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# OSMOTIC FRAGILITY AND FLUIDITY OF ERYTHROCYTE MEMBRANES FROM RATS RAISED ON AN ESSENTIAL FATTY ACID DEFICIENT DIET

## A SPIN LABEL STUDY

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**Erythrocyte membranes from rats raised on a diet with low content of essential fatty acids were studied by osmotic sensitivity tests and spin labeling techniques. This diet induced significant modifications in acylglycerophosphocholine fatty acid composition with regard to 16 : 1, 18 : 1, 18 : 2 (*n*-6), 20 : 3 (*n*-9), and 20 : 4 (*n*-6). No changes in membrane fluidity as monitored by spin label motion were found but the diet caused an increased osmotic sensitivity in essential fatty acid deficient erythrocytes. 50% hemolysis was obtained at a 51.0% dilution of saline with $H_2O$ as compared to a 57.0% dilution for the control material. Membrane fluidity was unaffected by $\gamma$-irradiation up to 80 krad.**

## Introduction

Rats and mice raised on a diet free of essential fatty acids have been reported to develop deficiency symptoms such as diminished growth, skin lesions, impaired control of water balance, decreased resistance to infections and an increased sensitivity to ionizing radiation [1,2]. Essential fatty acid deficiency during prenatal and during early postnatal development lead to profound changes in the normal fatty acid composition of the glycerophospholipids in liver [3] and muscles [4] while brain glycerophospholipids are almost not at all influenced [5], although impaired brain functions as a result of essential fatty acid deficiency have been reported [6–8].

The specific effects on membrane structure and function caused by modifications in fatty acid composition are poorly understood. In this work some basic properties of erythrocyte membranes from rats raised for three generations on a diet with low essential fatty acid content or on a diet supplemented with sunflower oil and linseed oil have been studied. The fluidity of the membranes was monitored by electron spin resonance (ESR) using lipid spin labels and correlated with osmotic sensitivity and analysis of the fatty acid composition.

## Materials and Methods

*Materials.* The spin labels 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (I(1, 14), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (I(12, 3)) and 17$\beta$-hydroxy-4,4-dimethyl-spiro-5$\alpha$-androstane-3,2-oxazolidin-3-yloxyl (ASL), were purchased from SYVA Research Chemicals and used without further purification. Inorganic chemicals were purchased from Merck. All other chemicals and biochemicals were purchased from Sigma Chemical Co.

*Animals.* Rats, Wistar strain R, were housed in plastic cages with stainless steel covers and 0.5 cm of wooden chips on the bottom. An artificial light

period of 12 h, a room temperature of 22°C and a humidity of 55% were maintained. The animals had free access to food and water. In all experiments blood was collected between 9 and 10 a.m. Animals used for the experiments were of the age of 5–7 months, experimental and control animals were always of the same age and sex, and had been fed the diets for more than two generations.

*Composition of the diet.* The two diets, low in essential fatty acids and high in essential fatty acids, had the following ingredients in common, here given in % (w/w): casein 18.0, fish protein 5.0, glucose 10.0, cellulose flour 3.0, corn starch 47.2, vitamin mixture * 1.0, salt mixture ** 4.0, trace elements *** 0.3, fodder lime 0.5 and calcium phosphate (dibasic) 1.0. The diet low in essential fatty acids also contained hydrogenated lard 10.0, while the content of hydrogenated lard was reduced to 7.8 in the diet high in essential fatty acids, that instead was supplemented with sunflower seed oil 1.65 and linseed oil 0.55. The content of essential fatty acids in the diets low and high in essential fatty acids was analyzed and found to be 0.3 and 3.8 energy %, respectively. The linoleic acid content in the diet high in essential fatty acids was in the normal range for essential fatty acid requirement in rats [9]. All ingredients were of a quality designed for human consumption. Salts were of purissimum or purum quality. The diets were given to the rats as pellets.

*Labeling of intact erythrocytes.* Erythrocytes were prepared from freshly drawn venous heparinized rat blood. The erythrocytes were isolated by centrifugation for 20 min at $1000 \times g$. The plasma and buffy-coat were removed by aspiration, and the cells were washed three times in phosphate buffered saline (pH 7.2) [10] and finally resuspended in equal volume of phosphate buffered saline. This erythrocyte suspension contained about $6 \cdot 10^9$ cells/ml. The spin labels were dissolved in ethanol. A thin film of label was prepared by placing adequate amounts of solution in a test tube with round bottom and evaporating the solvent under a stream of nitrogen. The erythrocyte suspension was added and incubated 30 min at 37°C in a water bath. After centrifugation for 10 min at $3000 \times g$ the cells were washed once with five volumes of phosphate buffered saline and resuspended in phosphate buffered saline. The amount of spin label incorporated into the erythrocyte membranes was estimated by double integration of the ESR spectra and comparison with a 1 mM Cu-EDTA standard solution. In all preparations studied 90–95% of input spin label were detected in the resuspended pellet. The molar ratio of spin label to membrane phospholipid was 1.1% or less.

*Labeling of erythrocyte ghosts.* 2 ml erythrocyte suspension was pipetted into 28 ml hypotonic buffer, pH 7.4 (4.4 mM $NaH_2PO_4 \cdot H_2O$, 0.4 mM $Na_2HPO_4 \cdot H_2O$) of an ionic strength corresponding to 10 isosM. The cells were collected by centrifugation at $20\,000 \times g$ for 40 min, resuspended and washed three times as above in the hypotonic buffer [11]. Finally, the erythrocyte ghosts were resuspended in an equal volume of phosphate buffered saline and spin labeled as described above by exchange with a thin film of label. Incubation conditions were the same. The labeled ghosts were centrifuged 30 min at $40\,000 \times g$, washed once with five volumes of phosphate buffered saline and resuspended in phosphate buffered saline.

*Labeling of lipids extracted from erythrocyte membranes.* Spin label dissolved in ethanol or chloroform was added directly to the lipid methanol-chloroform extract prepared as described below. The solvent was evaporated in a stream of nitrogen and then exposed to high vacuum for at least 1 h. The material was suspended in phosphate buffered saline by 3 min sonication in a Bransonic 32 waterbath. The molar ratio of the spin label to phospholipid was 0.75–0.95% as estimated by double integration of ESR spectra.

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* Vitamin mixture in % (w/w): Vit. A (500 000 I.E./g) 0.152, Vit. $AD_3$ (500 000/170 000 I.E./g) 0.086, Vit. B-1 0.04, Vit. B-2 0.124, Vit. B-6 0.05, calcium pantothenate (45%) 0.222, Niacin 0.4, Vit. B-12 (0.05%) 0.4, Vit. K-3 0.015, Biotin (1%) 0.3, Vit. C 5.0, Inositol 0.3, Vit. E (500 mg/g) 0.84, choline chloride (50%) 20.0, *p*-aminobenzoic acid 2.5, folic acid 0.005 and starch 69.566.

** Salt mixture in % (w/w): $KH_2PO_4$ 34.268, $CaCO_3$ 36.214, KCl 2.502, NaCl 18.045, $MgSO_4 \cdot H_2O$ 5.079, $FeC_6H_5O_7 \cdot 5H_2O$ 3.346, $MnSO_4 \cdot H_2O$ 0.400, $Cu_2C_6H_4O_7 \cdot 2.5H_2O$ 0.063, $Zn_3(C_6H_5O_7)_2 \cdot 2H_2O$ 0.035, $CoCl_2 \cdot 6H_2O$ 0.002, $KAl(SO_4)_2 \cdot 12H_2O$ 0.008, NaF 0.025, $KIO_3$ 0.009, $NaAsO_2$ 0.001, $Na_2B_4O_7 \cdot 10H_2O$ 0.002 and $Na_2MoO_4 \cdot 2H_2O$ 0.001.

*** Trace elements: 6% of a premixture composed of (values in % w/w) $Fe_2CO_3 \cdot H_2O$ 45.2, ZnO 20.8, $Mn_2O$ 24.3, $Cu_2O$ 5.2, $CoSO_4 \cdot 7H_2O$ 0.7, CaI $\cdot H_2O$ 0.7 and wheat feed meal 3.1, was mixed with 94% wheat feed meal.

*Electron spin resonance measurements.* ESR measurements were made with a Varian E-9, X-band spectrometer using a rectangular cavity and glass capillaries (inner diameter 0.97 mm) in a standard quartz ESR tube. The temperature was regulated with a heater-sensor system using cold nitrogen gas, and the sample temperature was measured with a platinum resistance to ±0.5°C. Preliminary experiments were performed with a JEOL ME-1X X-band spectrometer with a similar heater-sensor system.

ESR spectra obtained with the spin label I(1, 14) were analyzed by the ESR motional narrowing formalism [12]. Apparent correlation times for the spin probe were calculated from the ESR spectra using the formula

$$\tau_0 = 6.5 \cdot 10^{-10} W_0(\sqrt{h_0/h_{-1}} - 1)$$

where $W_0$ is the peak-to-peak width of the midfield line in the first derivative ESR spectrum, $h_0$ the height of the midfield line and $h_{-1}$ the height of the high-field line [13]. $\tau_0$ is an empirical motional parameter which reflects molecular movement assuming rapid isotropic rotation of the spin label and that the system does not impose order on the probe. The limits of validity for the formula are discussed by Cannon et al. [14]. ESR spectra for I(1, 14) in membranes of intact erythrocytes give $\tau_0$ values of the order of $2 \cdot 10^{-9}$ s. In this range the use of $\tau_0$ as a parameter is satisfactory for the purpose of comparison between different types of membranes. For the probe I(12, 3) the maximum hyperfine splitting $2T_{max}$ measured on the ESR spectra was used as a parameter reflecting the fluidity of the probe environment. The outermost spectral singularities were recorded separately with high gain and slow sweep rate. This allowed determination of the spacings between the high-field and the low-field extremas of the derivative spectra to within ±0.01 mT (1 mT = 10 gauss).

*Fatty acid composition of acylglycerophosphocholine from erythrocyte ghosts.* Lipids were extracted from the ghost suspension with 10 vol. chloroform/methanol (1 : 1, v/v) and the lipid extract was purified by phase partition [15]. This lipid extract was used for spin labeling studies as well as for the determination of fatty acid composition and cholesterol and total phospholipid content. The precision of the analysis of fatty acid composition has been described in detail elsewhere [16]. In essence, the main steps involve isolation of the major lipid fractions by thin-layer chromatography, preparation of the fatty acid methyl esters using sodium methoxide 0.1 M in methanol. The fatty acid methyl ester composition was analyzed by gas-liquid chromatography. The fatty acid composition is reported as molar percentage of methyl esters. Standard methods were used for the determination of cholesterol [17] and total phospholipid content [18].

*Osmotic fragility.* From a buffered saline, pH 7.4 (0.154 M NaCl, 0.085 M $Na_2HPO_4 \cdot 2H_2O$ and 0.016 M $NaH_2PO_4 \cdot H_2O$) a dilution series was prepared with $H_2O$ in 3-ml aliquots. The undiluted saline was denoted 100%. To each test tube was added 30 µl whole heparinized blood. After 30 min the samples were centrifuged at 500 × *g* for 5 min and the supernatant read in a spectrophotometer at 545 nm [19]. The degree of hemolysis is expressed in percentage, where 100% represents full hemolysis.

## Results and Discussion

*Fatty acid composition of erythrocyte membranes*

The influence of the diets with low or high content of essential fatty acids on the fatty acid composition of erythrocyte membranes is shown in Table I. Acylglycerophosphocholine was regarded most suitable for analysis since it is the largest fraction of the phospholipids in rat erythrocyte membranes [20,21] and has been shown to respond with a stronger correlation to variations in the dietary essential fatty acids than any of the other phospholipids [21]. Significant differences in the proportions of individual fatty acids were observed. The diet low in essential fatty acids caused more than a 40% decrease of 18 : 2 (*n*-6) * (linoleic acid) as well as of 20 : 4 (*n*-6) (arachidonic acid). The whole linoleic series 18–22 (*n*-6) was significantly reduced in the diet group low in essential fatty acids compared to the diet group high in essential fatty acids.

The lower proportions in the membranes of 18 : 2

* Fatty acids are designated by chain length: number of double bonds; (*n*-6) denotes that the first double bond from the methyl group occurs after the sixth carbon atom, the methyl group being counted as number one.

TABLE I

FATTY ACID COMPOSITION OF ACYLGLYCEROPHOSPHOCHOLINE IN ERYTHROCYTE MEMBRANES FROM RATS FED DIETS WITH A HIGH OR LOW ESSENTIAL FATTY ACID CONTENT

Values are expressed as mol % of methyl esters. n.d., non detectable.

| | High essential fatty acid content | | Low essential fatty acid content | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| 16 : 0 | 42.6 | 39.3 | 43.2 | 37.0 |
| 16 : 1 | 1.8 | 1.6 | 3.8 | 3.4 |
| 18 : 0 | 19.4 | 18.0 | 15.9 | 16.5 |
| 18 : 1 | 12.2 | 13.2 | 20.2 | 19.5 |
| 18 : 2 (*n*-6) | 12.2 | 12.0 | 6.5 | 7.0 |
| 18 : 3 (*n*-3) | 0.3 | 0.3 | 0.4 | 0.7 |
| 20 : 3 (*n*-9) | 0.1 | 0.7 | 3.6 | 4.6 |
| 20 : 3 (*n*-6) | 0.5 | 1.2 | 1.1 | 1.2 |
| 20 : 4 (*n*-6) | 8.4 | 10.1 | 3.9 | 6.4 |
| 20 : 5 (*n*-3) | 0.3 | 0.5 | 0.5 | 1.7 |
| 22 : 4 (*n*-6) | 0.3 | n.d. | n.d. | n.d. |
| 22 : 5 (*n*-6) | n.d. | 0.1 | 0.5 | 0.4 |
| 22 : 5 (*n*-3) | 0.7 | 0.6 | 0.3 | 0.5 |
| 22 : 6 (*n*-3) | 1.2 | 2.3 | 0.6 | 1.0 |
| 18–22 (*n*-6) | 21.4 | 23.4 | 12.0 | 15.0 |
| 18–22 (*n*-3) | 2.5 | 3.7 | 1.8 | 3.9 |

(*n*-6) and 20 : 4 (*n*-6) were compensated for in the group low in essential fatty acids by incorporation of significantly larger quantities of 16 : 1, 18 : 1 and 20 : 3 (*n*-9). However, some basic features of the fatty acid composition seemed to be kept unaltered. Thus, the molar percentage of saturated fatty acids was in the same range for the two groups, which in this respect differed by 3% only, and the sum of the fatty acids with the same chain length was kept constant. Furthermore, only a minor reduction (about 5%) in the total amount of unsaturated carbon bonds was found in the acylglycerophosphocholine fraction from the group low in essential fatty acids. Similar results have been reported by Rao et al. [22] with regard to the influence of diets with low and high contents of essential fatty acids on the fatty acid composition of acylglycerophosphocholine from rat erythrocyte membranes.

The analysis of the phospholipid/cholesterol ratio for the two types of membranes gave 1.2 mol/mol for the group high in essential fatty acids and 1.3 for the group low in essential fatty acids, respectively. This invariability of the ratio, in spite of the fat composition of the diets, has also been observed by others in related systems [23].

*Electron spin resonance measurements*

Fig. 1 shows the variation of the empirical motional parameter $\tau_0$ for the probe I(1, 14) in the membranes of intact erythrocytes as a function of temperature. No significant difference in membrane fluidity as reflected by the motion of the spin label could be detected when erythrocytes from rats raised on the diet high in essential fatty acids or on the diet low in essential fatty acids were compared. The Arrhenius type plot shows two regions with different slopes intersecting at about 28.5°C. The significance of this transition temperature is not clear but may

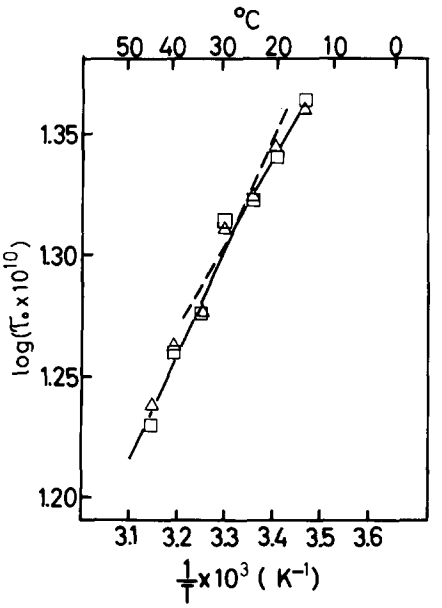


Fig. 1. Arrhenius type plot. Temperature dependence of the empirical rotational parameter $\tau_0$ for intact erythrocytes in phosphate buffered saline (pH 7.0), labeled with spin probe I(1, 14). The points are averages of two different experiments agreeing within ±1% on material from different animals, △, control rats, diet high in essential fatty acids. □, rats raised on a diet low in essential fatty acids. The curve is best fitted by two straight lines intersecting at 28.5°C. The correlation coefficients for temperatures above 28.5°C and below 28.5°C are 0.997 and 0.990, respectively. $\tau_0$ is expressed in seconds.

reflect a phase transition or a molecular reorganization in the membrane. The motion of the spin label increased more rapidly with temperature above the transition indicating that the membrane was in a more fluid state than below 28.5°C. A similar experiment was performed using a spin-labeled derivative of androstane (ASL). The temperature dependence of probe motion was the same in both types of membranes. With this probe however no break in the plot could be detected in the temperature range 10–60°C.

In Fig. 2 the maximum hyperfine splitting $2T_{max}$ of I(12, 3) in erythrocyte ghosts and lipids extracted from the membranes is given as a function of temperature. The spin label was also incorporated into intact erythrocytes. These results are not shown in the figure, but the values of $2T_{max}$ coincided with the membrane values at all temperatures studied. The

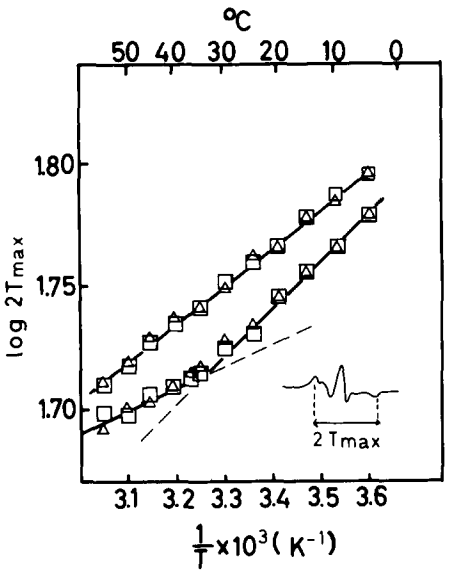


Fig. 2. Arrhenius type plot. Temperature dependence for the hyperfine splitting of spin label I(12, 3) in erythrocyte membranes (upper curve) and lipids extracted from the membranes (lower curve). The points are averages from determinations differing by less than 1% on three different membrane preparations from each of the groups high and low in essential fatty acids, respectively. The lipids were extracted from membranes of both diet groups in two experiments. △, control rats, diet high in essential fatty acids. □, rats raised on a diet low in essential fatty acids. Membrane curve: best fit with a straight line, correlation coefficient 0.998. Lipid curve: best fit with two straight lines intersecting at 33°C. Correlation coefficient: 10–25°C, 0.997; 40–55°C, 0.999. $2T_{max}$ is expressed in gauss.

variation of $2T_{max}$ with temperature was the same in erythrocyte membranes prepared from the two dietary groups. The Arrhenius type plot shows a straight line without any apparent transition points (upper curve). A slight inflexion may occur at about 30°C as suggested by Rigaud et al. [24] for human red cells. The slope is the same for rat and human erythrocyte membranes. The absence of transition temperature may partly be due to the presence of proteins which are known to form a rather rigid 'matrix' in the erythrocyte membrane [25]. Measurements on the extracted lipids (Fig. 2) from both dietary groups gave similar resuls within the limits of experimental error. The average of the results are therefore plotted. A best fit analysis of the experimental values gave two straight lines intersecting at a temperature around 33°C.

The results indicate that the diet-induced decrease of about 5% in total amount of unsaturated carbon bonds in acylglycerophosphocholine may be at the limit of detectability of the spin label technique [26]. Experiments using other methods for the study of membrane fluidity such as fluorescence polarization are planned.

The rigidity of the erythrocyte membranes has also been shown to depend on and vary with the cholesterol content [27,28]. In experiments with plasma membranes prepared from Ehrlich ascites cells, King et al. [29] revealed significant changes in fluidity, as measured by ESR, after feeding the tumor bearing mice a fat-free diet for 6–8 weeks before inplantation of the tumor. The phospholipid/cholesterol ratio in these membranes was 2.3–2.4 mol/mol as compared to 1.2–1.3 mol/mol found in the rat erythrocyte membranes (cf. above). The modifications of the fatty acid composition in their material is only given for total lipids and cannot be directly compared to those of acylglycerophosphocholine presented in Table I. We have made preliminary experiments on intact Ehrlich ascites cells obtained from mice fed diets either high or low in essential fatty acids. A significant influence on both the motional parameter $\tau_0$ and the order parameter S and I(1, 14) were detected (Harms-Ringdahl M., Ehrström, M. and Alling, C., unpublished). These observations indicate that the high cholesterol content of the erythrocyte membranes stabilizes the membranes to such an extent that alterations of the fatty acid composition

of the kind and degree seen in Table I do not influence the fluidity to a level detectable with the spin label technique. Besides the effect of cholesterol, the protein matrix in the erythrocyte membranes may further contribute to this quenching of fluidity variations [25].

*Irradiation of erythrocytes*

Impairment of membrane functions has been demonstrated in many system exposed to ionizing radiation, and it has earlier been suggested that the membranes may be targets contributing significantly to cellular radiation damage [30–32]. Observations of an extensive increase in radiosensitivity of rats and mice raised on a fat-free diet [33,34] motivated some experiments where the effects of γ-irradiation on fluidity of the membranes prepared from the two dietary groups were compared.

Irradiation was made under aerobic conditions at 0°C and a total dose of 80 krad was given at a dose rate of 3.3 krad/min. The results are shown in Fig. 3. The temperature dependence of $2T_{max}$ for the spin label I(12, 3) for irradiated and unirradiated membranes (ghosts) was determined. For clarity only the measurements of unirradiated membranes high in essential fatty acids and irradiated membranes low in essential fatty acids are shown in the figure as no significant differences could be detected between the four experimental groups. Although lipid peroxidation as well as protein-SH oxidation have been shown to occur in erythrocyte membranes at this dose level [35,36], the radiation-induced chemical modifications were not detectable as changes in membrane fluidity, possibly for the reasons mentioned in the section above. Effects on the fluidity of erythrocyte membranes have however been observed at the Mrad level using the ESR technique [37].

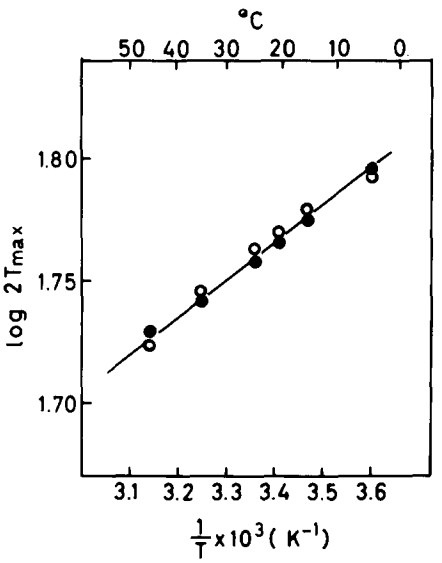


Fig. 3. Arrhenius type plot. Temperature dependence for the hyperfine splitting $2T_{max}$ of I(12, 3) in erythrocyte membranes irradiated with 80 krad γ-radiation as compared to unirradiated membranes. Only the results for unirradiated membranes high in essential fatty acids and for irradiated membranes low in essential fatty acids are included. Data for irradiated control membranes and unirradiated membranes low in essential fatty acids coincide within the limits of experimental error, ○, unirradiated membranes high in essential fatty acids. ●, irradiated membranes low in essential fatty acids. $2T_{max}$ is expressed in gauss.

*Osmotic sensitivity*

Although the differences in fatty acid composition between the two dietary groups did not alter the physical properties of the membranes to an extent that was detectable with ESR, the rigidity of the membranes was clearly influenced as shown by changes in osmotic sensitivity. A highly reproducible increased sensitivity to hypotonic treatment was seen for erythrocytes prepared from the group low in essential fatty acids. In a series of experiments, the mean value for the dilution of saline, at which 50%

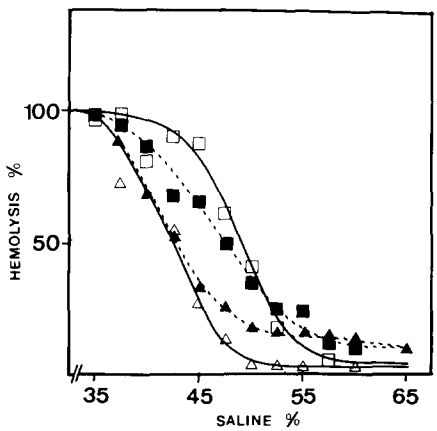


Fig. 4. Osmotic sensitivity of rat erythrocytes obtained from animals fed a diet high in essential fatty acids (△) and a diet low in essential fatty acids (□) for four generations. Erythrocytes irradiated with 80 krad γ-radiation, filled symbols, dotted lines.

hemolysis was obtained, differed between the two groups by 6% with a standard deviation of 0.5%. A typical experiment is shown in Fig. 4. For the group low in essential fatty acids, 50% hemolysis was obtained already at a 51% dilution of saline with $H_2O$, while the corresponding degree of hemolysis was not obtained until 57% dilution of saline for the group high in essential fatty acids.

Cholesterol and phospholipids are supposed to form complexes that stabilize the membranes [27, 38]. Decreases in the phospholipid/cholesterol ratio increases the osmotic sensitivity of erythrocytes [28, 39]. The present study suggests that the altered fatty acid composition contributes to the difference in osmotic sensitivity as the phospholipid/cholesterol ratio found in the membranes low in essential fatty acids is almost the same as in the membranes high in essential fatty acids. However, the interactions between proteins and lipids may also be affected and contribute to the changes in membrane rigidity.

In experiments with rats, which were fed a fat-free diet, an increased sensitivity for hypotonic treatments has been observed [21,40]. However, a direct comparison is not possible as the phospholipid/cholesterol ratios were not determined. Effects on the fatty acid composition similar to those reported above (cf. Table I) were seen in those experiments [21].

Irradiation aerobically at 0°C with 80 krad $\gamma$-radiation induced only minor modifications of the osmotic sensitivity as shown in Fig. 4. The more gradual slopes of the curves for irradiated erythrocytes indicate a broadened response to hypotonic treatment as compared to the steeper curves obtained for the unirradiated cells.

The fluidity of the erythrocyte membrane is determined by a number of factors among which cholesterol content, fatty acid composition and the protein matrix have significant influences [25,27]. The interaction of these factors seem to influence to a varying degree the physiological properties of the membranes which may be studied by fluidity changes and osmotic sensitivity. Due to these interactions, significant modifications of the fatty acid composition of the membranes as seen in one of the major phospholipids, acylglycerophosphocholine, were not detectable with the spin labeling technique, while the osmotic sensitivity was clearly changed.

The major part of the earlier work referred to above has been performed on membranes obtained from rats fed a fat-free diet. These animals may develop several types of biochemical abnormalities, not directly correlated to essential fatty acid deficiency, as a consequence of an altered ratio between carbohydrates and proteins in the energy supply [40]. In our experiments both diets contained 10 energy % fat but had different essential fatty acid content. The effects reported here are therefore due to influence of essential fatty acids deficiency and not to fat deficiency.

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