

Lipid composition and fluidity of the erythrocyte membrane in copper-deficient rats

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The influence of dietary copper on lipid composition, phospholipid fatty acid and protein profiles and fluidity of the erythrocyte membranes of rats is reported. In general Cu deficiency in rats induced some changes in the phospholipid-fatty acid profile of erythrocyte membranes when compared with Cu-adequate animals. Stearic (18:0) and docosadienoic (22:2n-3) acids contents, for example, were significantly increased ($P < 0.001$) while oleic (18:1n-9) and linolenic (18:3n-3) acid contents were significantly depressed ($P < 0.001$) as a result of Cu deficiency. Moreover the cholesterol:phospholipids molar ratio and the cholesterol (mol):membrane proteins (mg) ratio in Cu-deficient rats were, to different degrees, significantly lower than in animals fed on Cu-adequate diets. In addition, diets deficient in Cu led to a reduction in erythrocyte membrane fluidity ($P < 0.001$) as assessed by the intramolecular excimer fluorescence of 1,3-di(1-pyrenyl) propane. However, no significant alteration in the phospholipid:protein ratio was observed as a result of differences in dietary treatment. The pattern of erythrocyte membrane proteins obtained with sodium dodecyl sulphate-polyacrylamide gel electrophoresis did not seem to be influenced by Cu-deficient diets.

Cu deficiency: Erythrocyte membranes: Membrane lipids: Membrane fluidity: Rat

Hypercholesterolaemia associated with copper deficiency has been observed by numerous investigators, mostly in rats but also in other species including humans (for a recent review, see Lei, 1990). Lei (1977, 1978) suggested that a shift of cholesterol from the liver pool to the plasma pool appeared to be responsible. Shao & Lei (1980) demonstrated that cholesteryl ester, newly synthesized from [14 C]mevalonate, cleared the liver faster in Cu-deficient rats. In addition, a 2-fold increase in fatty acid synthesis from [14 C]acetate was observed in liver slices derived from Cu-deficient rats (Lei, 1977). Furthermore, an increase in hepatic cholesterol synthesis was indicated by a 2-fold increase in hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.88) activity (Yount *et al.* 1990) and by findings on in vivo cholesterol synthesis (Yount *et al.* 1991). Thus, the increased capacity of hepatic fatty acid and cholesterol synthesis and enhanced clearance of cholesteryl ester both may have contributed to the hypercholesterolaemia and hyperlipoproteinaemia observed in Cu-deficient rats (Croswell & Lei, 1985; Lei *et al.* 1989). Recently, Al-Othman *et al.* (1990), expressed plasma lipoprotein as the amount present in the vascular pool corrected for body-weight. The plasma pool size of protein, triacylglycerols (TG) and phospholipids (PL) and cholesterol of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were increased 2-fold or more by Cu deficiency. As for very-low-density lipoprotein (VLDL), a 6-fold increase in TG, a 36% reduction in cholesterol, and no change in PL and protein

pool sizes were observed in Cu-deficient rats. In addition, changes in HDL-PL fatty acid composition were represented by a 61% increase in stearate and reductions of 44% and 97% in oleate and linolenate respectively as a result of Cu deficiency. These alterations may exert a profound influence on the lipid composition of many important cellular membranes, such as the erythrocyte membrane, which are in constant contact with plasma lipoproteins. Alterations in membrane lipid composition such as the cholesterol content of the membrane (Ladbrooke *et al.* 1968) and length and degree of unsaturation of phospholipid (Schwarz *et al.* 1984) resulted in modifications in the membrane fluidity. In view of the changes in lipid metabolism induced by Cu deficiency, the influence of PL acyl groups and cholesterol on membrane fluidity and the importance of membrane fluidity on membrane activities (Abu-Salah *et al.* 1988; Abu-Salah, 1991) and cellular functions (Spector & Yorek, 1985), the present study was performed to determine the influence of Cu status on lipid composition, PL fatty acid profile, protein profile and fluidity of the erythrocyte membrane.

EXPERIMENTAL

Materials

1,3-di(1-pyrenyl)propane (DPP) was purchased from Molecular Probes, Inc, 4849 Pitchford Ave, Eugene, OR 97402. Dextran T500 was obtained from Pharmacia Fine Chemicals AB, Box 175, S-75104, Uppsala 1, Sweden. The Cu reference standard was provided by Fisher Scientific Co., Los Angeles, CA. Standard fatty acids were obtained from Supelco Inc., Bellefonte, PA. All other reagents were at least Analytical grade and were obtained from Sigma Chemical Co., St Louis, MO 63178.

Methods

Animals and diets. Weanling male Sprague-Dawley rats were housed individually in suspended stainless-steel wire cages in a laboratory maintained at 22° with a 12 h light-dark cycle. Rats were randomly allocated to the dietary treatments (Cu-deficient and Cu-adequate). The Cu-deficient diet was prepared according to the specifications of American Institute of Nutrition (1977), except that the Cu supplement was omitted from the mineral mix. The diet contained 0.7 mg Cu/kg diet as determined by atomic absorption spectrophotometry (Hitachi Ltd, 1980) using the certified reference standards of Fisher Scientific Co. Copper carbonate was added to the basal diet to achieve a level of 8.0 mg Cu/kg. Diet and distilled demineralized water were provided *ad lib*. The dietary treatment continued for 8 weeks before animals were exsanguinated.

Preparation of erythrocyte ghosts (membrane)

In a typical experiment, five male rats weighing 700–1000 g from each group of Cu-adequate and Cu-deficient animals were fasted for 12 h and exsanguinated under diethyl ether anaesthesia. Blood was withdrawn directly from the heart, by cardiac puncture after thoraco-abdominal incision, into syringes containing EDTA. Collected blood was centrifuged at 1000 g for 30 min at 4° in tubes containing EDTA (1 mg/ml). Plasma and the buffy coats were removed and the cells were resuspended in 0.1 M-Tris-hydrochloric acid buffer pH 7.6, then centrifuged again as described previously. The erythrocytes thus obtained were washed twice after removal of the buffy coats. Erythrocyte ghosts were prepared as described by Hanahan & Ekholm, 1974. Briefly, washed erythrocytes obtained from Cu-adequate and Cu-deficient animals were suspended in about seven times their volume of 20 mOsm-Tris-HCl buffer, pH 7.6, and centrifuged at 20000 g for 40 min at 4°. The supernatant fraction was aspirated carefully and with minimal contamination from

the small fibrin-like skin, which contained the ghosts, at the bottom of the tube. Membranes were washed thrice more as described previously.

Protein estimation. Protein concentrations of prepared ghosts were measured, after solubilizing the membranes in sodium dodecylsulphate (20 g/l; SDS), using the manual ninhydrin method (Hirs, 1967). Bovine serum albumin in SDS was used as a standard. Plasma membranes were stored frozen at -20° in 11.0 mM-Tris buffer, pH 7.6 at 4 mg protein/ml buffer.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes obtained from Cu-adequate and Cu-deficient rats were dissolved in SDS in 11.0 mM-Tris-HCl, pH 7.7, and incubated with or without 0.5 M-dithiothreitol for 40 min at 37° . Samples thus prepared were subjected to electrophoresis by the method of Weber & Osborn (1969) as described by Abu-Salah (1978). Samples of not less than 100 μ g membrane proteins were applied at pH 8.5 to 7.5% discontinuous polyacrylamide gels with 10 g methylenebisacrylamide/1-l g SDS/l. Bromophenol blue in glycerol (100 ml/l) was used as a tracking dye. Gels were pre-run for 1 h at 1 mA/gel then for 4 h at 7 mA/gel. Gels were stained in Coomassie brilliant blue R250 (2.5 g/l) in methanol (400 ml/l) in acetic acid (75 g/l) and the excess dye was washed out with methanol (400 ml/l) in acetic acid (75 g/l). Gels were scanned using a LKB 2202 UltraScan laser densitometer equipped with a helium-neon laser as a light source.

Lipid extraction and analyses

Total lipids were extracted from ghosts with a chloroform-methanol mixture (2:1, v/v) as described by Folch *et al.* (1957). Cholesterol and total PL contents were determined by the methods of DeHoff *et al.* (1978) and Fiske & Subbarow (1925) respectively. PL were separated by thin-layer chromatography developed with *n*-hexane-diethyl ether-acetic acid-methanol (90:20:2:3, by vol; Brown & Johnston, 1962). Base-catalysed transesterification (Morrison & Smith, 1960) was performed with sodium methoxide (Tahin *et al.* 1981) to convert fatty acids of PL to their fatty acid methyl esters, which were measured using a Shimadzu gas-liquid chromatograph, (Model 8A, equipped with a Supelco Wax 10 wide-pore capillary column 30 mm \times 0.75 mm). Measurements were performed at temperatures of 280° and 220° for the injection and column respectively and at nitrogen carrier gas pressure of 0.9 kg/cm². Peaks were identified by comparison of retention times with those of standard fatty acids, and integrated using a Hewlett-Packard 3390 A Integrator.

Excimer fluorescence. The method used was essentially that of Storch & Schachter (1984), modified for rat hepatocyte plasma membrane.

A stock solution of 0.15 mM-1,3 di(1-pyrenyl)propane (DPP) was freshly prepared in absolute alcohol by stirring in the dark, in a hot water-bath, for 45 min. Freshly-thawed membranes were resuspended by sonication for 10 s using Fisher Sonic Dismembrators Model 150 (Artek Systems Corporation), in 400 μ l Tris-buffered saline (TBS; 5 mM-Tris-146 mM-sodium chloride-4 mM-potassium chloride, pH 7.4). The incubation mixture containing (3.75 μ M DPP/mg protein) was prewarmed to 37° and vortexed vigorously for 20 s, and then incubated in the dark at 37° for 1.5 h with constant shaking. To each sample, 3 ml deoxygenated TBS (evacuated and flushed with highly purified N₂ for 30 min) was added. The diluted mixture was layered to the top of 2 ml Dextran T500 (200 g/l; Pharmacia) to separate the insoluble aggregate of DPP from the suspension. The upper phase containing the membrane was removed after it had stood for 10 min. Membranes were pelleted then washed twice, after resuspending them by sonication in 5 ml deoxygenated TBS, by centrifugation at 45 000 *g* for 25 min at 20° . The washed pellet was suspended in 4 ml deoxygenated TBS and allowed to stand at room temperature for 3 h.

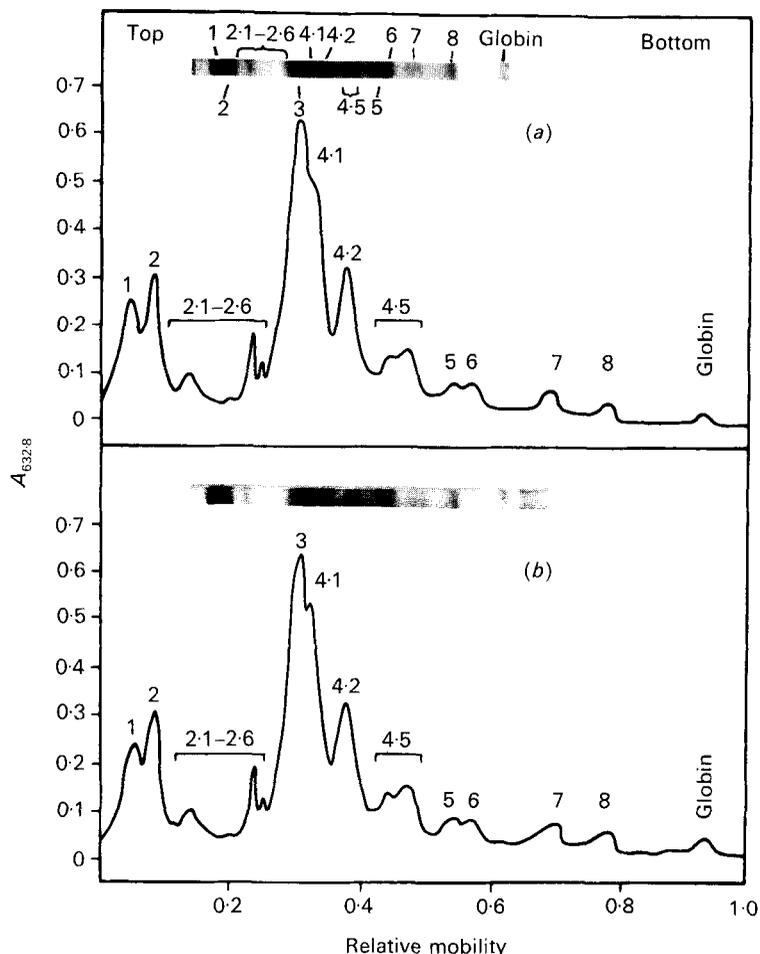


Fig. 1. Electrophoresis patterns and densitometric scans of Coomassie Blue-stained gels showing typical protein profiles of erythrocyte membranes from rats fed on (a) copper-adequate and (b) Cu-deficient diets. Gels were scanned using a LKB 2202 UltroScan laser densitometer at a wavelength of 632.8 nm. and absorbance of 1.0, a speed of 6.0 mm/min, a peak width of 0.2 and a reject area of 10^6 . The nomenclature used is based on that suggested by Steck (1974).

Fluorescence emission spectra were recorded at 20° with a Perkin-Elmer 650-15 fluorescence spectrophotometer at a sensitivity of 0.1 and at a time constant of 0.3. An excitation wavelength of 345 nm was employed. Excimer and monomer fluorescence peak intensities were monitored at 485 and 396 nm respectively. Fluorescence intensities were corrected for membranes carried under identical conditions without DPP and for the probe added to TBS alone.

RESULTS

The criteria of Cu deficiency such as the significant depression in weight gain, increased heart:body-weight ratio and depleted hepatic Cu stores were all clearly established compared with rats fed on Cu-adequate diets as described by Allen *et al.* (1982).

SDS-PAGE patterns demonstrated no difference in the membrane-protein profiles from Cu-adequate and Cu-deficient rats (Fig. 1). Nevertheless, the fatty acid profile of membrane PL derived from erythrocytes was influenced by dietary treatment. Among the individual

Table 1. *Fatty acid composition of erythrocyte membranes of copper-adequate and Cu-deficient rats**

(Mean values with their standard errors)

Component fatty acid	Phospholipids (mol/100 mol total fatty acids)				Statistical significance of difference†: $P <$
	Cu-adequate		Cu-deficient		
	Mean	SEM	Mean	SEM	
16:0	27.17	0.56	25.45	0.06	0.002
18:0	13.89	0.16	17.96	0.11	0.001
18:1 <i>n</i> -9	11.19	0.24	7.53	0.02	0.001
18:2 <i>n</i> -6	8.29	0.32	7.62	0.05	0.01
18:3 <i>n</i> -3	0.32	0.04	0.17	0.01	0.01
20:0	0.12	0.3	0.14	0.06	NS
20:1	0.18	0.008	0.19	0.01	NS
20:2	0.45	0.01	0.55	0.01	0.001
20:3	0.27	0.01	0.27	0.01	NS
20:4 <i>n</i> -6	27.09	0.35	26.74	0.16	NS
22:2	2.23	0.25	3.42	0.08	0.002
22:4	3.14	0.04	3.23	0.03	NS
22:5 <i>n</i> -6	1.80	0.05	1.80	0.06	NS
22:5 <i>n</i> -3	0.86	0.03	1.00	0.02	0.001
22:6	1.36	0.03	1.76	0.07	0.001
Unidentified	1.64		2.17		

NS, not significant.

* For details of diets and dietary regimen, see pp. 436.

† Student's *t* test; 28 df.

fatty acids, significant elevations in stearic (18:0, 29.3%), docosahexaenoic (22:6*n*-3, 29.4%) and docosadienoic (22:2*n*-3, 53.4%) acids were observed in Cu-deficient rats. On the other hand, reductions in oleic (18:1*n*-9, 32.7%) and linolenic (18:3*n*-3, 46.9%) acids were observed in Cu-deficient treatment (Table 1). Membrane fluidity was assessed by measurement of the fluorescence of excited complexes (excimers) of an intramolecular excimer-forming fluorophore, DPP. DPP has been used to monitor short-range lateral diffusion of pyrene substituents in the erythrocyte membrane (Zacharisse *et al.* 1982). The short-range lateral diffusion in plasma membranes was evaluated by the use of excimer:monomer fluorescence intensity ratio of DPP. The reduction in this ratio observed in the erythrocyte membranes of Cu-deficient rats indicated a decreased fluidity (Table 2).

In order to evaluate the degree of fatty acid unsaturation in the PL of the erythrocyte membranes of Cu-adequate and Cu-deficient animals, the double-bond index was derived from the sum of product of the proportion of each fatty acid multiplied by the number of double bonds in that acid. When the index was calculated up to and including arachidonic acid (20:4*n*-6) as described by Storch & Schachter (1984), a significant reduction was observed (Table 2) for membrane PL fatty acids from Cu-deficient compared with Cu-adequate rats. However, when the fatty acids up to 22:6 were used in the calculation the double-bond index was not significantly altered. On the other hand, a significant decrease ($P < 0.001$) in the unsaturated:saturated PL fatty acids was observed in Cu-deficient compared with Cu-adequate rats (Table 2). Moreover, the oleic:stearic PL fatty acid ratio was significantly depressed as a result of Cu deficiency. These two fatty acids together

Table 2. *Fluidity variables and lipid composition of erythrocyte membranes in copper-adequate and Cu-deficient rats**
(Mean values with their standard errors)

Variable	Cu-adequate		Cu-deficient		Statistical significance of difference†: $P <$
	Mean	SEM	Mean	SEM	
Excimer:monomer fluorescence intensity‡	0.335	0.003	0.274	0.011	0.001
Double-bond index up to and including arachidonic acid (20:4n-6)§	1.367	0.017	1.294	0.007	0.001
Double bond index for all phospholipid-fatty acids§	1.746	0.024	1.728	0.005	NS
Ratio of 18:1 to 18:0 phospholipids-fatty acids	0.806	0.007	0.419	0.0002	0.001
Percentage unsaturated:percentage saturated phospholipid-fatty acids	1.389	0.026	1.246	0.008	0.001
Cholesterol (μ mol/mg membrane protein)	0.480	0.013	0.457	0.003	0.1
Phospholipid (μ mol/mg membrane protein)	0.520	0.013	0.526	0.007	NS
Cholesterol:phospholipid molar ratio	0.923	0.005	0.870	0.008	0.01

NS, not significant.

* For details of diets and dietary regimen, see pp. 436.

† Student's *t* test; 28 df.

‡ 22 df.

§ The nature (no. of double bonds, if any) of the unidentified fatty acids was unknown, thus, they were excluded when calculating the double bond index.

constitute a significant proportion of PL fatty acids (approximately 25 mol/100 mol) (Table 1) in the erythrocyte membrane.

In the light of the important influence of the cholesterol:PL ratio on the fluidity of PL vesicles and biomembranes (Ladbrooke *et al.* 1968; Cooper *et al.* 1978; Giraud *et al.* 1981), the effect of Cu deficiency on the ratio was examined in the present study. The cholesterol:PL molar ratio was significantly decreased ($P < 0.01$, Student's *t* test) in rats fed on Cu-deficient diets (Table 2). Nevertheless, the cholesterol (mol):protein (mg) ratio was slightly lower in Cu-deficient rats ($P < 0.1$) and the PL:protein ratio did not seem to be affected at all by Cu deficiency.

DISCUSSION

Alterations in the PL fatty acid profile of the erythrocyte membrane are similar to those reported for HDL derived from Cu-deficient rats (Al-Othman *et al.* 1990). However, the magnitude of changes in the PL fatty acid profile of HDL appeared to be greater than that of the erythrocyte membrane, in terms of the increase in 18:0 and reductions in 18:3. These similarities may have resulted from equilibrium exchange of PL fatty acids, aided by the PL ester transfer protein and to a lesser extent by the cholesterol transfer protein between the erythrocytes and HDL (Tall, 1986).

Since the present study is the first to demonstrate the detailed PL fatty acid composition of the erythrocyte membrane in rats and the pattern by which it is influenced by Cu deficiency, the results are compared with a previous study (Lei *et al.* 1988) on the effect of Cu deficiency on rats hepatic plasma membranes. The present study revealed a different PL fatty acid profile and different trends in the changes in the relative proportions of erythrocyte membrane fatty acids in Cu-deficient rats compared with hepatic plasma membrane. Whereas Cu-deficient dietary treatments resulted in a reduction in the ratio of

18:0 to 18:1 fatty acids in the erythrocyte membrane, the ratio remained unchanged in hepatic plasma membranes. Moreover, while the proportion of arachidonic acid (20:4 n -6) was not affected at all in the PL of the erythrocyte membrane, it was reduced markedly in the PL of the hepatic membrane. The marked reduction in arachidonic acid in the Cu-deficient hepatic plasma membrane may have resulted from an enhanced turnover of this PL fatty acid. An increased release of 20:4 by phospholipase A₂ (EC 3.1.1.4) to form eicosanoids, or an increased removal of oxidized 20:4 in the detoxification of PL peroxides (Van Kuijk *et al.* 1987), are possible explanations. Alternatively, the supply of 20:4 for hepatic plasma membrane PL formation and regeneration may be depressed by Cu deficiency resulting in the reduction in 20:4.

The reduction in excimer:monomer fluorescence intensity ratio of DPP suggests a decreased lateral diffusion in the erythrocyte membranes of Cu-deficient rats. The significant reduction in the double bond index calculated up to and including arachidonic acid could be considered as a contributing factor in the observed decrease in membrane lateral fluidity. Nevertheless, the value of the double-bond index obtained when the fatty acids up to 22:6 were used showed no significant difference in the degree of unsaturation of PL fatty acids from Cu-deficient compared with Cu-adequate rats. Thus, the index may not be a good indicator of the degree of unsaturation when small amounts of fatty acids greater than 20:4 are included in the calculations. On the other hand, an increase (4% of the total PL fatty acid composition) in the saturated stearic acid accompanied by a large decrease (32.7%) in the unsaturated oleic acid and consequently a decrease (52%) in the ratio of 18:1 to 18:0 may be a major contributing factor in the observed reduction in membrane fluidity in Cu-deficient rats. The significant decrease in the ratio of 18:1 to 18:0 may be explained, at least in part, by a possible decrease in the activity of 9-desaturase (Wahle & Davies, 1975) which normally requires Cu for its activity. Moreover, further evaluation of the degree of unsaturation revealed a significant reduction in the total unsaturated:total saturated fatty acid ratio in Cu-deficient rats, thus, providing support for the reduction in the proportion of unsaturated fatty acids and the ratio of 18:1 to 18:0 fatty acids, which indicated a decrease in unsaturation. The reduced cholesterol (mol):PL (mg) ratio is a strong indication of a decrease in the molar proportions of cholesterol in the erythrocyte membranes in rats fed on Cu-deficient diets. The reduced amount of cholesterol is expected to result in an increase in the disordering effect and hence in the fluidity of the membrane (Ladbrooke & Chapman, 1969; Cooper *et al.* 1978; Giraud *et al.* 1981), provided the PL fatty acid composition is kept unchanged. Nevertheless, the reduction in the cholesterol:PL molar ratio in Cu-deficient rats was accompanied by a decrease in the degree of unsaturation of the fatty acids and in a significant increase in some saturated fatty acids, such as stearic acid. Therefore, the observed decrease in the fluidity of erythrocyte membrane could be accounted for, at least in part, on the basis of the combined effect cholesterol has on restricting the motion of unsaturated fatty acids and preventing saturated chains from crystallizing (Ladbrooke *et al.* 1968). The PL:protein ratio as a whole did not seem to have any influence on modulating the fluidity of the erythrocyte membranes as the ratio was not affected by different dietary treatments.

The small but significant reduction in cholesterol content observed in the erythrocyte membranes of Cu-deficient rats could be due to an impaired delivery of cholesterol to developing stem cells of bone marrow or a small efflux of cholesterol from the erythrocytes. This occurs despite the increases in both total plasma cholesterol (Lei, 1977, 1990) and HDL-cholesterol (Yount *et al.* 1990) in Cu-deficient rats. Alternatively, the decreased cholesterol content of erythrocyte membranes may have resulted from an impaired incorporation of cholesterol in the membranes of developing stem cells as a result of the observed alteration in their PL fatty acid composition.

SDS-PAGE membrane protein profile did not seem to be altered by Cu-deficient diets. This finding is not in agreement with that obtained by Johnson & Kramer (1987) who reported a significant increase in the amount of a 170000 Da protein in the membrane of erythrocytes derived from rats fed on Cu-deficient diets. The differences in SDS-PAGE protein profiles obtained from both studies could be due to differences in dietary composition used.

The results of the present study demonstrate that the lipid composition and fluidity of erythrocyte membranes are responsive to the animals' Cu status and suggest that other cellular functions such as the transport of ions and nutrients dependent on optimal membrane fluidity may also be altered.

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