Swelling of Liver Mitochondria from Rats Fed Diets Deficient in Essential Fatty Acids.* (25625)

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Liver mitochondria from rats fed diets deficient in essential fatty acids (EFA)‡ have been shown to uncouple oxidative phosphorylation(1); subsequently, Levin *et al.*(2) demonstrated that normal rat liver mitochondria resemble those from rats deficient in EFA with respect to succinate oxidizing capacity and oxidative phosphorylation when studied in a suspending medium of low osmolarity. These workers also showed that liver mitochondria from rats fed diets deficient in EFA were slightly larger than normal mitochondria, were less opaque and contained many crescent-shaped components. Furthermore, increased fragility of the red blood cells as well as increased permeability of the skin of rats has been demonstrated(3) in EFA deficiency. Such studies suggest that both the cellular and mitochondrial membrane of the rat fed diets deficient in essential fatty acids differ morphologically from those of normal rats. On the other hand, since the early observations by Cleland(4) and by Raaflaub (5), swelling phenomena have been frequently used to study structural alterations of mitochondria in relation to biochemical properties. This communication describes studies which show that liver mitochondria from rats deficient in EFA have characteristic patterns of swelling which are different in several ways from those of normal rats. The fatty acid composition of the mitochondrial, microsomal and supernatant fractions isolated from livers of rats fed diets deficient in essential fatty acids and from normal rats are also presented.

Methods. Weanling male albino rats housed in individual cages were divided into groups and maintained on experimental diets (6) *ad libitum* for 15 to 19 weeks. One group of rats was fed a purified diet including 5% by weight of corn oil; the other group received a diet devoid of fat. Liver mitochondria were isolated in 0.88 M sucrose by the method of Ziegler *et al.*(7). Glass-redistilled water and a glass-Teflon homogenizer were used throughout this study. One ml of the resulting mitochondrial preparation was equivalent to 1.0 g of fresh rat liver. The swelling of mitochondria was measured spectrophotometrically by the method of Lehninger *et al.*(8) as slightly modified. The standard test system was 3.0 ml of 0.30 M sucrose—0.02 M Tris buffer, pH 7.4, using a Beckman DU Spectrophotometer equipped with thermospacers to maintain temperature in the cuvette carriage chamber at 23-24°C. Studies of mitochondrial swelling under various experimental conditions were performed on liver mitochondria from 12 control and 12 rats deficient in EFA. Aliquots of the liver mitochondrial preparations, as well as microsomal and supernatant fractions isolated and purified by the method of Schneider and Hogeboom(8) were added to 5 volumes of acetone:ethanol (1:1 v/v). After heating the preparation at 40° for one hour, precipitated protein was removed by filtration. Aliquots of the filtrate were used for isolation of fatty acids. Fatty acid preparations were methylated by a modification of the method of Stoffel *et al.*(9). Analyses of the mixtures of fatty acid esters were carried out using gas-liquid chromatography. A 4-foot column employing ethylene glycol adipate polyester (EGA) as the stationary phase and an argon ionization detector (W. G. Pye and Co., Cambridge, England) was used.

Results. Mitochondrial swelling. Results of the studies of swelling rates are illustrated in Fig. 1. In contrast to the low initial swell-

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‡ Abbreviations: EFA, essential fatty acids; EDTA, ethylene-diaminetetraacetate; Tris, tris(hydroxymethyl) aminomethane; ATP, adenosine triphosphate.

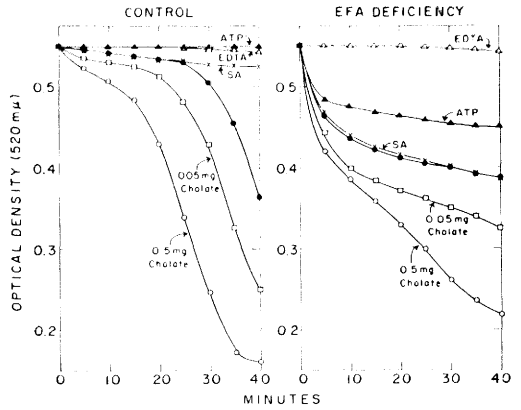

FIG. 1. Rates of swelling of liver mitochondria from rats fed a diet containing corn oil (5% of diet) and a diet deficient in essential fatty acids. Measurements were made at 520 mμ; temp., 23°-24°. The following additions were made to 0.30 M sucrose–0.02 M Tris, pH 7.4: ●——● no addition; ▲——▲ 0.003 M ATP; △——△ 0.001 M EDTA; ×——× 30 mg serum albumin per 3 ml media; □——□ 0.05 mg cholate per 3 ml media; ○——○ 0.5 mg cholate per 3 ml media.

ing rates of control mitochondria, markedly higher swelling rates during the first part of the observation period were seen for EFA deficient mitochondria. Relatively slower swelling rates of control and EFA deficient liver mitochondria were observed in 0.88 M sucrose than were seen using 0.30 M sucrose medium.

ATP prevented swelling of control mitochondria in 0.30 M sucrose medium, but not that of EFA deficient mitochondria. On the other hand, ATP appeared partially to inhibit swelling of control and EFA deficient mitochondria in a KCl media (results not indicated on figure). The different effects of ATP on mitochondrial swelling of EFA deficient mitochondria in sucrose and KCl media are reminiscent of the observations of Lehninger *et al.*(10) on thyroxine-treated mitochondria. EDTA, which was added to test the action of a metal chelating agent in the sucrose medium, showed a protective action against swelling of both groups of mitochondria. Thus, it appears that the mechanisms of action of ATP and EDTA as inhibitors of mitochondrial swelling are different. A similar conclusion was reached by Lehninger *et al.* (8) from studies of thyroxine-induced swelling.

Serum albumin (human, Cohn Fraction V) was added to the sucrose medium, to study the effect of a high molecular weight substance. Serum albumin prevented swelling of the control mitochondria during the latter part of observation period (Fig. 1); however, it did not prevent swelling of EFA deficient mitochondria. This observation suggested that permeability of the membrane of the liver mitochondrion was altered by feeding the fat-deficient diet. Serum albumin has been shown to act as a fatty acid binding agent; therefore, a possible explanation of the above phenomenon is that lack of EFA might influence permeability of the mitochondrial membrane by altering the binding sites for the albumin added to the test media.

Earlier studies have shown that certain detergents cause mitochondrial swelling(11,12). Potassium cholate, presumably a naturally-occurring anionic detergent in liver, accentuated the swelling pattern of mitochondria isolated from livers of each group of rats in this study. "Pluronic F68" (polyethylene propylene glycol), which is a non-ionic detergent, was also tested. The results were similar to those seen with cholate in that the shape of the swelling curves of the mitochondria from both groups of rats were merely accentuated.

The effects of various respiratory inhibitors, in presence of β-hydroxy-butyrate as a chain reductant, on the swelling of mitochondria from livers of the 2 groups of rats were also evaluated. Lehninger(13) has shown that cyanide, amytal and antimycin A inhibit mitochondrial swelling. All 3 agents were effective in preventing swelling of control mitochondria in these experiments and were only slightly less effective as inhibitors of swelling of EFA deficient mitochondria. All of the above phenomena were observed consistently, being characteristic of mitochondria isolated from 12 control and 12 EFA deficient rats.

Fatty acid analyses of mitochondria, microsomes and supernatant fractions. The distribution of fatty acids from total lipid extracts of mitochondria, microsomes and supernatant fractions isolated from livers of control rats and from rats fed diets deficient in essential fatty acids is indicated in Table I. Values are the mean figures for 2 animals from each group. The mitochondrial lipid from liv-

TABLE I. A Comparison of Effect of Feeding a Fat-Free and a Corn Oil Containing Diet to Rats on Fatty Acid Composition of Lipids of Mitochondria, Microsomes and the Supernatant Fraction Isolated from Liver.

Fatty acid Chain length: unsat.	Mitochondria		Microsomes		Supernatant	
	Control	EFA deficient	Control	EFA deficient	Control	EFA deficient
14:0	1.8*	.6	.3	.1	.5	.6
15:0	.0	.0	.1	.0	.4	.2
16:0	14.8	15.5	20.2	17.9	23.2	24.1
16:1	3.4	8.2	2.4	6.6	3.7	10.4
17:0	.0	.0	.3	.0	.0	.4
18:0	19.1	19.6	23.1	19.6	16.9	14.3
18:1	10.6	24.0	13.1	23.1	19.0	29.6
18:2	20.3	1.6	13.9	2.7	17.8	1.1
20:3(?)	.0	21.2	.0	22.2	.0	9.4
20:4	30.0	8.9	25.7	3.7	18.5	4.8

* Values expressed as a percentage of total fatty acids in the fraction.

ers of rats fed control (corn oil containing) diets had a higher percentage of fatty acids as linoleic and arachidonic acids than did either microsomal or supernatant fractions. These 2 acids made up 50% of the fatty acids of the control mitochondria. The feeding of diets essentially devoid of fat resulted in very low levels of linoleic and arachidonic acids in all 3 cellular subfractions. In agreement with the rather consistent observations of various workers in studies of essential fatty acid deficiency, the differences in total unsaturation of lipids from the 2 groups of rats were partially offset by the appearance of higher concentrations of endogenously derived monounsaturated fatty acids (*i.e.*, oleic and palmitoleic acids) in fractions from the rats fed fat-free diets. There were, furthermore, 2 twenty carbon acids in the chromatographs of methyl esters of fatty acids isolated from the liver cellular subfractions of rats deficient in essential fatty acids. The smaller of the 2 peaks had a retention time which was consistent with a 20:4 acid. The larger of the 2 peaks had a shorter retention time which was consistent with that of a 20:2 acid(14) but was probably 5, 8, 11-eicosatrienoic acid. After hydrogenation of lipid samples from the EFA deficient group, a single peak equivalent in size to the sum of the eicosatrienoic (20:3) and eicosatrienoic (20:4) peaks before hydrogenation appeared at the arachidic (20:0) acid locus.

Discussion. These studies indicate that *in vitro* patterns of swelling of normal mitochondria and mitochondria from rats fed diets deficient in essential fatty acids were different when a variety of suspending media and supplements were used. Fatty acid composition of lipids from control and deficient mitochondria were also strikingly different. The presence of high concentrations of linoleic and arachidonic acids in control mitochondria and of 5, 8, 11-eicosatrienoic in deficient mitochondria is largely consistent with the findings of Klein and Johnson(15) using the alkaline isomerization technic. Twenty-one per cent of the fatty acids in lipids of mitochondria from the deficient group appeared to be an eicosadienoic or eicosatrienoic acid of unknown specific structure.

It seems possible that differences in swelling of mitochondria from the 2 groups of rats may be related to differences in concentrations of the fatty acids in the lipids present in the mitochondrial membrane. The finding that anaerobiasis protects mitochondria against ageing(16) suggests that the protective effect of EFA may be related to their role as easily oxidizable fatty acids. The inhibitory effect of serum albumin, which has a strong affinity for free fatty acids, on swelling of normal, but not deficient, mitochondria could also be interpreted as resulting from the binding of albumin to sites on the mitochondrial membrane whose albumin binding capacities are influenced by their fatty acid composition.

Klein and Johnson(1) have shown that liver mitochondria from rats fed diets deficient in essential fatty acids uncouple oxidation from phosphorylation. Results of the present study demonstrated that ATP added to 0.30 M sucrose media inhibits swelling of mitochondria from the control but not the

swelling of mitochondria from the deficient group. On the other hand, EDTA inhibited swelling of liver mitochondria from both groups of rats. These data would indicate that ATP influences mitochondrial swelling in a manner related to its role as a phosphate donor.

Summary. Comparison of rates of *in vitro* swelling of liver mitochondria from control rats and from rats fed diets deficient in essential fatty acids were studied under a variety of conditions. Higher swelling rates of EFA deficient mitochondria were observed using 0.30 M sucrose medium. Addition of ATP or serum albumin inhibited swelling of normal but not that of EFA deficient mitochondria. Respiratory inhibitors prevented swelling of both groups of mitochondria. Cholate accentuated the swelling pattern of mitochondria from both groups of rats. The fatty acid composition of lipid from mitochondrial, microsomal and supernatant fractions from both groups of livers were also presented.

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Linoleic Acid as Causative Agent of Encephalomalacia in Chickens Fed Oxidized Fats.* (25626)

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Chickens fed certain partially oxidized fats manifest an edemic condition known as "toxic fat" disease(1,2,3). Our original purpose was to determine whether the feeding of heated cottonseed oil will result in this disease. It was found that ingestion of heated cottonseed oil does not cause "toxic fat" disease but does result in a high incidence of encephalomalacia even with dietary levels of Vit. E usually sufficient to prevent this disease. High levels of Vit. E or a supplement of the antioxidant Santoquin† (herein after referred to by generic term ethoxyquin) completely prevented encephalomalacia. Evidence is presented which demonstrates that increased requirement for Vit. E was caused by presence of linoleic acid in the diet. This is in agreement with other reports(4,5,6). Fats high in linolenic acid but

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† Registered trademark of Monsanto Chemical Co., for 1,2-dihydro-6-ethoxy, 2,2,4-trimethyl quinoline.