ABSTRACT

Wayne Warren Armstrong. UPTAKE OF PHOSPHOROUS-32 AND CALCIUM-45 BY THE LIVER MITOCHONDRIA OF RATS MADE DEFICIENT IN THE ESSENTIAL FATTY ACIDS.

Liver mitochondria were isolated from rats that had been deprived of the essential fatty acids in their diets. The isolated mitochondria were tested for respiratory control to make certain that they were capable of carrying out oxidative phosphorylation and ion accumulation.

Mitochondria were incubated at room temperature in the presence of Phosphorous-32 and Calcium-45. They were compared with mitochondria from rats that had received a parallel diet that had been supplemented with corn oil to restore essential fatty acids. Incubations were conducted in the presence and the absence of ADP for periods of one, two, five, and ten minutes.

The data collected appear to show decrease in Pi accumulation relative to Ca by mitochondria upon EFA deficiency. The data revealed no difference in the accumulation ratio between the presence and the absence of ADP in the incubation medium. Finally, there was no difference in the ratio between various incubation periods.

UPTAKE OF PHOSPHOROUS-32 AND CALCIUM-45 BY LIVER MITOCHONDRIA OF RATS MADE DEFICIENT IN ESSENTIAL FATTY ACIDS

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DEDICATION

This thesis is dedicated with much love and devotion to my wife, Bonnie.

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TABLE OF CONTENTS

INTRODUCTION

Although a considerable body of research has been compiled concerning the active accumulation of charged particles by mitochondria, the techniques most often used have involved either chemical methods of determination of the charged particles or a combination of chemical and radiotracer methods of determination. Few attempts have been made to utilize the methods of dual label radiotracer techniques.

Also, few studies have attempted to investigate the role of the essential fatty acids in the ion uptake phenomena associated with mitochondria.

This investigation is concerned with the comparison of the ratios for the active accumulation of calcium (Ca) and phosphate (Pi) by the liver mitochondria of rats made deficient in EFA with that of rats that have not been made deficient in EFA.

REVIEW OF LITERATURE

Mitochondria have long been known to bind and accumulate certain inorganic cations by processes dependent on respiration and oxidative phosphorylation. However, it was not possible to determine whether such mitochondrial ion uptake was a relatively insignificant process or a relatively massive process with a stoichiometric relationship to electron transport until it was first demonstrated that Ca could be accumulated in massive amounts by a reaction that required ATP or ADP, Mg, Pi, and respiratory substrate (Vasington and Murphy, 1961, 1962; DeLuca and Engstrom, 1961).

Since that time, much work has been done on this subject. Lehninger, Carafoli, and Rossi (1967) have extensively reviewed the literature on the topic of energy linked ion movements in mitochondrial systems. This massive loading process was found to be inhibited by 2,4-dinitrophenol (DNP) and other uncoupling agents. Oligomycin was found to have no inhibitory effect on this reaction.

Brierley et al. (1963) using beef heart mitochondria and Lehninger et al. (1963) using rat liver mitochondria reported that Ca accumulation was accompanied by accumulation of Pi. This concomitant accumulation of Pi and Ca could be supported by either of two separate pathways, one dependent pathway showing little dependence on the concentration of Pi in the reaction medium and requiring Mg, was sensitive to oligomycin while the respiration dependent pathway, requiring respiratory substrate and Pi in the medium, was sensitive to DNP and other uncouplers of oxidative phosphorylation but not to oligomycin.

These results have been interpreted as evidence for the existence of a DNP sensitive high energy intermediate common to both the process of oxidative phosphorylation of ADP and the process of ion accumulation. This high energy intermediate is believed to be generated at a site that lies between the electron transport chain and the site of oligomycin inhibition (Vasington and Murphy 1962; DeLuca and Engstrom, 1961; Brierley et al., 1963, 1964; Rossi and Lehninger, 1963b; Lehninger et al., 1967).

Ca is an uncoupling agent and ADP is not converted to ATP when Ca is in the medium (Lehninger et al., 1967). However, Vasington and Murphy (1962) have shown that ADP is active in supporting Ca accumulation but much less so than ATP. They speculated that some ADP was converted to ATP via the adenylate kinase pathway in the presence of succinate and under conditions of massive loading.

It has also been shown that Ca accumulation takes precedence over oxidative phosphorylation when it is in the medium simultaneously with ADP under limited loading conditions. In other words, they are alternative processes (Lehninger et al., 1967).

Accumulation of 1.00 molecule of Pi and 1.67 atoms of Ca occurred per pair of electrons traversing each energy conserving site of the respiratory chain. This is consistent with a Ca:O ratio of 5, and the ratio represents stoichimetry between ion accumulation and electron flux in the respiratory chain (Lehninger et al., 1963; Rossi and Lehninger, 1963a).

The large amounts of Pi and Ca taken up during the massive loading reaction far exceed their solubility, and precipitation of calcium

phosphate occurs as hydroxyapatite which has a Ca:Pi ratio of 1.67, similar to the ratio for stoichiometric accumulation of these ions (Rossi and Lehninger, 1963a; Lehninger et al., 1963; Weinbach and von Brand, 1965).

Recent evidence has been reported for the existence and isolation of binding sites in mitochondria that are capable of binding Ca with a very high affinity. The isolated binding sites possessed a similar affinity with Ca in actively respiring mitochondria (Reynafarje and Lehninger, 1969). It has been shown that the relatively small number of these binding sites may be favorably compared to the number of respiratory assemblies and atractyloside sensitive ATP-binding sites. Also, the high affinity binding of Ca is completely inhibited by DNP but not by oligomycin. Finally, Mn and Sr, but not Mg, have been shown to be bound by the high affinity binding sites.

Lehninger (1970) has reported that this high affinity binding factor has been isolated as part of an extract from mitochondria disrupted by osmotic shock in distilled water. This factor has been found to have two types of binding sites, one having low affinity for Ca and the other having high affinity.

It has been suggested that the high affinity binding sites may be supplies with either a high energy intermediate generated by electron transport or a specific divalent cation carrier molecule in the membrane (Reynafarje and Lehninger, 1969; Lehninger, 1970).

Little work has been done with regard to the active transport of ions by the mitochondria of rats made deficient in the essential fatty acids. Guarnieri and Johnson (1970) have included this topic in their

review concerning the essential fatty acids. They state that the preponderance of evidence at this time seems to indicate "that the EFA function as moieties of one or another of the phospholipids which are an integral part of membrane function."

Several fatty acids have been shown to have well defined activities in curing EFA deficiency symptoms. They include γ-linolenic acid, linoleic acid and arachidonic acid. Usually it is the unsaturated fatty acids with double bonds at the ω6 and ω9 positions (counting from terminal, $-CH_3$, methyl group) that are associated with such biological activity.

The general symptoms of EFA deficiency in rats include scaling of the skin, stunted growth, increased water consumption, and increased susceptability to infectious disease. Also, various lesions in the energy coupling mechanism have been reported such as impaired capacity for oxidative phosphorylation and increased swelling *in vitro*.

A somewhat less respiratory control has been reported by Ito and Johnson (1964) when deficient mitochondria are exposed to digitonin, *C. adamanteus* venom, and Ca. The conclusion was made that deficiency in EFA results in structural alteration of liver mitochondria and that energy coupling *per se* is not significantly altered. Stancliff *et al.* (1969) have also reported that energy dependent accumulation of a variety of ions was essentially unaffected by deficiency. Ca-induced swelling was measured and found to be no different than in control animals. Also, Pi-induced volume oscillations were initially of the same amplitude in normal and deficient mitochondria. Only after repeated O_2 pulses were the amplitudes different. However, the periods

of oscillation were increased in the deficient mitochondria which might indicate some involvement of lipid. Williams et al. (1972) again noticed that deficient mitochondria underwent volume oscillations of longer periods than in normal mitochondria. They concluded that this increased period of oscillation was not the result of a decreased rate of ion accumulation. This is somewhat in contrast to Tinberg et al. (1972) who concluded that there was active involvement of lipid in the motochondrial oscillatory state.

MATERIALS AND METHODS

Animals

Two groups of rats of the Holzman strain were weaned onto a fat free diet. For one group, the diet was supplemented with corn oil (Mazola) to a level of 5% by weight. This was the normal group. For the other group, the diet was supplemented with tristearin at a level of 5% by weight to accelerate the onset of deficiency symptoms and to make the diets as nearly the same as possible without adding EFA. This latter group was the deficient group.

The fat free diet contained 58% dextrose, 21% "vitamin free" casein, 16% inert cellulose, 4% salt mixture U.S.P. XIV, and 1% vitamin fortification mixture (General Biochemicals, Inc.). The rats were maintained on these diets for a minimum of twelve weeks, after which those on the tristearin supplemented diet were beginning to show symptoms of EFA deficiency such as scaling and discoloration of the skin, increased water consumption and stunted growth.

Isolation of Mitochondria

For each experiment, a rat was sacrificed by stunning followed by decapitation. Then the whole liver was rapidly removed, placed in a small beaker where it was washed twice with cold preparation medium (0.25 M sucrose) and sliced into pieces with clean, sharp scissors. The chopped liver was rapidly homogenized using a glass teflon homogenizer containing approximately 40 ml of the preparation medium with the aid of a small motor. The homogenate was diluted to approximately 120 ml total volume with cold preparation medium and distributed evenly among four

50 ml centrifuge tubes. It was then centrifuged for eight minutes at approximately 600 X G in a Sorvall refrigerated centrifuge to remove all unbroken cells, nuclei, and other debris. Most of the supernatant was carefully removed and distributed to four more 50 ml centrifuge tubes. Difference in volume between the tubes were adjusted by adding more cold preparation medium as necessary. The sediment was discarded and diluted supernatant was centrifuged for eight minutes at approximately 8700 X G after which the supernatant was discarded along with the pink, fluffy layer of broken or damaged mitochondria. The tightly packed pellet that remained in each tube was then dispersed evenly into approximately 45 ml of cold preparation medium and centrifuged again for eight minutes at 8700 X G. The supernatant was discarded and the pellets from all four centrifuge tubes were combined and dispersed evenly into approximately 10 ml of cold preparation medium. This suspension was then stored on an ice bath prior to incubation.

Respiratory Control

Respiratory control rations were determined by means of a Clark Oxygen Electrode. Measurements of oxygen consumption were made in a reaction medium pH 7.4 containing 0.1 M sucrose, 0.04 M KCl, 10 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, and 0.1 mM EDTA. State 4 respiration was initiated by addition of a 25 µl aliquot of .335 M sodium succinate to 3.1 ml of the reaction medium. State 3 respiration was stimulated by the further addition of either a 5 µl or a 10 µl aliquot of 40 mM ADP at pH 7.4.

The rate of oxygen consumption was determined as oxygen consumed

per minute. Respiratory control ratios were determined by dividing the rate of oxygen consumption under conditions of State 3 respiration by the rate of oxygen consumption under conditions of State 4 respiration.

Incubations

Incubations at room temperature were commenced upon addition of 0.5 ml of mitochondrial suspension to test tubes containing 4.5 ml of previously prepared phosphorylation medium containing the radioisotopes phosphorous-32 (P-32) as phosphoric acid and calcium-45 (Ca-45) as $CaCl_2$ obtained from New England Nuclear Laboratories. The radioisotopes were added in neglibible amounts to give a specific activity of approximately 200 counts/ml/minute. The phosphorylation medium pH 7.4 was composed of 0.1 M sucrose, 0.04 M KCl, 10 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 2.5 mM $CcCl_2$, 3 mM sodium succinate as substrate, and 3.0 mM ADP, pH 7.4, where applicable.

Incubations at room temperature were conducted under conditions of State 3 respiration (in the presence of ADP) or under conditions of State 4 respiration (in the absence of ADP) as defined by Chance and Williams (1956). Incubations under conditions of State 3 or State 4 respiration were conducted for periods of 1, 2, 5, and 10 minutes commencing upon addition of mitochondria. At the end of each incubation period, a 0.5 ml aliquot was removed and subjected to a rapid filtration through a 0.45 μ millipore filter followed by washing with a 0.5 ml aliquot of the non-radioactive phosphorylation medium.

Counting

The filters bearing the mitochondria were placed in scintillation counting vials after the washing. Each vial contained 0.75 ml of water and 15 ml of scintillation counting fluid prepared beforehand as follows: 800 ml toluene, 230 ml triton X100, 7 gm PPO (2,5-diphenyloxazole) as primary fluor, and 0.3 gm POPOP (1, 4-bis-2-[5-phenyloxazolyl]-benzene) as secondary fluor.

Samples thus prepared were placed in a Packard Instrument Co. Series 3000 Liquid Scintillation Counter at 0° C which had been prepared for counting samples with a dual label by the channels ratio method.

Two channels were selected to be optimized, one for P-32 and the other for Ca-45. One channel was optimized for each radioisotope by adjusting the potential of the photomultiplier tubes of each channel until the maximum counting rate was achieved for the desired radioisotope.

For this purpose, two incubations under conditions of State 4 respiration were conducted for one minute intervals under conditions identical with those previously described. However, one of the incubation tubes contained only P-32 as the radioactive component while the other incubation contained only Ca-45. Each sample was used to optimize one channel.

After the channels had been optimized in this manner, the sample containing only Ca-45 was counted in the channel that had been optimized for P-32. By appropriate adjustment of the lower gate of the window, the counts due to Ca-45 in this channel were eliminated.

The sample containing only P-32 was then counted in both channels for ten minutes. A ratio of counts due to P-32 in the Ca optimized

channel to counts due to P-32 in the P-32 optimized channel was determined from these data. This ratio was multiplied by the number of counts in the P-32 optimized channel when P-32 and Ca-45 were present in the reaction medium simultaneously to give the number of counts in the Ca-45 optimized channel due to P-32. The number of counts due to Ca-45 in the Ca-45 optimized channel, when P-32 and Ca-45 were present simultaneously, was then obtained by subtracting this product from the total count in the channel.

Then ratios of P-32:Ca-45 were determined by dividing the number of counts registered in the channel optimized for P-32 by the number of counts calculated to be due to Ca-45 in the Ca-45 optimized channel.

All samples thus prepared were counted for ten minute intervals.

RESULTS AND DISCUSSION

Statistical analysis of data obtained by the experiments conducted for this thesis was not made because of the small number of determinations involved.

Respiratory control ratios as presented in Appendix A indicate that the mitochondria were well coupled although the deficient mitochondria used were slightly less well coupled than were the normal mitochondria, as found previously (Ito and Johnson, 1964).

The data presented in Appendix B represent the numbers of counts due to P-32 and Ca-45 on the filters bearing mitochondria after the filtrations were completed. Each horizontal row represents the counts per minute (CPM) for the mitochondria of one rat that had been subjected to an incubation period of 1, 2, 5, or 10 minutes as indicated in the table. Each horizontal row respectively shows the P-32 counts and Ca-45 counts for an incubation that took place under conditions of State 3 respiration and P-32 counts and Ca-45 counts that took place under conditions of State 4 respiration. The data presented in Appendix B were used to calculate the ratios of P-32:Ca-45 shown in Appendix C by dividing the P-32 counts by the counts due to Ca-45.

It is evident from Appendix C that there is a difference in the ratios of counts due to P-32 to counts due to Ca-45 in mitochondria between the two diets. The difference seems to indicate according to Appendixes B and C that there is a decrease in the amount of Pi accumulated relative to the amount of Ca accumulated upon EFA deficiency. The difference could be the result of a relative decrease in the number of binding sites for Pi on the mitochondrial membrane or a relative decrease

in the permeability of the mitochondrial membrane to Pi upon EFA deficiency.

A decrease in the rate of Pi uptake or a decrease in the efficiency of Pi retention by deficient mitochondria seems to be the most probable cause of the difference of Pi:Ca ratios between normal and deficient mitochondria, for an isolated high affinity binding factor for Ca in mitochondrial membranes is a protein rather than a lipid (Lehninger, 1970; Reynafarje and Lehninger, 1969). If it is assumed that the affinity for Ca binding of the protein factor is not affected by the change in lipid, so that the factor continues to bind Ca--such assumption is quite plausible--it is reasonable to conclude either that the capacity of the mitochondria to take up Pi is reduced, or that the ability of the mitochondria to retain the transported Pi is decreased upon EFA deficiency.

The data of Appendix C also indicate that there are no differences between State 3 and State 4 respiration with respect to the ratios of P-32 to Ca-45. DeLuca and Engstrom (1961) found that ADP in concentrations of 1 μM or less was active in supporting Ca accumulation when succinate was present in the reaction medium. However, it was found to be only 50-60% as active as ATP when used in these concentrations. They also reported that concentrations of ADP greater than 1 μM tended to reduce such ion accumulation. Concentrations of 3 mM ADP used in the experiments reported in this thesis could have reduced the ion accumulation to the level that occurs under the conditions of State 4 respiration. Perhaps that is why ADP had no effect on the accumulation reactions. More work is necessary to determine the effect of ADP on ion accumulation.

Finally the data in Appendix C indicate that there is no difference in the accumulation ratio of P-32 to Ca-45 as a result of different time lengths of incubation. Ion uptake is a very rapid process under conditions of massive loading, such as were used in the experiments reported in this thesis.

Although an evidence was obtained as to the decrease in Pi accumulation relative to Ca upon EFA deficiency as shown by the data presented in Appendix C, in view of the fact that the number of experimental animals used was rather small, it may be appropriate to state that more work is needed to substantiate such evidence.

SUMMARY

Accumulation ratios for P-32:Ca-45 in liver mitochondria were determined using the channels ration counting method.

Accumulation ratios of P-32:Ca-45 were determined following appropriate incubations of liver mitochondria of rats that had been fed a diet that was deficient in unsaturated fatty acids or a similar diet that was supplemented with corn oil. Incubations were conducted under conditions of state 3 and state 4 respiration (in the presence and the absence of ADP) for periods of 1, 2, 5, and 10 minutes.

The data appear to indicate that accumulation of Pi relative to Ca by mitochondria is reduced upon EFA deficiency. Accumulation ratio of Pi to Ca by mitochondria was neither altered by the presence of added ADP, nor by the period of incubation time.

APPENDIX A

Respiratory Control Ratios

Respiratory control ratios were obtained as described in text by dividing oxygen consumed per minute in State 3 respiration by oxygen consumed per minute in State 4 respiration.

APPENDIX A (Continued)

Respiratory Control Ratios

Mitochondria	Respiratory Control Ratios
Normal	4.67
	3.52
	5.16
Deficient	3.31
	2.56

APPENDIX B

P-32 and Ca-45 Found in Mitochondira Under Different Conditions

Counts per minute (X_1) of P-32 in an unknown sample containing P-32 and Ca-45 were obtained directly from the channel optimized for P-32 in which counts due to Ca-45 were eliminated appropriately. Counta of Ca-45 in the same unknown were corrected as follows: (See text for details.)

$$\text{Counts due to Ca-45 in the unknown} = X_2 - (C_2/C_1) \text{ times } X_1$$

Where

C_1 is the counts of a sample containing only P-32 in the channel optimized for P-32,

C_2 is the counts of the same sample in the channel optimized for Ca-45,

X_1 is the counts of an unknown containing P-32 and Ca-45 in the channel optimized for P-32,

X_2 is the counts of the same unknown in the channel optimized for Ca-45.

APPENDIX B (Continued)

NORMAL DIET

	State 3		State 4	
	P-32 CPM	Ca-45 CPM	P-32 CPM	Ca-45 CPM
	397	228	341	193
1 min.	242	172	260	193
	332	184	249	170
	322	283	281	190
2 min.	232	148	218	141
	268	180	186	118
	399	242	272	198
5 min.	242	174	252	185
	310	198	166	100
	494	294	182	136
10 min.	350	209	190	148
	436	236	141	92

DEFICIENT DIET

	State 3		State 4	
	P-32 CPM	Ca-45 CPM	P-32 CPM	Ca-45 CPM
	184	220	183	229
1 min.	133	182	128	157
	134	158	155	199
2 min.	136	172	141	200
	159	191	148	188
5 min.	128	155	147	192
	146	172	154	188
10 min.	150	185	136	119

APPENDIX C

Ratio of counts due to P-32 to counts due to Ca-45

The data in Appendix B was used to calculate a ratio of P-32 to Ca-45.

APPENDIX C (Continued)

Ratio of Radioactive Count of P-32 to That of Ca-45
(Accumulation Ratio of P-32:Ca-45 in Rat Liver Mitochondria)

NORMAL DIET

1 Minute		2 Minutes		5 Minutes		10 Minutes	
State 4	State 3	State 4	State 3	State 4	State 3	State 4	State 3
1.83	1.80	1.53	1.16	1.41	1.40	1.37	1.62
1.70	1.40	1.55	1.57	1.36	1.39	1.28	4.94
1.46	1.81	1.58	1.49	1.66	1.57	1.53	1.85

DEFICIENT DIET

1 Minute		2 Minutes		5 Minutes		10 Minutes	
State 4	State 3	State 4	State 3	State 4	State 3	State 4	State 3
0.84	0.82	0.85	0.79	0.83	0.82	0.85	0.80
0.73	0.82	0.79	0.71	0.83	0.97	0.81	1.14

APPENDIX D

The abbreviations used throughout the text are as follows.

APPENDIX D (Continued)

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
Ca-45	Calcium-45
Ca	Calcium ion
CPM	Counts Per Minute
DNP	2,4-dinitrophenol
EDTA	Ethylene Diamine Tetracetic Acid
EFA	Essential Fatty Acids
Mn	Manganese ion
Mg	Magnesium ion
P-32	Phosphorous-32
Pi	Inorganic Phosphate
Sr	Strontium ion

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