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THE EFFECT OF CHOLINE DEFICIENCY ON THE OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA

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The outer membranes of mitochondria prepared from the liver of rats kept 12 days on a choline-deficient diet were analyzed for changes in phospholipid and protein content. The total amount of phospholipid in the outer membranes was not affected by the deficiency. There was, however, a significant decrease in the amount of phosphatidylcholine and an increase in phosphatidylethanolamine. The alterations in the membrane phospholipids were reflected in a reduction in the fluorescence of the membrane probe, 8-anilino-1-naphthalene sulfonate. Choline deficiency also affected the protein composition of the outer membranes as judged by electrophoretic analysis; however, the activity of several enzymes which serve as markers for the outer membrane was not affected by the deficiency.

Shortly after rats have been placed on a diet deficient in choline, it is possible to observe rather substantial structural and compositional changes in the hepatocyte [1,2]. The most obvious manifestation of the diet is the hepatic accumulation of triacylglycerols due, in part, to an impaired ability of the cell to secrete serum lipoproteins [1,3]; the changes in triacylglycerol secretion are, however, preceded by alterations in the phospholipid composition of the cell. While the amount of total cellular phospholipid may not change, the proportion of cellular phosphatidylethanolamine (PE) increases and the amount of phosphatidylcholine (PC) diminishes [4,5]. We have studied the effect of 12 days of choline deficiency on the outer membranes from rat liver mitochondria; the parameters which have been measured include phospholipid and protein constituents as well as the activities of several enzymes typical of the outer membranes.

Male Wistar rats weighing approximately 100–125 g received a commercially prepared choline-deficient diet (Nutritional Biochemicals, Cleveland, OH), ad libitum while the controls were fed the same diet which had been supplemented with approx. 0.17% choline chloride. After 12 days on the experimental diets, there were no obvious differences in appearance or behavior between the choline-supplemented and -deficient animals. The livers of deficient animals had a fatty infiltration which was discernible on gross examination; however, there were no other obvious pathologies, nor did the deficient animals weigh significantly less than the choline-supplemented group (176 ± 44 g for choline deficient group, $n = 9$; 185 ± 37 g for control group, $n = 9$). Mitochondrial outer membranes were prepared from the livers [6].

Analysis of the phospholipid content of the outer membranes according to a modification of

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; ANS, 8-anilino-1-naphthalene sulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE I

THE EFFECT OF CHOLINE DEFICIENCY ON THE PHOSPHOLIPID CONTENT OF MITOCHONDRIAL OUTER MEMBRANES

	Choline-supplemented	Choline deficient
Total phospholipids (PL) [a] (mg PL/mg protein)	0.52 ± 0.1	0.57 ± 28
Component phospholipids [a] (mg specific PL/mg total PL)		
Phosphatidylcholine [b]	0.58 ± 0.04	0.48 ± 0.04
Phosphatidylethanolamine [b]	0.21 ± 0.09	0.36 ± 0.03
Phosphatidylinositol	0.07 ± 0.02	0.04 ± 0.03
Phosphatidylserine	0.15 ± 0.05	0.13 ± 0.01
Ratio of phosphatidylethanolamine to phosphatidylcholine [b]	0.38 ± 0.18	0.76 ± 0.12

[a] Each value is the mean ± S.D. of three separate determinations of three pooled livers.
[b] Difference between supplemented and deficient phospholipid is significant at the level of $P < 0.05$.

the Folch method [7–9] showed that while there was no significant effect of the deficiency on either the amount of phosphatidylserine or phosphatidylinositol, there was a significant reduction in the amount of PC. In deficient rats, the decreased PC content was offset by a corresponding increase in PE so that the total amount of outer-membrane phospholipid was not changed, but the ratio of PE to PC was substantially increased (Table I). Since PC and PE comprise 70–80% of the outer-mem-

TABLE II

THE EFFECT OF CHOLINE DEFICIENCY ON THE ANS FLUORESCENCE OF MITOCHONDRIAL OUTER MEMBRANE LIPIDS [a]

	Choline-supplemented	Choline-deficient
Outer membranes:		
Fluorescence (arbitrary units)/mg protein [b]	12.1 ± 0.4	10.5 ± 1.0 [f]
Maximum wavelength of fluorescent emission [c]	474 nm	474 nm
Liposomes from outer membranes lipids [d]		
Fluorescence (arbitrary units)/mg lipid	16.5 ± 0.9	9.5 ± 0.2 [f]
Maximum wavelength of fluorescent emission [c]	470 nm	470 nm
Lipid contribution to outer membrane [e]		
Fluorescence (arbitrary units)/mg protein	6.4 ± 0.7	4.7 ± 1.6

[a] Each value is the mean ± S.D. of three separate determinations.
[b] Outer membranes were suspended to a concentration of 200 μg protein per ml in 75 KCl/5 mM Hepes (7.5) containing $2 \cdot 10^{-5}$ M ANS. The fluorescence of this mixture was compared to a similar solution without the outer membranes using a Farand filter fluorometer. Exciting light was filtered with a Corning 3-60 filter and emitted light was filtered with a Corning 3-71 filter.
[c] Emission maxima were determined using a Perkin-Elmer 650-10S scanning fluorescence spectrophotometer. Outer membranes samples of 200 μg protein per ml or liposomes at 100 μg phospholipid per ml were suspended in 75 mM KCl/5 mM Hepes [7–5] containing $2 \cdot 10^{-5}$ M ANS. These samples were excited at 370 nM and fluorescence emission was scanned between 410 nM and 520 nM. Each value is representative of three determinations.
[d] Liposomes were prepared from lipids obtained by chloroform/methanol extraction of outer membranes [7–9]. The ANS fluorescence was estimated as described above using 100 μg phospholipid per ml.
[e] Lipid contribution was calculated using the ANS fluorescence for liposomes prepared from choline-deficient and -supplemented outer membranes and the phospholipid content of these same membranes (0.49 ± 0.18 and 0.39 ± 0.03 mg lipid/mg protein, respectively).
[f] Difference between supplemented and deficient PL is significant at the level of $P < 0.05$.

brane phospholipids, one might expect the deficiency would also influence the membrane-induced effect on the fluorescence of the membrane probe 8-anilino-1-naphthalene sulfonate (ANS). In fact, as can be seen in Table II. Outer membranes from deficient rats exhibited less fluorescence at nearly saturating concentrations of ANS (Fig. 1) than the outer membranes from supplemented controls. This effect is much more pronounced when liposomes prepared from deficient outer membranes are compared with liposomes from supplemented outer membranes (Table II). It should be noticed that the maximum wavelengths for ANS fluorescence in outer membranes and liposomes prepared from outer membranes was unaffected by the deficiency. Also in Table II, the ANS fluorescence of liposomes prepared from outer-membrane phospholipids and the amount of phospholipid per mg of outer-membrane protein has been used to estimate the contribution of phospholipids to the fluorescence enhancement caused by the outer membranes. From this calculation, it seems that the phospholipids account for about half of the total outer membranes fluorescence but are entirely responsible for the lessened fluorescence of deficient outer membranes. In Fig. 1, the ANS fluorescence of outer membranes from rats fed choline-deficient and -supplemented diets were compared over a range of ANS concentrations. Under these conditions, the maximum ANS fluorescence in outer membranes from deficient animals was about 60% of that of outer membranes from supplemented rats. The apparent dissociation constants were $6.4 \cdot 10^{-5}$ M and $8.2 \cdot 10^{-5}$ M for deficient and supplemented outer membranes, respectively.

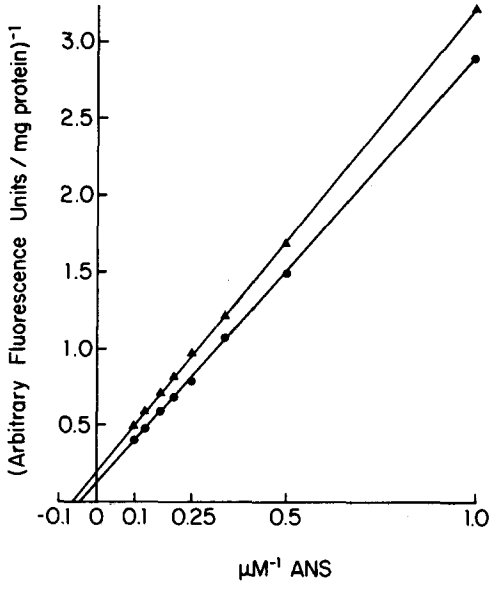

Fig. 1. ANS fluorescence in outer membranes as a function of ANS concentration. Outer membranes from choline-deficient (△) and supplemented (○) rat liver were suspended to 200 μg protein per ml in 75 mM KCl/5 mM Hepes (pH 7.5) and amounts of ANS between 10^{-6} M and 10^{-5} M were added. The samples were excited at 370 nM and fluorescence emission at 474 nM was measured with a Perkin-Elmer 650-10S scanning fluorescence spectrophotometer. The data have been plotted based on computer-calculated ordinate and abcissa intercepts.

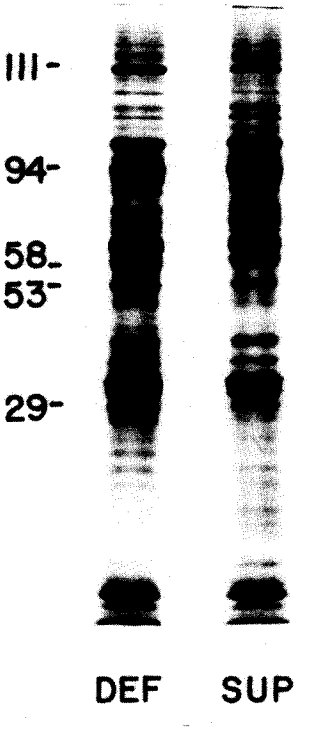

Fig. 2. The effect of choline deficiency on the polypeptide composition of the mitochondrial outer membrane. 50-μg samples of outer membranes from animals fed a choline-deficient diet and a similar diet supplemented with choline are compared using the sodium dodecyl sulfate gel system described by Laemmli [10]. These gel patterns are representative of five similar analyses. Molecular weights are based on comparisons with standards of known size.

In addition to the altered lipid content of outer membranes from choline-deficient rats, there was a substantial alteration in the protein components of the membrane. This change is evident in Fig. 2, which compares analyses of the outer-membrane proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis [10]. This experiment was performed on five separate occasions and the gel patterns were identical. The proportions of several of the outer-membrane proteins are changed by the deficiency, as judged by the intensity of the Coomassie brilliant blue staining. The most striking examples are proteins that migrate with apparent molecular weights of 94000 and 58000; the former protein is so scarce in outer membranes from choline-deficient animals as to be missing, while the 58 kDa protein is much enriched in deficient outer membranes. These are not the only examples of outer-membrane proteins affected by the deficiency; species at 111000, 53000 and 29000 are also enriched in deficient outer membranes. In fact, a careful inspection of Fig. 2 indicates that, although there are regions of the gel which stain with approximately the same intensity, there are a large number of proteins which are apparently present in different amounts in the supplemented and deficient outer membranes. An attempt was made to identify changes in enzyme activity in outer membranes from deficient animals and so several activities typical of the outer membranes were measured. As can be seen in Table III, none of these was affected by the altered lipid composition of the outer membranes.

It is quite clear that choline deficiency causes disturbances in both the lipid and protein components of the outer membranes. It cannot be determined from the data presented here whether the changed protein composition of the outer membranes is a consequence of an altered lipid milieu or a result of some other effect of the deficiency. It should also be noted that the lack of an effect of the deficiency on monoamine oxidases A and B confirms earlier observations which suggest that lipids do not play a role in the regulation of these enzymes [15].

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TABLE III

THE EFFECT OF CHOLINE DEFICIENCY ON ENZYMATIC ACTIVITIES IN THE MITOCHONDRIAL OUTER MEMBRANE

Enzymatic activities were measured by previoulsy published methods [11–13] and specific enzymatic activities are based on estimates of protein according to the method of Lowry et al. [14]. Maximum velocities and K_m values were estimated by extension of a double-reciprocal plot of enzyme velocity against substrate concentration.

	n	Choline-supplemented	Choline-deficient
NADH: ferricyanide reductase			
Maximum velocity (A_{450}/min per mg)	9	40.6 ± 20	36.9 ± 6.8
K_m (mM) for NADH		0.28 ± 0.19	0.20 ± 0.07
Cytochrome b_5 (nmol/mg)	3	0.22 ± 0.03	0.35 ± 0.15
NADH: cytochrome c reductase			
Maximum velocity (nmol/min per mg)	6	2460 ± 1030	2070 ± 710
K_m (μM) for NADH		4.5 ± 2.7	3.9 ± 1.6
Monoamine oxidase A (serotonin oxidation)			
Maximum velocity (nmol/10 min per mg)	9	403 ± 250	431 ± 190
K_m (mM) for serotonin		0.039 ± 0.022	0.040 ± 0.016
Monoamine oxidase B (benzylamine oxidation)			
Maximum velocity (nmol/min per mg)	12	68 ± 30	77 ± 33
K_m (mM) for benzylamine		0.34 ± 0.15	0.41 ± 0.13

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