Effects of a Nutritional Deficiency of Unsaturated Fats on Rat Liver Mitochondria

I. RESPIRATORY CONTROL AND ADENOSINE TRIPHOSPHATE-INORGANIC ORTHOPHOSPHATE EXCHANGE ACTIVITY

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It has been known for some time that mitochondria from livers of rats rendered deficient in the essential unsaturated fatty acids exhibit an impaired oxidative phosphorylation capacity (1-4) and a marked tendency to swell in vitro (5, 6). These observations have led to the suggestion that the essential unsaturated fatty acid deficiency results, in the rat at least, in liver mitochondria that are altered morphologically and are structurally less stable than normal ones (5, 6). However, the relation between such changes and altered respiration-linked reactions in the deficient mitochondria is not known. In the present investigation, this was explored further by measuring respiratory control (7-9) of deficient and normal mitochondria that had been treated in various ways. The investigation also included further studies of adenosine triphosphate-inorganic orthophosphate exchange activity in normal and deficient mitochondria.

EXPERIMENTAL PROCEDURE

Male Holtzman rats were weaned at 18 days of age and kept either on a fat-free diet (3) or on one in which corn oil was substituted at a level of 5% for an equal weight of dextrose. After 12 to 16 weeks, the rats fed the fat-deficient diet exhibited decreased weight gains and scaliness of paws and tails, and were considered to be in a state of fat deficiency (3). Liver mitochondria were prepared in a medium of 0.25 M sucrose and 1 mM EDTA, pH 7.4 (0.1 M KOH was used to adjust the pH), according to the method of Schneider (10). They were finally suspended in 0.25 M sucrose. In some of the experiments, a medium containing 0.3 M d-mannitol, 1 mM Tris, and 0.1 mM EDTA was used in the preparation and the final suspension of mitochondria (11).

Regardless of the preparation medium, the larger particles and cell debris were removed at a centrifugal speed of 2000 r.p.m., and mitochondria were collected at 8500 r.p.m. in a Servall refrigerated RC-2 centrifuge, size 40 rotor. The loosely packed particles were liberally discarded in order to obtain mitochondria possessing maximum respiratory control activity. Final suspensions contained mitochondria equivalent to 1 g of fresh liver per ml. Unless otherwise stated, the sucrose medium was employed to prepare and suspend mitochondria, and succinate was employed as substrate.

Respiratory control and P:O ratios were measured polarographically (8, 12, 13). The Clark oxygen electrode (Yellow Springs Instruments Company, Yellow Springs, Ohio) was employed in these measurements. A closed reaction cell was constructed to accommodate the electrode (13). Oxygen consumption was recorded directly on a Sargent model SR recorder. Oxygen concentration in the reaction mixtures was assumed to be the same as in pure water, 236 μM at 25°C.

ATP-Pi exchange was measured according to the methods of Cooper and Lehninger (14), Lindberg and Ernster (15), and Martin and Doty (16). The reaction was carried out in a glycoll-glycine buffer, in both the presence and absence of cyanide, at 23°C for 30 minutes. ATPase activity was measured at the same time by determining initial and final concentrations of Pi. ATP-Pi exchange activity was corrected for the dilution caused by hydrolysis of ATP according to Cooper and Lehninger. It was expressed as millimoles of Pi per liter exchanged per min (average) Pi per min (average) ATP per 30 minutes. The radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. ATP and ADP were products of Sigma Chemical Company. The concentrations of these compounds in solutions were determined spectrophotometrically at 260 μM. Thyroxine was a product of Nutritional Biochemicals Corporation. Dr. H. A. Laedt kindly gave us oligomycin. Lyophilized Crotalus adamantea venom was obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Florida.

Respiratory control is defined in this paper as the ratio of the respiratory rate in State 3 (in the presence of ADP) to the respiratory rate in State 4, wherein the added ADP has been completely depleted (8). Conditions which resulted in 50% inhibition of the maximal respiratory control, i.e. half-maximal inhibition, were determined by the equation

Respiratory control at 50% inhibition = \[ \frac{(\text{maximum respiratory control} - 1)}{2} + 1 \]

where maximum respiratory control is the respiratory control observed in the control experiment.

1 B. Hagiwara, personal communication.

2 C. Cooper and A. L. Lehninger, personal communication.
Table I

Respiratory control and P:O ratio of normal and fat-deficient rat liver mitochondria

The reaction mixture was 0.25 M d-mannitol, 0.025 M potassium phosphate buffer (pH 7.4), 0.006 M MgCl_2, 0.01 M KCl, and 0.0001 M EDTA. To 2.6 ml of reaction mixture in the oxygen electrode cell was added 0.2 ml of mitochondria, followed successively by 50 ~1 of 1 M sodium succinate and two to three portions of 10 ~1 of 0.05 M ADP. Respiratory control was calculated from the slopes of State 3 and State 4 (S) respiration rates. M and S indicate mannitol and sucrose, respectively, for the preparation media. The numbers in parentheses are the number of individual mitochondrial preparations used.

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Respiratory control</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.46 (M, 5)</td>
<td>1.75 (M, 5)</td>
</tr>
<tr>
<td></td>
<td>5.31 (S, 16)</td>
<td>1.82 (S, 16)</td>
</tr>
<tr>
<td>Deficient</td>
<td>5.22 (M, 6)</td>
<td>1.78 (M, 6)</td>
</tr>
<tr>
<td></td>
<td>4.47 (S, 13)</td>
<td>1.79 (S, 13)</td>
</tr>
</tbody>
</table>

![Graph: Effect of aging at various temperatures on respiratory control of normal and fat-deficient rat liver mitochondria.

FIG. 1. Effect of aging at various temperatures on respiratory control of normal and fat-deficient rat liver mitochondria. See Table I for the experimental details. Mitochondria were incubated at 10°, 20°, 30°, and 40° and used for the determination of respiratory control. The preparation medium was mannitol.

- - - - normal; ×--×, deficient.

RESULTS

Respiratory Control and Oxidative Phosphorylation

When normal liver mitochondria were compared with mitochondria from deficient rats on the basis of respiratory control and oxidative phosphorylation, no differences were observed in P:O ratios between the two preparations whether mitochondria were prepared in d-mannitol or sucrose. The liver mitochondria from deficient animals, when prepared in sucrose, however, exhibited a low respiratory control ratio, while mitochondria from deficient rats, when prepared in mannitol, exhibited a high respiratory control ratio as did similarly prepared normal mitochondria (Table I).

Effect of Aging at Different Temperatures on Respiratory Control

Mitochondria were prepared and suspended in the d-mannitol medium, incubated for 30 minutes at various temperatures, and transferred to an ice bath. Respiratory control measurements were performed on each incubated suspension. Fig. 1 describes the results of a typical experiment. Half-maximal inhibition of respiratory control occurred at 33° with the normal mitochondria, and at 22° with the fat-deficient mitochondria. When both types of mitochondria were prepared and suspended in the preparation medium containing 0.5 mg of bovine serum albumin per ml and incubated in the same manner as above, few or no differences due to the effect of the bovine serum albumin were seen. It may be inferred from this that the difference seen in the deficient mitochondria was not due to the possible presence of free fatty acids, which might have decreased respiratory control as the mitochondria were aged.

When the above experiment was performed with mitochondria prepared in sucrose, the differences were not as great between the normal and deficient mitochondria as were seen with the d-mannitol preparations. For example, the temperature which gave the half-maximal inhibition was 19° for the deficient mitochondria and 23° for normal ones.

Effect of Aging at 30° for Different Periods of Time on Respiratory Control

Mitochondria were kept at 30° for different periods of time. The results of respiratory control measurements are shown in Fig. 2. Aging the mitochondria at this temperature resulted in a more rapid loss of respiratory control in the deficient than in the normal mitochondria. This was observed when either succinate, β-hydroxybutyrate, or α-ketoglutarate was employed as substrate.

![Graph: Effect of aging at 30° for different periods of time on respiratory control of normal and fat-deficient rat liver mitochondria. See Table I for the experimental details. - - - - normal; ×--×, deficient.

![Graph: Effect of aging at different temperatures on respiratory control of normal and fat-deficient rat liver mitochondria.

FIG. 2. Effect of aging at 30° for different periods of time on respiratory control of normal and fat-deficient rat liver mitochondria. See Table I for the experimental details. - - - - normal; ×--×, deficient.
Effect of pH on Respiratory Control and P:O Ratio

In view of reports (17-19) that multiple pH optima were exhibited in mitochondrial ATPase and oxidative phosphorylation, the effect of pH on the respiratory control and P:O ratio in both deficient and normal mitochondria was determined. The deficient mitochondria exhibited pH-respiratory control and pH-P:O ratio relationships that were similar to those seen with normal mitochondria. However, they possessed somewhat lower respiratory control in the acid pH range than did the normal ones (Fig. 3). The pH-respiratory control relationship appeared graphically to have a skewed bell shape, with a maximum at pH 7.4. However, the P:O ratio was not altered appreciably over the wide range of pH employed.

Effect of Various Chemicals on Respiratory Control

Digitonin—In some of the experiments, respiratory control was measured immediately after mitochondria were added to the reaction mixture containing digitonin; in others, the mitochondria were allowed to remain in the oxygen electrode cell for 3 minutes in the medium containing digitonin before measurements were made.

When the measurements were made immediately, little difference was observed between the deficient and normal mitochondria. The concentration of digitonin producing half-maximal inhibition in deficient mitochondria was 0.014%, and it was 0.012% with normal mitochondria (Fig. 4). When the measurements were made following a 3-minute preincubation in the presence of digitonin, the normal mitochondria exhibited respiratory control nearly identical with those that had not been preincubated. However, the deficient mitochondria exhibited a relatively marked instability toward digitonin under the latter conditions. For example, although 0.014% digitonin produced half-maximal inhibition with unincubated mitochondria, only 0.006% digitonin was required to produce a similar inhibition after the 3-minute preincubation (Fig. 4).

Inasmuch as a short period of contact between the mitochondria and digitonin seemed to be required to produce an effect of the latter on respiratory control, both preparations were incubated at 0° for 30 minutes in the presence of various concentrations of digitonin, and respiratory control measurements were carried out. The results were similar to those obtained when mitochondria were incubated for 3 minutes in the reaction cell; i.e. the digitonin concentration that produced half-maximal inhibition for the deficient mitochondria was less than half of that observed in the case of normal mitochondria (0.083% digitonin was required for normal mitochondria and 0.031% for the deficient).

2,4-Dinitrophenol—The effect of different amounts of dinitrophenol on respiratory control in normal and deficient mitochondria was determined, and the concentration of dinitrophenol which resulted in half-maximal inhibition of the respiratory control was estimated. The results, summarized in Table II, indicate that the concentration of dinitrophenol required to produce half-maximal inhibition of respiratory control in deficient mitochondria was considerably higher than was required for the normal mitochondria.

Ca++—The concentration of Ca++ required to produce half-maximal inhibition of respiratory control was determined as in the case of dinitrophenol. It was found to be 134 μM for the normal mitochondria and 84 μM for the deficient.

Oligomycin—Oligomycin has been reported to block phosphorylation at the terminal phosphate transfer steps (20). When this substance was studied in the same manner as Ca++ and dinitrophenol above, its effect was found to be similar to that of Ca++ (Fig. 5).

Thyroxine—Reports (21-24) that thyroxine uncouples oxida-
tive phosphorylation only in the intact mitochondria and not in the submitochondrial particles, while dinitrophenol does so in the intact mitochondria as well as in the submitochondrial systems (22), led us to examine its effect on respiratory control and oxidative phosphorylation in normal and deficient mitochondria.

Fig. 6 shows the effect of different concentrations of thyroxine on the respiratory control and P:O ratio of the normal mitochondria that were preincubated in the oxygen electrode cell in the presence of thyroxine for 0, 4, and 5 minutes. In each case, the changes in respiratory control with increasing concentrations of thyroxine appeared to be a cubic function of the latter. Respiratory control decreased rapidly with increasing concentrations of thyroxine until the level reached 70 μM. When the concentration exceeded this value, respiratory control increased temporarily, with increasing amounts of thyroxine, and then gradually decreased to unity. A thyroxine concentration of 70 μM with an incubation period of 5 minutes resulted in a complete loss of respiratory control, but a larger concentration restored respiratory control, and a further increase again resulted in the complete loss of respiratory control. P:O ratios changed in relation to thyroxine concentrations, in a qualitatively similar manner, particularly in the case of the 4- and 5-minute incubations.

When mitochondria from deficient rats were employed, the depression of respiratory control with the low concentration of thyroxine, and a partial restoration of respiratory control at intermediate concentrations (100 to 200 μM thyroxine), were much less pronounced than those observed with normal mitochondria. The partial restoration of respiratory control at intermediate concentrations sometimes could not be observed in the deficient mitochondria. Higher concentrations of thyroxine resulted in a complete loss of respiratory control, similar to that with normal mitochondria.

Effects of Snake Venom

Mitochondria were incubated in the presence of various amounts of lyophilized C. adamanteus venom, and respiratory control was determined. The amount of the venom which produced half-maximal inhibition with deficient mitochondria was 5.5 μg per ml; 8.1 μg per ml were required to produce a similar effect on normal mitochondria (Fig. 7).

P:O ratios obtained with mitochondria treated with intermediate amounts of the venom appeared to be higher than those obtained with untreated mitochondria, although respiratory control of the treated ones was depressed (Fig. 7). This was constantly observed with both normal and deficient mitochondria and could not be ascribed to an experimental error, which might have resulted, for example, from an error in ADP calibration. It suggests that an ADP-consuming reaction occurred, in addition to phosphorylation of ADP to form ATP. One possibility was that the venom unmasked other ADP-consuming reactions from mitochondria. Another was that the venom possessed an ADP-hydrolyzing activity similar to that found in potatoes (25) and bovine seminal fluid (26).

To test the latter possibility, ADP was subjected to the action of the venom for various periods of time, and the remaining ADP

![Fig. 5. Effect of oligomycin on respiratory control of normal and fat-deficient rat liver mitochondria. See Table I and Fig. 4 for the experimental details. Oligomycin was dissolved in 1% aqueous ethanol at a concentration of 100 μg per ml. The control experiments, with 1% aqueous ethanol containing no oligomycin, showed no decrease of the respiratory control. O—O, normal mitochondria preincubated for 2 minutes as described in Fig. 4; •—•, normal mitochondria with no preincubation period; △—△, deficient mitochondria preincubated for 2 minutes; •—•, deficient mitochondria with no preincubation period.](http://www.jbc.org/)

![Fig. 6. Effect of thyroxine on respiratory control and P:O ratios of normal rat liver mitochondria. See Table I and Fig. 4 for the experimental details. The mitochondria were preincubated for 0, 4, and 5 minutes as described in Fig. 4. O—O, no preincubation period; •—•, 4-minute preincubation; □—□, 5-minute preincubation.](http://www.jbc.org/)
mitochondria, is similar to that of the normal ones. Little was observed, indicating that endogenous phosphorylation did not occur in either preparation to any marked degree.

The reaction tube contained 4.8 mM ATP; 8 mM glycyglycine buffer, pH 6.8; 0 or 10⁻⁴ M KCN; 0.4 mM Pi containing approximately 2 × 10⁶ c.p.m. as ³²P; 0.05 ml of mitochondria; and 0 to 30 μM dinitrophenol. The total volume was 0.5 ml. Mitochondria were incubated at 25° for 30 minutes. The concentration of dinitrophenol that gave half-maximal inhibition of ATP-P₁ exchange reaction toward dinitrophenol, in the deficient mitochondria, is similar to that of the normal ones. Little difference due to the presence of KCN in the reaction medium was observed, indicating that endogenous phosphorylation did not occur in either preparation to any marked degree.

Effect of Aging at 30° for Different Periods of Time on ATP-P₁ Exchange

Mitochondrial suspensions were kept at 30° for different periods of time, and ATP-P₁ exchange activity was measured on each. The data, shown in Fig. 8, indicate that the aging time required for half-maximal inactivation of deficient mitochondria was approximately 13.5 minutes, both in the presence and in the absence of KCN.

Table III

<table>
<thead>
<tr>
<th>Time on ATP-P₁ Exchange</th>
<th>ADP found in incubation mixture</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no venom)</td>
<td>598</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>401</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>215</td>
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<tr>
<td>15</td>
<td>140</td>
<td></td>
</tr>
</tbody>
</table>

Table IV

Concentration of 2,4-dinitrophenol that gave half-maximal inhibition of ATP-P₁ exchange activity of normal and fat-deficient liver mitochondria

The reaction tube contained 4.8 mM ATP; 8 mM glycyglycine buffer, pH 6.8; 0 or 10⁻⁴ M KCN; 0.4 mM Pi containing approximately 2 × 10⁶ c.p.m. as ³²P; 0.05 ml of mitochondria; and 0 to 30 μM dinitrophenol. The total volume was 0.5 ml. Mitochondria were incubated at 25° for 30 minutes. The concentration of dinitrophenol that gave half-maximal inhibition was determined from a plot of the data similar to Fig. 1. The values are the average of the number of experiments indicated in parentheses.

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Without KCN</th>
<th>With KCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (2)</td>
<td>9.1</td>
<td>7.4</td>
</tr>
<tr>
<td>Deficient (2)</td>
<td>8.9</td>
<td>9.6</td>
</tr>
</tbody>
</table>
unsaturated fatty acid deficiency in rats. i

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the conditions were the same as described in table iv, except that dinitrophenol was not used. mitochondria were aged at 10°, 20°, and 30° for 30 minutes and then were used for the determination of the exchange activity. the values are the average of the number of experiments indicated in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>without kcn</th>
<th>with kcn</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal (2)</td>
<td>24.2°</td>
<td>20.8°</td>
</tr>
<tr>
<td>deficient (4)</td>
<td>17.2</td>
<td>17.3</td>
</tr>
</tbody>
</table>

fig. 8. effect of aging at 30° on atp-pi exchange activity of normal and fat-deficient rat liver mitochondria. see table iv for the experimental details. mitochondria were aged at 30° for different periods of time, after which they were returned to an ice bath, then used for the determination of the exchange activity.

absence of cyanide, and 25 (plus cyanide) and 32 minutes (minus cyanide) for the normal mitochondria.

discussion

respiratory control is considered by slater and hülsmann (27), chance (28), and klingenberg and bücher (29) to be one of the most sensitive indications of physiological intactness of mitochondria. according to this criterion, the present data show that when mitochondria are prepared in a mannitol medium (11), the deficient mitochondria are as intact, physiologically, as the normal ones. whether or not the depressed respiratory control seen in the deficient mitochondria that had been prepared in sucrose is associated with an effect of the latter on possibly related systems (14, 30) is not known.

the degree to which physiological intactness is dependent on, or otherwise related to, morphological intactness is not completely clear. information concerning this would be germane, in the light of the present data, to any discussion of the defect that exists in deficient mitochondria, inasmuch as several of the observations, which can be explained on the basis of differences in structural stability (5, 6) of the two preparations, seem to have a respiratory chain-linked dependency.

for example, the fact that deficient mitochondria exhibited less stable respiratory control than normal ones in the presence of digitonin, c. adamantheus venom, and ca++ (31) suggests that the former have a relatively labile structural organization. this is further indicated by the aging experiments in which atp-pi exchange was measured. the action of both digitonin and c. adamantheus is presumed to be on lipid (lipoprotein) structures, and it may be inferred, therefore, that such structures in the deficient mitochondria are more vulnerable to the action of these agents than corresponding structures in the normal mitochondria.

the relative instability of respiratory control in deficient mitochondria in the presence of oligomycin probably indicates that structural instability is involved, rather than a defect associated with terminal phosphate transfer per se, since the oxidative phosphorylating capacity of deficient mitochondria appeared normal.

the action of c. adamantheus venom (presumably phospholipase a) on mitochondria might be considered to be a compound one. a part of its effect may be an uncoupling action of the fatty acids (32) and the lysophosphatides (33-35) released by the venom. a second portion of the effect may be associated with changes in the mitochondrial structure per se. bovine serum albumin, which is known to bind strongly with fatty acids (36-39), failed to protect either the normal or the deficient mitochondria against the action of the venom. this suggests that the decrease in respiratory control is due to the loss of the structural integrity, partly occasioned by the loss of fatty acids from the mitochondrial phospholipids and partly by some other causes, rather than to a direct action of released fatty acids, the amount of which may be too small to affect respiratory control.

the relative stability of the deficient mitochondria toward dinitrophenol, in respiratory control, was unexpected and of considerable interest. it may have resulted from the unmasking of sites which bind dinitrophenol, and which are irrelevant to coupled phosphorylation. such sites may be present to a greater degree in the deficient mitochondria, or they may be more readily unmasked as a result of the relatively labile structures of these mitochondria. perhaps the most significant aspect of the observations associated with the effect of dinitrophenol is the suggestion that deficient mitochondria also are less sensitive to adp; i.e. more adp is required for rapid oxidation and phosphorylation. it is conceivable that the structural lability in these mitochondria could give rise to a diminished sensitivity to adp in the coupled phosphorylation system.

on the other hand, the effect of dinitrophenol on the atp-pi exchange reaction in deficient mitochondria was quite similar to that of the normal ones. the explanation for the two types of response to dinitrophenol, depending on whether respiratory control or the exchange reaction is being measured, may reside in the fact that the conditions for measurement of the atp-pi exchange require a sucrose-free hypotonic medium, in order to avoid an inhibition of the exchange by sucrose (14). the hypotonicity undoubtedly had an effect on the structure of both the normal and deficient mitochondria, and thus may have exposed dinitrophenol-binding sites to such an extent in both preparations that the action of dinitrophenol no longer discriminated between the two.
The reported effect of thyroxine (21-24, 40-44) on oxidative phosphorylation in both intact and fragmented mitochondria, as well as the data presented here, indicates that the action is complex. The present experiments, however, show that respiratory control in the deficient mitochondria is less influenced by changes in thyroxine concentration than is that of the normal mitochondria. The mechanism by which thyroxine affects respiratory control is perhaps somewhat defective in the structurally labile, deficient mitochondria.

Although an uncoupled oxidative phosphorylation can be demonstrated in deficient mitochondria under conditions in which phosphorylation is still well coupled in normal mitochondria (1-3), the present and other data (4-6, 45) suggest that uncoupling in the deficient mitochondria results from relatively easily induced structural changes in the latter. Such uncoupling can be prevented, for example, by exerting special care in the preparation of the mitochondria (45) and in their handling during respiration measurement. It now appears likely that the primary effect of the deficiency is on mitochondrial structure, wherein a "latent" lability results that does not necessarily involve a defect in the coupling mechanism. Whether or not the normal swelling and shrinking associated with respiratory chain electron flow gives rise to change in phosphorylation efficiency and perhaps in other systems in the deficient mitochondria, which differ from those that would be produced in normal mitochondria, is not known.

Another possible effect produced by the unsaturated fatty acid deficiency may be due to liver lysosomes, which probably contain mitochondrial preparations (46). The deficiency conceivably could cause a greater extent of membrane disruption in the deficient than in the normal lysosomes, releasing lysosomal enzymes which would result in more extensive digestion of the deficient than of the normal mitochondria.

SUMMARY

1. Liver mitochondria prepared from rats rendered deficient in the essential unsaturated fatty acids exhibited satisfactory P:0 ratios, but less respiratory control, if a sucrose medium was employed rather than n-mannitol.

2. Losses of respiratory control and adenosine triphosphate-inorganic orthophosphate exchange activity occurred much more readily with aging in deficient mitochondria than in normal ones. Respiratory control in fat-deficient mitochondria was lost more readily in the presence of digitonin, Ca++, Crotalus adamanteus venom, or oligomycin than was observed in normal mitochondria. On the other hand, 2,4-dinitrophenol produced less effect on respiratory control in deficient mitochondria than it did in normal ones.

3. The deficient mitochondria were similar to normal ones in the way in which oxidative phosphorylation and respiratory control were influenced by changes in pH.

4. These observations are interpreted as indicating that a nutritional deficiency of the essential unsaturated fatty acids in rats results in liver mitochondria that are altered structurally. The changes are manifested, in vitro, in aberrations in respiratory chain-linked reactions.

Acknowledgment—The technical assistance of Mrs. Ofelia Hernandez is gratefully acknowledged.
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