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Erythrocyte Fatty Acid Composition and Apparent Permeability to Non-Electrolytes.* (29126)

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The presence of lipids in the cell membrane was inferred from permeability studies(1), because the ability of non-electrolytes to penetrate the cell increased as the oil-water partition coefficients increased. Thus the composition of the membrane lipids might be expected to exert some control over the rate of penetration of solutes into the cell. DeGier and his co-workers(2) subjected the lipids extracted from the erythrocytes of various animal species to analysis and attempted to correlate the lipid composition with permeability towards certain non-electrolytes. The results indicate a possible correlation between the mixed fatty acid composition of the erythrocyte membrane and the permeability of the cell to non-electrolytes(3). The response of the cellular fatty acids to dietary fat(4,5) provides a means of testing this hypothesis in a single species.

Materials and methods. Male weanling albino rats were fed a diet containing 68% casein, 10% dextrose, salts, vitamins(6) and 10% corn oil, castor oil, butterfat, or hydrogenated coconut oil. The fatty acid compositions of the erythrocytes were determined by gas liquid chromatography of the methyl esters after the animals had received the diets for a period of 22 weeks(5).

Permeability to 3 non-electrolytes, glycerol, thiourea, or diethylene glycol, was determined by following the rate of hemolysis of the cells in isotonic solutions of the non-electrolytes. The procedure was based on that employed by Jacobs *et al*(7). Isotonic solutions of the non-electrolytes (0.3 M) were prepared using water distilled in an all glass apparatus. The temperature of the solution was adjusted to 25°C and 3.0 ml were pipetted into a 1 cm glass cuvette. The tails of representative rats were washed with absolute ethanol, 20 mm^3 of blood was drawn from a fresh cut in the tail, added to the solution of the non-electrolyte and mixing accomplished by gently blowing through the pipette employed for making the addition. The cuvette was immediately placed in the well of a Cary recording spectrophotometer, the chart set in motion, and the change in optical density of the cell suspension at 600 $m\mu$ recorded against time. This graph represented the clearing of the cell suspension

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TABLE I. Effect of Dietary Fat on Major Erythrocyte Fatty Acids.

Acid*	Coconut oil	Butterfat	Castor oil	Corn oil
	(%)	(%)	(%)	(%)
16:0	22.4	20.6	25.2	24.2
16:1	2.7	2.3	1.7	.4
18:0	14.2	9.8	13.5	13.5
18:1	15.7	16.9	13.5	8.6
18:2	2.2	4.3	5.3	11.5
20:3	16.0	6.4	2.3	.6
20:4	15.4	24.5	26.3	31.0
Total† EFA	20.2	30.7	34.6	47.6

* Numbers before and after colon represent carbon chain length and number of double bonds respectively.

† Total essential fatty acids include 20:5 and 22:4 of linoleate series (8) in addition to 18:2 and 20:4.

due to hemolysis of the cells which resulted from penetration of the non-electrolyte into the cell. A sigmoid curve was obtained, the slope of this curve at the point of inflection was determined and used for comparing the maximum rates of hemolysis of cells obtained from animals which had received the various dietary fats. Five animals each from the groups fed corn oil, castor oil or butterfat and 6 from the coconut oil group were used for this study. The major changes induced by the various dietary fats are reproduced (5) in Table I.

Results. The increasing amounts of dietary linoleic acid as supplied by the coconut oil, butterfat, castor oil and corn oil (0.6, 2.2, 6.6 and 54.0% respectively) resulted in increased incorporation of this fatty acid into the cell and also to increased arachidonic acid incorporation. Where dietary linoleate was restricted, more palmitoleic and oleic acids were incorporated into the cellular lipids, and the eicosatrienoic acids characteristic of essential fatty acid deficiency were also found in increasing amounts, comprising over 16% of the total fatty acids when hydrogenated coconut oil was the dietary fat.

The relative mean hemolysis rates for erythrocytes in isotonic solutions of the non-electrolytes are given in Table II. With each solute investigated, cells from the animals which had received hydrogenated coconut oil were hemolyzed most rapidly. As the dietary linoleic acid intake increased, rate of hemolysis decreased. This was most evident when isotonic glycerol was the hemolytic agent, the difference between the corn oil and coconut oil diets being statistically significant as indicated by the t-test ($t = 4.25$, $p = 0.01$). When thiourea or triethylene glycol was employed, the relative hemolysis rates of cells from the butterfat and castor oil groups were reversed; erythrocytes from the animals fed butterfat were less susceptible to hemolysis than those from the rats fed castor oil. The difference between corn oil and coconut oil was statistically significant for the thiourea induced hemolysis ($t = 5.89$, $p = 0.01$), but the difference between these 2 groups for triethylene glycol induced hemolysis was less significant ($t = 1.06$, $p = 0.3$). The apparent correlation between the relative rates of glycerol induced hemolysis and the essential fatty acid content of the cell is shown in Fig. 1. Curve A represents the relationship of hemolysis to cellular linoleic acid, Curve B to cellular arachidonic and linoleic acids, and Curve C to total cellular essential fatty acids (18:2, 20:4, 20:5 and 22:4). Curve C is essentially linear.

Discussion. The rate of hemolysis of rat erythrocytes was found to be related to the nature of the dietary fat received by the animals. The relationship appeared to be one involving essential fatty acid deficiency, since an increase in linoleic acid content of the dietary fat resulted in a decreased susceptibility to hemolysis in isotonic solutions of

TABLE II. Effect of Dietary Fat on Relative Hemolysis Rates of Erythrocytes in Isotonic Non-Electrolyte Solutions.

Solute	Coconut oil	Butterfat	Castor oil	Corn oil
Glycerol	12.34 ± 1.65*	10.86 ± .54	10.45 ± 1.35	8.58 ± 1.16
Thiourea	5.71 ± .75	3.98 ± .30	4.43 ± .45	3.27 ± .60
Triethylene glycol	5.79 ± .83	4.51 ± .40	5.28 ± .78	4.80 ± .65

* Mean slope of hemolysis curve at point of inflection ± standard deviation of mean.

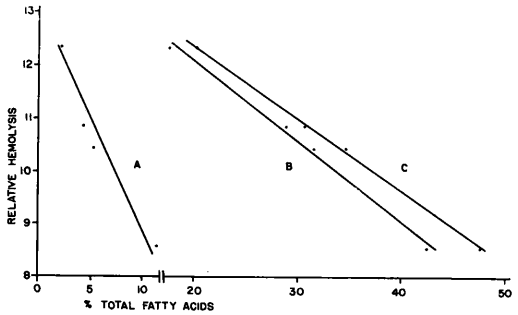

FIG. 1. Effect of cellular essential fatty acids on relative hemolysis of erythrocytes in isotonic glycerol. Curve A, linoleic acid; Curve B, linoleic and arachidonic acids; Curve C, linoleic, arachidonic, eicosapentaenoic and docosatetraenoic acids.

non-electrolytes. It is possible that these changes in hemolysis reflected structural changes arising in the erythrocyte membrane from the incorporation of specific fatty acids. Increasing dietary linoleate led to the incorporation of the so called "essential fatty acids," linoleic and arachidonic acids, while the palmitoleic, oleic and eicosatrienoic acids were deposited when diets low in linoleic acid (coconut oil and butterfat) were fed.

The rate of hemolysis or hemolysis time of red cells in an isotonic solution of a non-electrolyte has been taken as a measure of the permeability of the cells to the non-electrolyte(7). In this case the cell is assumed to act as a perfect osmometer and permeability is inversely proportional to hemolysis time. Jacobs *et al*(7) determined hemolysis times for red cells of various species in different non-electrolytes. Kogle *et al*(3) attempted to correlate these hemolysis times with the fatty acid composition of the erythrocyte stroma from the same species. They suggested that oleic acid or the unsaturated acids in general might be involved in controlling the rate of entry of non-electrolytes into the cell. When the various species under consideration were arranged in increasing order of permeability to glycol, the oleic acid content of the cell and the total unsaturated fatty acids in the cell decreased in the same order.

In the present study, the relative hemolysis rates of the cells were compared with their essential fatty acid content. The relationship between hemolysis in isotonic glycerol and linoleic acid content was not quite linear. If arachidonic and linoleic acids were considered together, a more linear relationship resulted, and inclusion of the eicosapentaenoic and docosatetraenoic acids of the linoleic acid series(8) resulted in an almost linear relationship of the essential fatty acid composition of the cell with the hemolysis rate. This decrease in hemolysis rate with increasing cellular essential fatty acids is the reverse of that observed by Kogle *et al*(3), where an increase in oleic acid and a decrease in linoleic plus arachidonic acids(9) was associated with a decreased rate of hemolysis and a decrease in the permeability of the cell to non-electrolytes.

Reports in the literature indicate that restriction of dietary essential fatty acids can result in a decreased erythrocyte survival time(10). It is possible that the more rapid rate of hemolysis observed in the cells from rats deficient in dietary linoleate in the present study was due to an increase in osmotic fragility and that the cells were unable to sustain as large an increase in volume without hemolyzing as those taken from the control animals. However, there are also reports indicating that essential fatty acids are indeed involved in permeability phenomena, in the case of mitochondria(11) and of the skin and kidney(12). Thus the present results should be noted with caution, since the actual phenomenon studied was the hemolysis of the cell. These results do not indicate conclusively that essential fatty acid deficiency caused an increase in permeability of the rat erythrocyte membrane to non-electrolytes, but there was an increase in rate of hemolysis of the cells from the deficient animals and this may result from an increased permeability to the non-electrolyte. Further studies are presently underway using other solutes and non-hemolyzing techniques.

Vandenheuvel advanced a model for biological organization at the molecular level (13). This model involved a complex resulting from the association of cholesterol with sphingomyelin or glycerophosphatide and was applied specifically to the structure of the myelin sheath. It is interesting, however, to consider the possibility of such complexes

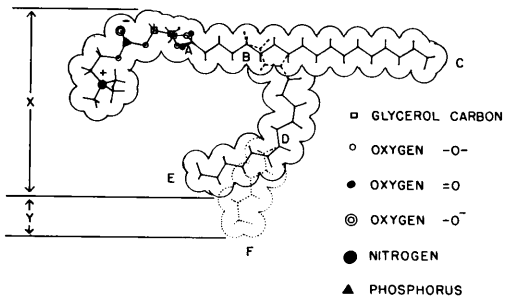

FIG. 2. Orthogonal projection of lecithin (top view), containing stearic acid (ABC) and arachidonic (ABDE) or 5,8,11-eicosatrienoic acid (ABDF).

existing in other membranous structures, the electron micrographs of which bear some similarity to that of the myelin sheath.

Unlike myelin, which contains predominantly saturated and monoenoic acids, the erythrocyte phospholipids are high in polyunsaturated acids, particularly arachidonic acid, or, as a result of restricted dietary linoleic acid, 5,8,11-eicosatrienoic acid. Fig. 2 is a representation of lecithin constructed geometrically from the parameters given by Vandenheuvel(13). The α-position of the glycerol moiety is esterified with stearic acid (ABC), the β-position with arachidonic (ABDE) or 5,8,11-eicosatrienoic acid (ABDF). In a complex such as that proposed by Vandenheuvel (13), the curvature of the arachidonic acid chain would result in greater stearic hindrance to the cholesterol than would the mono- or dienoic acids of the erythrocyte lipids. However, the substitution of the trienoic acid for the arachidonic acid also results in an increase in the overall width of the lecithin moiety (Fig. 2). This increase, Y, is about 20% of the width, X, of the arachidonyllecithin. It is tempting to speculate that some of the phenomena observed in cases of essential fatty acid deficiency result from the structural changes which arise when the eicosatrienoic acid is incorporated into the cell at the expense of arachidonic acid. The increase in bulk of the molecules may result in a looser packing of the phospholipid complexes in the membrane thus altering its stability and permeability. It is also interesting to note that *cis* 9, *trans* 12-octadecadienoic acid, although it is metabolized by chain lengthening and desaturation to 5,8,11, 14-eicosatetraenoic acid, will not prevent the symptoms of essential fatty acid deficiency (14). The resulting metabolite is a geometric isomer of arachidonic acid with a *trans* double bond in the 14-position. The orthogonal projection of a phosphatide containing this acid would be very similar to that of the eicosatrienoic acid phosphatide.

The above speculation is undoubtedly an oversimplification of the very complex situation existing within the erythrocyte membrane, but it is offered in an attempt to correlate the observed experimental results with recent theories of membrane structure, and may serve as a basis for a more sophisticated treatment as more information becomes available.

Summary. Variations in the nature of the dietary fat resulted in changes in the mixed fatty acid composition of rat erythrocytes. An increase in linoleate content of the diet led to an increased cellular linoleate and arachidonate content whereas diets deficient in linoleate resulted in accumulation of palmitoleic, oleic and eicosatrienoic acids. The changes in cellular essential fatty acids were accompanied by changes in the apparent permeability of the erythrocytes, as measured by rate of hemolysis of the cells in isotonic solutions of non-electrolytes. Increasing levels of cellular essential fatty acids resulted in decreases in hemolysis rates of the erythrocytes. The relationship between the relative rate of hemolysis in isotonic glycerol and the concentration of essential fatty acids was approximately linear. An attempt has been made to correlate the increased hemolysis of the cells from the deficient rats with the changes in fatty acid composition and the possible modification of the phosphatide structure resulting from these changes.

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Effects of Sodium Fluoride on Calcium Homeostasis.* (29127)

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Previous investigations in this laboratory have been directed toward defining the parameters of plasma calcium homeostasis(1,2). The data obtained support the hypothesis that plasma calcium is maintained by two processes. The first is a basic equilibration process which appears to be a continuous attempt to bring the calcium ion into equilibrium between bone and blood. The second is a hormonal process by which a continuously secreting parathyroid gland maintains the normally observed circulating calcium values above the basic level by influencing bone resorption. Osteoclastic activity has been correlated with this second process(3), though not proven necessary for hormonal action. These studies and conclusions are based on the prevention of a drop in plasma calcium below normal levels and are not necessarily related to the problem of the existence of a second parathyroid hormone, calcitonin, which has been postulated to be concerned with prevention of hypercalcemia(4).

Whether or not the solubility of the bone crystal could be affected *in vivo* was investigated by Talmage and Kraintz(2) using acidotic animals. Such treatment increased the amount of calcium removed by peritoneal lavage in the parathyroidectomized rats. In contrast, subsequent studies showed that administration of sodium fluoride by peritoneal lavage decreased the amount of calcium removed by this procedure(5). The investigations reported here are a continuation of the NaF studies and were carried out to clarify whether or not the decreased calcium levels observed in animals receiving sodium fluoride were a consequence of a reduction in solubility of the hydroxyapatite crystal of bone. Such an effect of fluoride on bone crystal has been suggested by others(6,7).

Materials and methods. A description of the technique of peritoneal lavage and of the constituents of the buffered, isotonic lavage fluid has been given(8). In the basic procedure, a calcium-free isotonic buffered solution was introduced into the peritoneal cavity and removed after a one-hour equilibration period through a stainless steel plug sewn into a midventral incision. This procedure was repeated hourly as desired. NaF, when used, was added to the lavage fluid in amounts (0.036 g/l or 0.072 g/l) so that each animal received either 0.5 mg or 1.0 mg F^- per hour.

Holtzman rats weighing 180 to 200 g were used throughout these studies. All surgery was performed under ether anesthesia. Nephrectomy, if done, and the insertion of the stainless steel plug were carried out 18 to 20

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