate of respiration of the mitochondria.

This study does not provide additional insight on whether or not vitamin E functions as an antioxidant or as an enzyme regulator or cofactor in rat liver mitochondria. Vitamin E may have both of these functions in the mitochondria rather than just one or the other.
REFERENCES


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Twelve weanling male rats averaging 50 g were used in this study. One half of them were fed a vitamin E-deficient (E-def) diet and the remainder a vitamin E-sufficient (E-suff) diet. All animals were killed after a 4-week feeding period, and their liver mitochondria isolated. State 4 and state 3 oxidative metabolism of E-def and E-suff rat liver mitochondria using three substrate conditions: endogenous, pyruvate plus malate, and a-ketoglutarate, were measured by oxygen electrode analysis. Statistically significant differences were seen among state 4 respiration with a-ketoglutarate, state 4 respiration (combined data with endogenous, pyruvate plus malate and a-ketoglutarate), state 3 endogenous respiration, and state 3 respiration (combined data with endogenous, pyruvate plus malate, and a-ketoglutarate) of E-def and E-suff rat liver mitochondria.

In vitro vitamin E addition studies were carried out to measure any toxic or beneficial effects of the vitamin on liver mitochondrial respiration from both groups. No significant effect of in vitro additions of vitamin E on liver mitochondrial respiration was found.

Liver mitochondria from E-def rats generally had faster respiration rates than those from E-suff animals. It is possible that the effect
of vitamin E deficiency on the membranes of the mitochondria accounted for these results.

If so, then those mitochondria deficient in the vitamin could become swollen and broken allowing substrates to enter the mitochondria at a faster rate and thus be utilized faster. It is postulated that vitamin E may play an important role in the body by maintaining proper liver mitochondrial respiration through the vitamin's beneficial influence on mitochondrial membranes.