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THE UNSATURATED FATTY ACID CONTENT OF MITOCHONDRIA IN RELATION TO OXIDATION OF EXOGENOUS REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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SUMMARY

Liver mitochondria isolated from essential fatty acid-deficient rats oxidize in hypotonic solution exogenous NADH more slowly than mitochondria isolated from normal rats. Because this difference becomes apparent with the onset of the essential fatty acid deficiency, and disappears when the deficient animal is fed a normal (essential fatty acid-sufficient) diet it appeared that measurement of NADH oxidation rates might be the basis of an *in vitro* assay for essential fatty acid deficiency. However, no close correlation between the pattern of mitochondrial fatty acid unsaturation and the ability to oxidize NADH could be observed. Indeed, diets could be manipulated in several ways to produce conditions wherein mitochondria presumably sufficient in essential fatty acids exhibited either a low capacity to oxidize NADH, similar to essential fatty acid-deficient mitochondria, or the reverse. It is concluded that the pattern of mitochondrial fatty acid unsaturation may vary widely without affecting the capacity to oxidize NADH, and presumably, therefore, the specific stability of the membrane.

INTRODUCTION

Earlier work[1] has shown that liver mitochondria from rats reared on diets deficient in essential fatty acids were able to oxidize exogenous NADH at appreciable rates in an isotonic medium, whereas normal ones were not. In hypotonic media, on the other hand, mitochondria from essential fatty acid-deficient rats carried out the oxidation of NADH more slowly than did normal mitochondria. The two mitochondrial preparations apparently contained the components of the system required for the oxidation, as indicated by the fact that sonicated mitochondria from normal and deficient animals yielded the same oxidation rates. We suggested, as a possible interpretation of this, that for normal mitochondrial membranes to become permeable to NADH in hypotonic media, steric factors were required that appeared to be dependent on the presence of essential fatty acids. Mitochondria from deficient rats did not

possess the necessary complement of these fatty acids, and could not demonstrate the membrane permeability changes under conditions of hypotonicity that were observed in normal mitochondria.

The present experiments investigate more closely a possible relationship between mitochondrial fatty acid composition and membrane permeability to NADH. LEHNINGER[2] has suggested that the oxidation of added NADH by mitochondria *in vitro* is an indication of the permeability of mitochondria to NADH. This view seems to be held generally, and in the present report NADH oxidizability is assumed to indicate mitochondrial membrane permeability*.

EXPERIMENTAL

Diets

Male Holtzman or Wistar rats were weaned at 18 days of age and fed, either a normal or essential fatty acid-deficient diet for 3 months. Normal diets fed were either a standard chow or a fat-free diet in which corn oil was substituted at a level of 5 % for an equal weight of dextrose (11 % of dietary calories). The essential fatty acid deficient-diet is described elsewhere[3]. Ethyl arachidonate was a gift from Hoffmann-La Roche, Nutley, N. J. Ethyl linolenate and ethyl linoleate were prepared from linseed oil or corn oil according to MCCUTCHEON[4].

Mitochondrial reactions

Mitochondria were isolated and NADH oxidation specific activities were measured as previously described[1]. Mitochondrial lipids were prepared[5] and fatty acids analyzed either on whole lipid extracts by a modification of the method of ENDRES[6] or on phospholipid preparations[5,7] by gas–liquid chromatography. Before addition of the saturated NaCl solution, 2–4 drops of redistilled benzene were added to the reaction flask. After centrifugation the fatty acid methyl esters concentrated in the benzene layer were immediately analyzed by gas–liquid chromatography[7].

RESULTS

In the first experiment to be reported, rats were reared for 3 months on either the fat-deficient diet or the diet containing 11 % of the dietary calories as corn oil. The essential fatty acid-deficient animals were then starved for 48 h and re-fed for 13 days either the 11 % corn oil diet or the fat-deficient diet to which had been added ethyl arachidonate (2.2 % of the dietary calories) or ethyl linolenate (4.4 % of the dietary calories).

The effect of dietary lipids on mitochondrial fatty acid content and NADH oxidation specific activity are summarized in Table I. The NADH oxidase activity is 0.51 for normal rats; polyenoic acids and essential fatty acids each comprise about 40 % of the total mitochondrial fatty acids. For mitochondria isolated from deficient rats, NADH oxidation specific activity is 0.19; the polyenoic acids and essential fatty acids comprise 32 and 12 %, respectively, of the total fatty acids.

* In making this assumption, the authors recognize the possibility of other explanations for the differences in NADH oxidation rates, depending on the nature of the diet, *e.g.* an inhibition or de-inhibition of NADH dehydrogenase related to the free fatty acid content of the liver cells.

TABLE I

MITOCHONDRIAL FATTY ACIDS AND NADH OXIDATION SPECIFIC ACTIVITY

*Experiment**	*Diet*	*Weight gain (g/13 days)*	*NADH oxidation specific activity***	*Mitochondrial fatty acids****		
				Unsaturated	*Polyenoic*	*EFA*
1	11 % corn oil	—	0.51 ± 0.03	61.0	41.7	38.9
2	Fat-deficient	—	0.19 ± 0.05	58.2	32.3	11.8
Fat-deficient rats supplemented with polyenoic acids for 13 days:						
3	11 % corn oil	13	0.75 ± 0.11	56.4	47.1	45.6
4	2.2 % Arachidonate	16	0.60 ± 0.05	57.3	44.2	41.1
5	4.4 % Linolenate	1	0.57 ± 0.04	58.0	34.1	3.7

* All animals, 4 in each experiment, were fed deficient or corn oil diets for 3 months.

** Values are the average of 4 experiments ± S.D.

*** The weight % of each mitochondrial fatty acid (C_{12}–C_{22}) was calculated[15]. Unsaturated fatty acids represent the sum, in weight %, of all fatty acids having one or more double bond; polyenoic acids represent the sum of all fatty acids having two or more double bonds; EFA represent the sum of all fatty acids having two or more double bonds and belong to the linoleate family: 9, 12–18:2; 8, 11, 14–20:3; 5, 8, 11, 14–20:4; 4, 7, 10, 13, 16–22:5. The values represent the average of 4 determinations.

When either arachidonate or linolenate was added to deficient diets NADH oxidation rates characteristic of normal mitochondria were re-established in 13 days (Table I). The mitochondrial essential fatty acid levels in the deficient rats that had been supplemented with corn oil or arachidonate were normal while the mitochondrial essential fatty acid content and the growth rate of animals fed linolenate was depressed. RAHM AND HOLMAN[8] observed that members of the linolenate family of acids are not fully effective as essential fatty acid; when fed to weanling rats they support growth but fail to prevent essential fatty acid deficiency dermatitis. Further, mitochondrial fatty acids from rats reared on a fat-free diet to which had been added linolenate differed from controls (animals reared on a fat-free diet) only in having less 5,8,11–20:3 and more 4,7,10,13,16,19–22:6 acid. There were no differences in essential fatty acid (linoleate and arachidonate) content. These results are confirmed by the data of Table I and, taken together, suggest that if indeed mitochondrial fatty acids have anything to do with mitochondrial permeability to NADH, it probably does not concern essential fatty acids.

In the second experiment to be reported rats were fed a fat-deficient diet for 3 months, and were then placed on fat-free diets containing graded amounts of linoleate for 3 weeks. Other rats were similarly re-fed for 2 weeks the diet to which had been added corn oil in varying amounts. At the end of these periods the animals were sacrificed and mitochondria were prepared, and NADH oxidation specific activities and phospholipid fatty acid contents were determined.

The results, shown in Table II, indicate that diets containing 0.15 % linoleate partially induced a return to normal mitochondrial fatty acid patterns and weight gain when fed to deficient rats for 3 weeks. The NADH oxidation specific activity, however, indicated no restoration of membrane integrity. A 10-fold increase in the dietary linoleate to 1.6 % resulted in normal mitochondrial fatty acid contents, normal weight gain, but mitochondrial permeability to NADH still resembled that of an essential fatty acid-deficient animal.

A similar lack of correlation was observed in the animals fed varying levels of linoleate, as corn oil. In this group a level of 2.1 % linoleate resulted in a NADH oxidation specific activity of 0.23, and the mitochondrial fatty acid level was normal. A NADH oxidation specific activity approximately that of normal controls was not observed until a level of 8.4 % linoleate was fed. It is interesting to note that in this group of animals, one sub-group was fed a diet containing 3.2 % purified linoleate, and the data obtained fit very well between the group fed 2.1 and 4.2 % linoleate, as corn oil, despite the fact that the latter groups were fed approx. 10–35 % more fat calories than the group fed 3.2 % linoleate.

TABLE II

THE EFFECT OF GRADED LEVELS OF LINOLEATE ON MITOCHONDRIAL FATTY ACIDS AND NADH OXIDATION SPECIFIC ACTIVITY*

Diet (% *linoleate*)	*Weight* (*g*)	*NADH oxidation specific activity*	*Mitochondrial fatty acids* §		
			Unsaturated	*Polyenoic*	*EFA*
0.0	329	0.11 ± 0.08	67.5	28.0	10.6
6.6 (as corn oil)	448	0.37 ± 0.08	66.3	41.2	41.2
Fat-deficient rats supplemented with linoleate for 21 days:					
0.15	367	0.10 ± 0.03	72.6	33.4	22.4
0.30	370	0.11 ± 0.03	71.9	37.7	27.2
0.40	376	0.17 ± 0.02	65.6	33.4	22.1
0.60	412	0.12 ± 0.03	69.3	37.4	29.9
0.80	395	0.18 ± 0.02	65.5	41.9	32.9
1.60	432	0.18 ± 0.02	62.8	42.0	37.0
Fat-deficient rats** supplemented with linoleate for 14 days:					
3.20	N.D.***	0.25 ± 0.06	48.1	31.5	28.6
1.05 (as corn oil)	N.D.	0.19 ± 0.05	66.4	40.8	32.1
2.10 (as corn oil)	N.D.	0.23 ± 0.04	46.4	28.7	25.9
4.20 (as corn oil)	N.D.	0.28 ± 0.06	54.5	40.7	40.0
8.40 (as corn oil)	N.D.	0.34 ± 0.03	52.6	42.3	42.3

* Five rats, Holtzman strain except where indicated, were used for each experiment. All results are expressed as the average or the average ± S.D. of 5 determinations.

** Wistar rats.

*** ND, not determined.

§ See footnote***, Table I.

In a third set of experiments, rats reared on an essential fatty acid sufficient diet were fasted for 2 days following which they were fed either 5 g/day of the essential fatty acid deficient-diet, or 5 g/day of the 11 % corn oil diet for 16 days. Such dietary regimens not only change the fatty acid composition of membrane lipids but also lead to alterations in activity of various enzymes (*cf.* ref. 8). The results of this experiment are shown in Table III. Mitochondria isolated from rats fed the restricted amount of the corn oil diet for 16 days had a normal fatty acid pattern (42 % polyenoic acids; 36 % essential fatty acids) and exhibited normal NADH oxidizability (NADH oxidation specific acitvity, 0.44), whereas mitochondria isolated from rats fed the essential fatty acid deficient diets exhibited normal NADH oxidizability, but had a fatty acid pattern characteristic of an essential fatty acid deficiency (38 % polyenoic acids; 15 % essential fatty acids).

TABLE III

EFFECT OF RESTRICTED DIETS ON MITOCHONDRIAL FATTY ACIDS AND NADH OXIDATION SPECIFIC ACTIVITY*

Diet (*5 g/day*)	*Weight loss*	*NADH oxidation specific activity*	*Mitochondrial fatty acids***		
			Unsaturated	*Polyenoic*	*EFA*
11% corn oil (16 days)	102	0.44 ± 0.07	54.3	42.1	39.6
Fat-free (16 days)	130	0.46 ± 0.02	60.9	38.2	15.0

* Three rats were used in each experiment. Results are expressed as the average or the average ± S.D. of 3 determinations.

** See footnote***, Table I.

DISCUSSION

Investigations in several laboratories[9–12] have led to the assumption that membranes of liver mitochondria from essential fatty acid deficient rats are in some way labilized and it has been suggested that essential fatty acids endow membranes with certain structural properties which are essential to the overall function and integrity of the membrane. The present experiments are an attempt to investigate this further. The data demonstrate that if essential fatty acids do indeed endow mitochondrial membranes with any special property that affects NADH permeability, such an effect must operate at a level which these experiments could not measure. The possibility still remains, unexplored in the present experiments, that relatively small amounts of 5, 8, 11–20:3 acid which increase in an essential fatty acid deficiency, have a deleterious effect on membrane stability, or there is a critical "threshold" amount of an essential fatty acid that is necessary for membrane integrity, as measured by NADH oxidation specific activity.

If the permeability of the mitochondrial membrane to NADH may serve as a model for membrane permeability the results of our experiments indicate that fatty acid unsaturation *per se* has little effect on membrane permeability. In all dietary experiments reported here, the total amount of fatty acid unsaturation remained remarkably constant.

Moreover, because the most sensitive indicators of mitochondrial function: oxidative phosphorylation, ATP–P_i exchange and ATPase activity, respiratory control, the capacity for energized translocation of mono- and divalent cations and NADH oxidation are similar in mitochondria isolated from normal rats and unstressed mitochondria from essential fatty acid-deficient rats[13], we suggest that the fatty acid content of the mitochondrial membrane may vary considerably without a corresponding change in membrane enzymic activity, or the specific stability of the membrane, namely: the ability of the membrane to support electrochemical gradients or the transport systems based on these gradients.

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