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ESSENTIAL FATTY ACIDS AND GLUCOSE PERMEABILITY OF LECITHIN MEMBRANES

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SUMMARY

Lecithins were isolated from egg yolk, and from internal organs of fish, essential fatty acid-deficient rats and normal rats. Fatty acid composition of each preparation was determined by gas–liquid chromatography.

From each lecithin, liposomes were formed in 0.3 M glucose. Extraparticulate glucose was removed by dialysis, and the subsequent rates of glucose efflux from within the particles were determined at various temperatures.

The liposomes prepared from essential fatty acid-deficient lecithin permitted a slower rate of glucose diffusion than those of normal rats and fish at all temperatures, and had a comparable rate with that of egg lecithin. Energies of activation for diffusion were 26.3 ± 1.6, 28.9 ± 5.8, 26.2 ± 3.9 and 26.7 ± 2.4 kcal/mole glucose, for lecithins from egg, essential fatty acid-deficient rats, normal rats and fish, respectively.

Differences in glucose diffusion rates were largely dependent upon the degree of unsaturation of the fatty acid residues in the lecithin molecules.

INTRODUCTION

The intimate involvement of essential fatty acids with membrane phospholipids has been well documented[1–5]. Many studies offer evidence of marked alteration in the stability and fatty acid composition of essential fatty acid-deficient membranes[3,6–9]; however, the precise function of essential fatty acids is not as yet known. Since it is generally believed that lipids have a definite role in the permeability barrier of biological membranes[10–13], a function of essential fatty acids might be related to diffusion rates of electrolytes and nonelectrolytes across essential fatty acid-deficient membranes compared to normal membranes.

BANGHAM *et al.*[14] have used phospholipid liquid crystals (liposomes) as model membrane systems to study the diffusion of ions and molecules. MOORE *et al.*[15] compared diffusion rates of Na^+ out of liposomes prepared from lecithin isolated from egg yolk and from internal organs of fish, essential fatty acid-deficient rats and normal rats. These workers reported that the membrane of essential fatty acid-deficient lecithin permitted a faster rate of Na^+ efflux at 25° and a slower rate at 50° compared to egg, fish or normal rat lecithin. Activation energies for efflux were 15.5, 11.5, 9.5 and 4.5 kcal/mole Na^+ for normal rat, fish, egg and essential fatty acid-deficient

rat lecithin membranes, respectively. MOORE *et al.*[15] also observed that essential fatty acid-deficient lecithin contained more saturated fatty acids than normal rat lecithin.

DE GIER *et al.*[16] and DEMEL *et al.*[21] studied the diffusion of glucose out of liposomes prepared from synthetic lecithins in which the unsaturation of the fatty acid residues was varied. Increased unsaturation resulted in an enhanced glucose permeability. These observations coupled with the observations of MOORE *et al.*[15] prompted this study of the diffusion of a nonelectrolyte out of liposomes prepared from various naturally occurring lecithins, including essential fatty acid-deficient lecithin.

MATERIALS AND METHODS

Chemicals

Lecithins were isolated from the internal organs of normal rats, essential fatty acid-deficient rats, Rainbow trout and egg yolk. Dicetyl phosphate was purchased from Nutritional Biochemical Co. Hexokinase, glucose-6-phosphate dehydrogenase, $NADP^+$ and ATP were obtained from Calbiochem. Magnesium acetate was a product of Baker Chemical Co., and NaCl and KCl were purchased from Mallinkrodt. Tris was obtained from Fisher Scientific Co., whereas Triton X-100 was purchased from Rohm and Haas Co. Standard methyl esters of fatty acids were purchased from the Hormel Institute.

Preparation of lecithin

The lecithins used in these studies were isolated essentially as described by MOORE *et al.*[15]. However, longer aluminum oxide columns were used, and the columns were washed exhaustively with chloroform prior to elution of the lecithin. This resulted in lecithin fractions of greater purity than that observed by MOORE *et al.*[15]. But there was still a minute quantity of non-polar contaminant apparent in the lecithins which traveled with the solvent front.

Thin-layer chromatography of column fractions was used to verify the purity of the lecithins. Appropriate column fractions were combined and the solvent was removed at room temperature under vacuum. The residue was dissolved in chloroform and the lecithin concentration in each preparation was determined using the phosphorus method of MORRISON[17].

Fatty acid composition

The fatty acid components of each lecithin preparation were determined as described by MOHRHAUER AND HOLMAN[9]. Methyl esters were prepared by treating each lecithin with methanol–boron trifluoride as described by MORRISON AND SMITH[18] and were chromatographed isothermally on an Aerograph 1520 equipped with a flame ionization detector essentially as described by MOORE *et al.*[15]. Comparison of retention times relative to standard methyl esters and comparison of equivalent chain length data with that of HOFSTETTER *et al.*[19] allowed qualitative identification of most peaks. For quantitative analysis, the area of each peak was estimated by multiplying the height by the width at half-height. Overlapping peaks were measured by approximating triangles as described by ETTRE[20]. For peaks comprising 1 % of the

area or more, the average deviation from the mean for duplicate determinations was 2.9 %. The area percent for individual components was found to equal weight percent, as judged from standard ester mixtures, to within 10 %. The weight percent methyl esters was then converted to mole percent for comparative purposes.

Lecithin membranes (liposomes)

The procedure for forming the lecithin–dicetyl phosphate liquid crystal dispersions was generally as described by MOORE *et al.*[15]. For each ml of dispersion 15 μmoles of lecithin, 3 μmoles of dicetyl phosphate and 0.045 μmole of butylated hydroxytoluene, all in chloroform, were transferred to a 50-ml round-bottom flask covered with aluminum foil. Solvent was removed under vacuum at room temperature with the last traces being removed with a jet of high purity N_2. 5 ml of 0.3 M D-glucose and several glass beads were added. The flask was flushed with N_2, tightly closed and shaken by hand until all the lipid was removed from the wall of the flask. The suspension was then shaken for 1 h at 28° on a rotary shaker. The liposomes were allowed to equilibrate overnight at 4°. 0.5-ml aliquots of the dispersion were dialysed at room temperature against 55-ml portions of a solution containing 0.1 M Tris–HCl buffer (pH 7.4), 0.03 M NaCl and 0.03 M KCl for five consecutive 1-h periods. During each dialysis period high purity N_2 was bubbled through the external liquid to provide agitation and retard oxidation.

Diffusion rates

The method for studying the diffusion of glucose out of the liposomes was basically the same as used by DEMEL *et al.*[21]. The system contained the following reagents in a 1-ml cuvette (added in order). 0.28 ml of 0.1 M Tris buffer (pH 7.4); 0.5 ml double strength isotonic salt mixture (0.143 M KCl and 0.143 M NaCl prepared in 0.1 M Tris buffer (pH 7.4)); 0.1 ml of 0.02 M magnesium acetate; 0.05 ml of 0.02 M ATP; 0.05 ml of 0.01 M $NADP^+$; 5 μl hexokinase (2500 I.U./ml diluted with one-half volume of deionized distilled water) and 5 μl glucose-6-phosphate dehydrogenase (200 I.U./ml). Control cuvettes were identical to the above except that $NADP^+$ was omitted. The reaction was started by the addition of 5 μl of the appropriate liposome preparation. The absorbance was measured at 340 nm using a DU-2 spectrophotometer equipped with dual thermospacers to maintain constant temperature. The temperature in the cuvette was monitored with thermocouples sprayed with vinyl copolymer resin to obviate metal contamination of the enzyme system. Initially the increase in absorbance was recorded every 10 sec over the first 3 min of diffusion then every 5 min for an additional 50 min. At the end of the diffusion measurements, 0.1 ml of 10 % Triton X-100 was added to the cuvette with mixing to lyse the particles. The absorbance increased rapidly and the final constant absorbance was recorded. The method for calculating diffusion rates of glucose was essentially as described by DEMEL *et al.*[21].

Peroxide values

Lecithins before the preparation of liposomes and after the diffusion measurements were analyzed for peroxide content using the microiodometric technique of HEATON AND URI[22].

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TABLE I

RATE CONSTANTS FOR GLUCOSE DIFFUSION AT VARIOUS TEMPERATURES

The diffusion rate constants were calculated by the least squares method. Mean value ± S. E. of four replicate measurements for egg, normal rat and fish and six replicate measurements for essential fatty acid-deficient rat lecithin preparations.

Egg		*Rat*				*Fish*	
		Essential fatty acid deficient		*Normal*			
temp.	$k \times 10^3$ (min^{-1})	*temp.*	$k \times 10^3$ (min^{-1})	*temp.*	$k \times 10^3$ (min^{-1})	*temp.*	$k \times 10^3$ (min^{-1})
19.9° ± 0.2	0.18 ± 0.04	20.2° ± 0.2°	0.17 ± 0.06	21.2° ± 0.3°	0.67 ± 0.10	20.8° ± 0.2°	0.69 ± 0.07
27.1° ± 0.2°	0.50 ± 0.13	26.8° ± 0.8°	0.88 ± 0.18	26.4° ± 0.3°	1.15 ± 0.09	26.9° ± 0.1°	1.58 ± 0.37
36.5° ± 0.4°	1.98 ± 0.15	36.6° ± 1.0°	2.45 ± 0.10	35.1° ± 0.1°	4.94 ± 0.34	35.8° ± 0.3°	6.00 ± 0.48

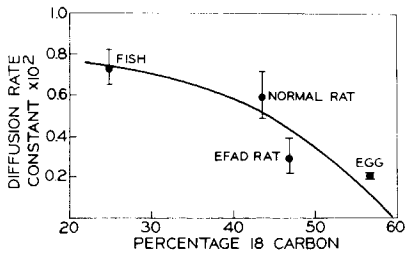


Fig. 1. Dependence of diffusion rate constant upon the percentage of C_{18} fatty acids. The diffusion rate constants for each lecithin preparation at 37° were estimated from Arrhenius plots. The C_{18} fatty acids represent the sum of the mole percentage of all C_{18} fatty acids. EFAD, essential fatty acid deficient.

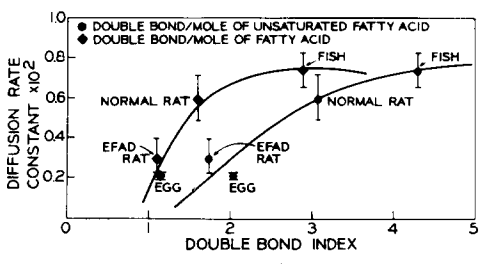
RESULTS AND DISCUSSION

The introduction of small amounts of butylated hydroxytoluene into the liposomes as an antioxidant proved effective in preventing peroxidation. Usually less than 1 mole percent of peroxidation was detected after the diffusion measurements. Studies in this laboratory indicated that as much as 10 mole percent peroxidation was evident unless antioxidant was added to the system. Consequently the observed variations in diffusion rates are not the results of peroxidative changes.

In the diffusion measurements, there was an immediate increase in the absorbance after addition of the liposomes. This apparently was due to light scattering and to enzymatic action on interparticle glucose.

Plots of the logarithm of the percentage of glucose remaining in the liposomes *versus* time indicated that the diffusion of glucose followed first-order kinetics in all cases. First-order diffusion rate constants calculated from the data are given in Table I. Diffusion rates of glucose were comparable for liposomes prepared from lecithins isolated from egg yolk and essential fatty acid-deficient rats. Lecithin from normal rats yielded liposomes characterized by diffusion rates which were greater than those from essential fatty acid-deficient rat lecithin. Liposomes prepared from fish lecithin exhibited the highest diffusion rates for glucose.

The fatty acid compositions of the lecithins were essentially the same as described by MOORE *et al.*[15]. For the most part the fatty acids of lecithin from egg yolk were shorter in chain length and highly saturated. The fatty acids of lecithin from essential fatty acid-deficient rats were relatively saturated and were high in oleic and eicosatrienoic acids characteristic of essential fatty acid deficiency[1-5,15]. Lecithin from normal rats contained more unsaturated fatty acids, especially linoleic and arachidonic acids. Lecithin from fish contained the most unsaturated fatty acids with a high concentration of docosahexaenoic acid characteristic of fish lipids[25].



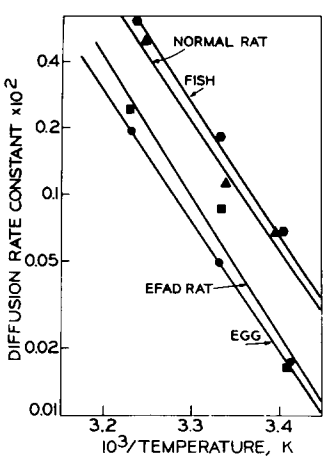


Fig. 2. Relationship between diffusion rate constants and double bond indices of fatty acid residues in the various lecithin preparations. The diffusions rate constants for each lecithin preparation at 37° were estimated from Arrhenius plots. EFAD, essential fatty acid deficient.

Fig. 3. Arrhenius plots of diffusion rate constants. Activation energies calculated from these plots are 26.6 ± 1.6 (12 measurements), 28.9 ± 5.8 (18 measurements), 26.2 ± 3.9 (12 measurements), and 26.7 ± 2.4 (12 measurements), for egg lecithin, essential fatty acid-deficient rat lecithin, normal rat lecithin and fish lecithin, respectively. EFAD, essential fatty acid deficient.

The average number of double bonds per molecule of fatty acid in lecithins from egg yolk, essential fatty acid-deficient rats, normal rats and fish were 1.14, 1.09, 1.60 and 2.90, respectively.

An inverse relationship between the mole percent of C_{18} fatty acids and rate constants for glucose diffusion was observed as shown in Fig. 1. This would infer that the C_{18} fatty acids may in some way control the diffusion rate of glucose, possibly by governing the membrane thickness.

Fig. 2 shows the effect of unsaturation on the rate of diffusion of glucose out of liposomes. Clearly the more unsaturated lecithins exhibit faster diffusion rates. This is true at all temperatures as indicated by Arrhenius plots (Fig. 3). Since the introduction of double bonds would change the direction and length of the fatty acid chain, the more unsaturated lecithins would yield less tightly packed membranes with higher diffusion rates. These data with natural lecithin preparations are in agreement with the data of DE GIER *et al.*[16] and DEMEL *et al.*[21] who demonstrated a faster diffusion of glucose from liposomes prepared from more unsaturated synthetic lecithins.

Activation energies calculated from Arrhenius plots (Fig. 3) yielded values of 26.6 ± 1.6, 28.9 ± 5.8, 26.2 ± 3.9 and 26.7 ± 2.4 kcal/mole glucose for egg yolk, essential fatty acid-deficient rat, normal rat and fish lecithins, respectively. Calculation of activation energies for glucose diffusion out of liposomes using literature values reveals a tendency toward high activation energies for this system. The data of DEMEL *et al.*[21] yields activation energies of 22.8 kcal/mole glucose and 19.5 kcal per mole glucose for lecithin to dicetyl phosphate ratios of 7:2 and 19:1, respectively.

These data indicate that in the liposome model system, essential fatty acid-deficient membranes are less permeable to glucose than control membranes. This seems to be related largely to the higher degree of saturation of the fatty acids in the deficient membranes. However, a precautionary note should be sounded here in that some of the differences in diffusion rates may be due to differences in average particle size or surface area of the liposomes which in turn may reflect the fatty acid composition of the lecithins.

Recently, IMAMI *et al.*[24] studied the transport of α-methyl-D-glucoside across everted intestinal sacs from normal and essential fatty acid-deficient rats. The transport of α-methyl-D-glucoside was about 30% slower in the essential fatty acid-deficient tissue. Carriers have been postulated as essential components for facilitated diffusion and active transport of solutes across cell membranes[25]. Presumably, the carrier or carrier–solute complex must diffuse through the interior of the cell membrane to facilitate transport.

In the present system the passive diffusion rate of glucose for essential fatty acid-deficient rat lecithin was about 50% that of normal rat lecithin. These data coupled with the observations of IMAMI *et al.*[24] would suggest that solute or carrier and carrier-solute diffusion in the essential fatty acid-deficient membrane might be retarded.

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