*Biochimica et Biophysica Acta*, 433 (1976) 150–163


BBA 77268

# LIVER PLASMA MEMBRANES FROM ESSENTIAL FATTY ACID-DEFICIENT RATS

## ISOLATION, FATTY ACID COMPOSITION, AND ACTIVITIES OF 5′-NUCLEOTIDASE, ATPase AND ADENYLATE CYCLASE

R. P. BRIVIO-HAUGLAND, S. L. LOUIS, K. MUSCH, N. WALDECK and M. A. WILLIAMS

*Department of Nutritional Sciences, University of California Berkeley, Calif. 94720 (U.S.A.)*

(Received August 25th, 1975)
(Revised manuscript received November 12th, 1975)

## SUMMARY

To determine whether changes in unsaturation of fatty acids in rat liver plasma membranes might alter activities of membrane-associated enzymes, liver plasma membranes were prepared from rats fed purified diets lacking or supplemented with essential fatty acids. Two methods of membrane purification were used. A similar degree of purification was obtained with both methods for both depleted and control membranes, as indicated by marker enzyme purification. The proportion of essential fatty acids of the linoleate series was significantly lower in phospholipids from depleted rats. The specific activity of 5′-nucleotidase was lower, and the activity, $V$ and apparent $K_m$ for total $(Na^+ + K^+ + Mg^{2+})$-ATPase were higher in the depleted liver plasma membranes. Arrhenius plots of total ATPase activity showed a discontinuity at the same temperature for both the depleted and control membranes. Activity with the depleted membranes was higher at all temperatures tested. Supplementation of deficient rats with a source of essential fatty acids (corn oil) restored $V$ and apparent $K_m$ values to normal. Adenylate cyclase activity in the presence of fluoride, glucagon or glucagon plus GTP was significantly lower in the depleted plasma membranes.

## INTRODUCTION

Considerable evidence indicates the importance of phospholipids in the activity of plasma membrane-associated enzymes such as $(Na^+ + K^+)$-ATPase [1, 2], $Mg^{2+}$-ATPase [3–6] and adenylate cyclase [7–10]. Phosphatidylserine and phosphatidylinositol appear to have specific roles in the function of $(Na^+ + K^+)$-ATPase [1, 6, 11], and variations in chain length and degree of unsaturation of phospholipid fatty acids have been shown to affect the activity of this enzyme [12]. The evidence

Abbreviations: $(Na^+ + K^+)$-ATPase: $Na^+$ and $K^+$-stimulated and $Mg^{2+}$ requiring ATPase or ouabain-sensitive ATPase; $Mg^{2+}$-ATPase is $Mg^{2+}$-stimulated, ouabain-insensitive ATPase.

for phospholipid effects has been obtained in vitro with isolated preparations of $(Na^+ + K^+)$-ATPase from which lipids have been removed and replaced with specific phospholipids. The function of $Mg^{2+}$-ATPase is not definitely known, but it constitutes a large portion of the plasma membrane ATPase activity [3, 5] and may be involved in the primary steps of insulin action in adipose tissues [13] or in lymphocyte transformation [14]. Hormonal activation of adenylate cyclase is phospholipid dependent [7–10], and phosphatidylserine and phosphatidylinositol may be specifically involved.

As yet, we do not know how fatty acid structures affect the activity of membrane-associated enzymes or the physiological effects that might result from changing these activities. In animals, including humans, significant changes can be made in the unsaturated fatty acid composition of cell membranes by varying dietary intakes of essential fatty acids [15]. Maximum changes occur in animals made essential fatty acid-deficient by feeding a diet lacking essential fatty acids. Consequently, if these membrane-bound enzymes were influenced by the unsaturated fatty acid composition of the associated lipids, then one would expect large changes in the activity of these enzymes in liver plasma membranes from essential fatty acid-deficient animals.

Evidence for this hypothesis has been obtained recently. An increase in the specific activity of $(Na^+ + K^+)$-ATPase has been found in brain homogenates and in isolated synaptosomal membranes from essential fatty acid-deficient mice [16]. A decrease in the specific activity of 5′-nucleotidase has been observed in brain homogenates from essential fatty acid-deficient rats [17]. The ability of $Na^+$ and $K^+$ to activate ATPase of erythrocyte ghosts is decreased in cells from rats fed a fat-free diet [18]. The results with erythrocyte ghosts led to the suggestion that the interaction of $Na^+$ and $K^+$ with the enzyme might be affected by the fatty acid composition of the plasma membrane, with subsequent modification of the structure of the membrane-bound ATPase.

Previously, Chandrasekhara and Ananth Narayan [19] reported a lower 5′-nucleotidase activity in liver plasma membranes from essential fatty acid-deficient rats fed a purified diet, but no difference was found in total $(Na^+ + K^+ + Mg^{2+})$-ATPase activity. Unfortunately, this experiment was not properly controlled, since the deficient rats were compared with those fed a chow diet. Laboratory "chow" diets differ from purified diets in so many respects, in addition to essential fatty acid content, that it is very possible that the experimental results might reflect these other differences rather than specific effects of essential fatty acid deficiency. It is clearly and abundantly documented that metabolic responses differ between rats fed complete purified diets and those fed "chow" diets [20].

Membrane preparations from essential fatty acid-deficient rats offer considerable potential for studying relationships between membrane fatty acid composition and properties of membrane-associated enzymes. Therefore, it was important (a) to eliminate the possibility that the results of Chandrasekhara and Ananth Narayan [19] arose from differences in dietary components other than essential fatty acids and (b) to determine whether other enzymatic differences in liver plasma membranes might exist between deficient rats and control rats fed the same purified diet supplemented with a source of essential fatty acids. In the present report, we have made such a comparison. Results for 5′-nucleotidase, ATPase and adenylate cyclase are reported.

METHODS

*Rats and diets.* Essential fatty acid deficiency was produced by feeding weanling male Sprague-Dawley rats a 20 % casein-sucrose diet containing 5 % hydrogenated coconut oil. Control rats were obtained by feeding the same 20 % casein-sucrose diet, supplemented with 5 % corn oil as the source of essential fatty acid [21]. The rats were caged in pairs in galvanized, suspended wire-bottom cages. Food and tap water were supplied ad libitum.

*Preparation of liver plasma membranes.* After at least 6 weeks of essential fatty acid-depletion, the deficient rats were sacrificed by decapitation without anesthesia. A control rat of the same age was sacrificed at the same time, so that deficient and control plasma membranes were always prepared in sets of two. The rats had access to food up to the time of sacrifice. The livers were perfused with ice-cold saline (0.15 M) through the portal vein, then excised and processed as described by Ray [22]. The resulting pellets from the low-speed centrifugation were then purified by the sucrose gradient method of Ray [22] or the two-phase polymer method of Lesko et al. [23].

*Gel electrophoresis.* Slab-sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by techniques similar to those described by Ames [24]. The discontinuous buffer system of Laemmli [25] was used. The stock solution of acrylamide and *N,N'*-bis-methylene-acrylamide (30 %, 0.8 %) was diluted to 12.5 % acrylamide in the running gel for this electrophoresis procedure.

*Enzyme assays*

5'-Nucleotidase (EC 3.1.3.5) was assayed at 30 °C as described by Solyom and Trams [26]. Inorganic phosphate was determined as described [27] with ferrous sulfate as reducing agent.

*ATPase* (EC 3.6.1.3). In the preparation of plasma membranes, ATPase activity was measured by inorganic phosphate release from ATP at 30 °C. For determination of temperature dependence, $V$ and $K_m$, ATPase was assayed by the method of Barnett [28] in which ADP formation is coupled with NADH oxidation. In both assays, the incubation medium contained 3 mM ATP, 3 mM $MgCl_2$, 80 mM NaCl, 10 mM KCl, and 50 mM Tris · HCl buffer, pH 7.5. In the NADH oxidation method, the incubation medium contained also 2 mM NADH, 2 mM phosphoenolpyruvate, 20 units lactate dehydrogenase (EC 1.1.1.27) and 200 units pyruvate kinase (EC 2.7.1.40) per ml.

*Adenylate cyclase.* The assay system [29, 30] contained in total volume of 0.20 ml: 30 mM Tris · HCl pH 7.5, 4 mM $MgCl_2$, 0.2 mM 1-methyl-3-isobutylxanthine, 1 mM EDTA, 1.25 mM ATP, 0.01 mg bovine serum albumin, 10 mM phosphocreatine and 7.5 units creatine phosphokinase (EC 2.7.3.2). Reactions were started by adding 0.04 mg membrane protein and stopped after 15 min at 34 °C by adding sodium acetate/acetic acid buffer, pH 4.5, to a final concentration of 50 mM. Samples were frozen at −20 °C until analysis for cyclic AMP [31].

*Alkaline phosphatase* (EC 3.1.3.1) was measured by hydrolysis of *p*-nitrophenylphosphate at pH 9 [22]. The assay system contained 1 mM *p*-nitrophenylphosphate, 2.5 mg $MgCl_2$, 0.1 % Triton X-100, and 100 mM Tris · HCl, pH 9.0. The change in absorbance at 410 nm was recorded with a Gilford spectrophotometer.

*Glucose-6-phosphatase* (EC 3.1.3.9) was assayed at 30 °C according to Hüb-

scher and West [32]. Succinate-cytochrome *c* reductase was used as a marker for mitochondrial membranes [33]. The degree of contamination for microsomes or mitochondria was calculated, according to Newkirk and Waite [34], as the ratio of the recovery of either enzyme in the plasma membrane fraction to the recovery of 5′-nucleotidase in the plasma membrane fraction.

*Protein* was determined by the method of Lowry et al. [35], with bovine serum albumin as standard.

*Lipid analysis.* Plasma membranes (10–12 mg protein) were extracted with chloroform/methanol (2 : 1, v/v) containing 0.001 % hydroquinone as described previously [36]. Phospholipid fractions were separated by thin-layer chromatography on silica gel H plates with chloroform/methanol/glacial acetic acid/water (25 : 15 : 4 : 2, v/v) as developing solvent [37]. Aliquots of membrane lipids containing about 2 mg fatty acid were applied to the plates and compared with known standards (0.5 mg of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine spotted adjacent to the sample). The phospholipid bands were located by spraying with 2′,7′-dichlorofluorescein (0.4 % in methanol) and viewing under ultraviolet light. The bands were scraped from the plates and transmethylated directly [36] with an appropriate amount of heptadecanoic acid as an internal standard. The methyl esters were extracted into petroleum ether, and analyzed by gas-liquid chromatographic analysis, as previously described [36].

*Materials.* Phosphoenolpyruvate, ATP, GTP, NADH (disodium salt), phosphocreatine, glucagon, lactate dehydrogenase, pyruvate kinase (salt-free) and creatine phosphokinase were from Sigma. Omnifluor was from New England Nuclear, and cyclic [$^3$H]AMP from Amersham.

## RESULTS

*Isolation of plasma membranes.* Initially both the sucrose gradient method of Ray [22] and the two-phase polymer method of Lesko et al. [23] were used for liver plasma membrane preparation in order to determine how membranes from essential fatty acid-deficient rats responded to different conditions of purification. Both methods produced a similar degree of purification (Table I) for membranes from either deficient or control rats, based on the values for 5′-nucleotidase, alkaline phosphatase, and ATPase as marker enzymes. The yield of membrane protein per weight of liver was greater with the two-phase polymer method, in agreement with the results of Lesko et al. [23]. The degree of contamination by mitochondria and microsomes (Table II) was similar in both deficient and control membranes. The values for specific activities, purification and recovery data for our preparations are similar to previously reported purification values (10–25 times) based on 5′-nucleotidase activity of sucrose gradient preparations [38–44]. Pohl et al. [38], assaying 5′-nucleotidase at 30 °C, obtained specific activity values of 32 and 38, with a 17- and 19-fold purification. With both methods of preparation, the specific activity of the 5′-nucleotidase was significantly lower in membranes from the deficient rats. ATPase activity, when measured by inorganic phosphate release, was higher in deficient than in control membranes, but the difference was not statistically significant.

The lower specific activity of 5′-nucleotidase could be attributed in part to the lower degree of purification of the depleted membranes, i.e. 14-fold cf. 17-fold in the

TABLE I

ENZYME ACTIVITIES IN RAT LIVER PLASMA MEMBRANES PREPARED BY SUCROSE GRADIENT OR TWO-PHASE POLYMER METHODS. ENZYMES ASSAYS WERE DONE AT 30 °C. VALUES ARE MEAN ±S.D. NUMBERS IN PARENTHESES ARE THE NUMBER OF PREPARATIONS ANALYZED

| Enzyme | −essential fatty acid | | | | −essential fatty acid | | | |
|---|---|---|---|---|---|---|---|---|
| | Spec. act. ($\mu$mol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ | | Recovery (%) | Purification (RSA)* | Spec. act. $\mu$mol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ | | Recovery (%) | Purification (RSA)* |
| 5′-Nucleotidase | | | | | | | | |
| homogenate | 2.70 ± 0.50 | (6) | | | 2.21 ± 0.76 | (6) | | |
| plasma membranes | | | | | | | | |
| Ray | 36.8 ± 8.4 | (3) | | | 29.6 ± 5.7 | (3) | | |
| Lesko | 45.1 ± 6.3 | (6) | 9.0 | 17 | 31.2 ± 6.2 | (6) | 12 | 14 |
| ATPase | | | | | | | | |
| homogenate | 5.92 ± 1.25 | (6) | | | 7.55 ± 1.26 | (6) | | |
| plasma membranes | | | | | | | | |
| Ray | 50.4 | (1) | | | 69.8 ± 15.1 | (2) | | |
| Lesko | 57.8 ± 13.8 | (6) | 5.4 | 10 | 67.2 ± 22.5 | (6) | 6.1 | 9 |
| Alkaline phosphatase | | | | | | | | |
| homogenate | 0.14 ± 0.03 | (6) | | | 0.23 ± 0.08 | (6) | | |
| plasma membranes | | | | | | | | |
| Ray | 1.70 ± 0.69 | (3) | | | 1.20 ± 0.31 | (3) | | |
| Lesko | 1.43 ± 0.66 | (6) | 6.8 | 10 | 2.55 ± 0.89 | (6) | 8.7 | 11 |

* RSA is the ratio of specific activity of enzyme in the plasma membrane fraction to the specific activity in the homogenate.

TABLE II

MITOCHONDRIAL AND MICROSOMAL CONTAMINATION OF LIVER PLASMA MEMBRANE PREPARATIONS MADE BY THE TWO-PHASE POLYMER SYSTEM (LESKO ET AL.) ENZYME ASSAYS WERE DONE AT 30 °C. VALUES ARE THE MEAN ±S.D. THE NUMBERS IN PARENTHESES ARE THE NUMBER OF PREPARATIONS ANALYZED

| Enzyme | +essential fatty acid | | | | − essential fatty acid | | | |
|---|---|---|---|---|---|---|---|---|
| | Spec. act. $\mu$mol $\cdot mg^{-1}$ protein $\cdot h^{-1}$ | | Recovery (%) | Contamination (%) | Spec. act. $\mu$mol $\cdot mg^{-1}$ protein $\cdot h^{-1}$ | | Recovery (%) | Contamination (%) |
| Glucose-6-phosphatase | | | | | | | | |
| homogenate | 5.77 ± 0.82 | (6) | | | 7.50 ± 1.1 | (6) | | |
| plasma membranes | 3.72 ± 1.30 | (6) | 0.4 | 4 | 5.16 ± 1.9 | (6) | 0.5 | 4 |
| Succinic-cytochrome reductase | | | | | | | | |
| homogenate | 0.026 ± 0.010 | (6) | | | 0.022 ± 0.011 | (6) | | |
| plasma membranes | 0.015 ± 0.015 | (6) | 0.5 | 5 | 0.012 ± 0.005 | (6) | 0.6 | 6 |

controls, if the ratio of the specific activity of the membrane preparation to the homogenate specific activity is used to calculate purification. However, when allowance is made for lower purification, the calculated specific activity of 5′-nucleotidase in the deficient membranes remains lower than in control membranes (37.0 cf. 45.1 μmol/mg protein/h). Chandrasekhara and Ananth Narayan reported a 50 % decrease in 5′-nucleotidase or only a 7-fold purification in the deficient liver plasma membranes, in contrast to a 13-fold purification in liver plasma membranes of the non-deficient rats. Based on ATPase activity (measured by inorganic phosphate release), their purification was about 6-fold with both deficient and control preparations.

Fig. 1 shows the slab gel electrophoresis patterns for membranes prepared by the two methods. Pairs of one deficient and one control rat were sacrified together, and the membranes were prepared under identical conditions, i.e., lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6. These preparations were made from rats fed the experimental diets for 4 months.

With either method of preparation, the patterns of the membranes from deficient rats were very similar to the patterns from the control rats. The bands observed in the control membranes were found in the deficient membranes, although some bands in the deficient preparation stained more intensely. However, it is obvious that the two methods of preparation did not give identical patterns. Membranes made by the two-phase polymer method showed more bands at the front of the gel. The nature

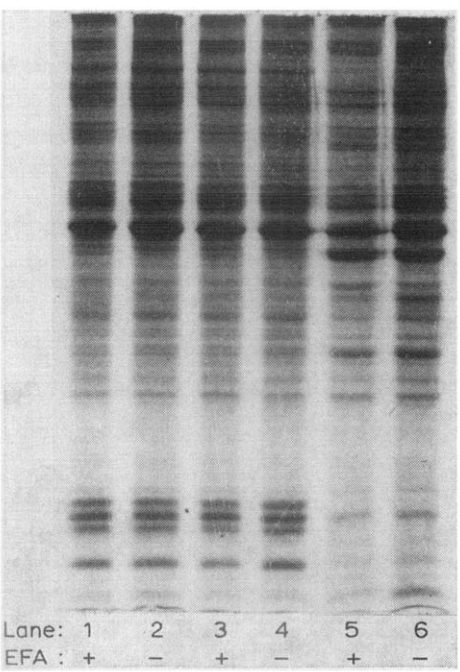


Fig. 1. Slab gel electrophoresis patterns of liver plasma membranes prepared from essential fatty acid-deficient (EFA−) and control rats. Lanes 1–4 represent preparations made by the two-phase polymer method. Lanes 5–6 are preparations made by the sucrose gradient method. Lanes 1, 3 and 5 are essential fatty acid-supplemented (EFA+) control preparations. Lanes 2, 4 and 6 are the corresponding essential fatty acid-depleted preparations made at the same time.

and significance of these lower molecular weight bands are unknown, but this difference in electrophoretic pattern did not measurably affect the specific activity of the enzymes considered as "marker" enzymes for liver plasma membranes. Since the two-phase polymer method appeared satisfactory for the plasma membrane preparation from both deficient and supplemented rats, this method was used subsequently because of its rapidity and greater yield of membranes.

*Fatty acid composition of liver plasma membrane phospholipids.* The glycerophospholipid fractions from deficient rats all showed the expected decreases in the proportions of linoleate and arachidonate (Table III). The greatest difference between deficient and control preparations in archidonate was in phosphatidylcholine, and the least difference was in phosphatidylserine. In preparations from deficient rats, the proportions of arachidonate still present in phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were definitely higher than in phosphatidylcholine. The values for arachidonate were approximately the same for phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol for control preparations, i.e. 33 %, 37 %, and 37 %.

TABLE III

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE LIPIDS*

| Lipid class | EFA | Fatty acid, % by weight | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 14 : 0 | 16 : 0 | 16 : 1 | 18 : 0 | 18 : 1 | 18 : 2 | 20 : 3 | 20 : 4 |
| Total lipids | + | 1.0 | 21.0 | 2.9 | 25.2 | 11.4 | 10.5 | – | 26.0 |
| | | ±0.3 | ± 1.8 | ±1.0 | ± 3.0 | ± 1.3 | ± 1.8 | | ± 1.4 |
| | – | 1.2 | 19.8 | 5.8 | 23.3 | 19.5 | 1.6 | 15.7 | 7.0 |
| | | ±0.2 | ± 2.0 | ±1.3 | ± 2.4 | ± 2.4 | ± 0.4 | ± 2.8 | ± 3.0 |
| Phosphatidyl-choline | + | 0.6 | 24.2 | 3.3 | 18.9 | 10.8 | 10.8 | – | 27.2 |
| | | ±0.8 | ± 3.4 | ±0.2 | ± 3.0 | ± 1.3 | ± 1.0 | | ± 2.5 |
| | – | 0.7 | 21.2 | 7.2 | 18.9 | 21.8 | 1.8 | 19.0 | 3.4 |
| | | ±0.1 | ± 0.8 | ±0.2 | ± 2.5 | ± 4.5 | ± 0.2 | ± 1.1 | ± 0.6 |
| Phosphatidyl-ethanolamine | + | 0.5 | 17.2 | 1.5 | 26.3 | 7.6 | 5.9 | – | 33.3 |
| | | ±0.5 | ± 1.4 | ±0.4 | ± 1.8 | ± 1.6 | ± 1.2 | | ± 4.0 |
| | – | 0.6 | 15.4 | 3.3 | 23.2 | 13.0 | 1.1 | 25.1 | 11.2 |
| | | ±0.3 | ± 3.1 | ±0.5 | ± 1.5 | ± 3.1 | ± 0.2 | ± 4.8 | ± 4.3 |
| Phosphatidyl-serine | + | 0.5 | 8.9 | 3.3 | 38.9 | 4.2 | 3.2 | – | 36.7 |
| | | ±0.4 | ± 2.3 | ±4.0 | ± 6.7 | ± 0.3 | ± 0.9 | | ± 5.9 |
| | – | 0.5 | 8.0 | 2.0 | 39.3 | 6.0 | 0.8 | 17.6 | 18.0 |
| | | ±0.2 | ± 1.0 | ±0.4 | ± 2.7 | ± 0.6 | ± 0.2 | ± 7.5 | ± 7.6 |
| Phosphatidyl-inositol | + | 0.8 | 9.8 | 1.0 | 36.6 | 5.2 | 2.5 | – | 37.2 |
| | | ±0.4 | ± 3.5 | ±0.2 | ± 2.0 | ± 1.2 | ± 2.0 | | ± 3.6 |
| | – | 1.6 | 8.3 | 2.0 | 33.3 | 7.4 | 0.8 | 27.7 | 9.6 |
| | | ±1.3 | ± 3.3 | ±0.9 | ± 4.7 | ± 1.9 | ± 0.2 | ± 3.1 | ± 6.6 |

* Average and S.D. of three membrane preparations, except for total lipids which are from five preparations. EFA = essential fatty acid. Minor fatty acids or fatty acids with retention times greater than 20 : 4 have been omitted because these measurements were less reliable due to the limited amounts of membrane available for analysis. The data show clearly that the rats were essential fatty acid-deficient, as indicated by the decrease in 18 : 2 and 20 : 4 and the increases in 20 : 3.

*ATPase*. Since ATPase activity in deficient membrane preparations tended to be higher than in controls, as assayed by inorganic phosphate release, we wondered whether a significant increase in phosphate release from ATP might be obscured by variations in phosphate released from hydrolysis of the ADP formed in the reaction. Formation of AMP by ADPase action would also provide substrate for 5′-nucleotidase activity, which was higher in the control membranes. ADPase activity has been detected in liver plasma membranes [45]. Assay of inorganic phosphate released from ADP showed somewhat more activity in control membranes, i.e. 0.85 $\mu$mol $P_i$/min/mg protein for control cf. 0.50 for the deficient membranes. Therefore, it was possible that the higher 5′-nucleotidase activity, coupled with an equivalent ADPase activity, could elevate $P_i$ release from ATP by control membranes, even though the ATPase activity of deficient membranes might be higher.

Consequently, we assayed ATPase by the coupled NADH oxidation method [28]. This method has the following advantages over methods based on $P_i$ formation: (a) formation of ADP is recorded continuously, and a linear time course is obtained as long as NADH and phosphoenolpyruvate are in excess; (b) the hydrolyzed ATP is regenerated, and the initial velocity at any substrate concentration is maintained throughout the reaction until NADH becomes limiting. The coupled enzymatic assay is therefore less sensitive to the action of enzymes releasing inorganic phosphate from ADP or AMP. Some ATP could be hydrolyzed by adenylate cyclase, but the activity of this enzyme in liver plasma membranes is much less ($\approx$ 1 %) than that of ATPase.

ATPase assay by the coupled oxidation method showed that the activity was significantly higher in the essential fatty acid-deficient membranes. Estimation of $V$ and apparent $K_m$ by Lineweaver-Burk plots showed that these values were significantly greater for membrane preparations from deficient rats in comparison with membranes from paired control rats of the same age (Table IV). When deficient rats were fed on the 5 % corn oil diet for one week, the $V$ and $K_m$ values decreased to the levels obtained with membranes from control rats of the same age.

TABLE IV

$K_m$ (mM ATP) AND $V$ ($\mu$MOL ADP/MG PROTEIN/MIN) FOR TOTAL ATPase ACTIVITY OF SIX PAIRED PREPARATIONS OF LIVER PLASMA MEMBRANES FROM CONTROL AND ESSENTIAL FATTY ACID-DEFICIENT RATS. EACH PAIR OF CONTROL AND DEFICIENT RATS WERE SACRIFICED AT THE SAME TIME

| Pair | Control membranes | | Deficient membranes | |
|---|---|---|---|---|
| | $K_m$ | $V$ | $K_m$ | $V$ |
| 1 | 0.37 | 0.85 | 0.67 | 1.36 |
| 2 | 0.32 | 1.25 | 0.69 | 1.86 |
| 3 | 0.37 | 1.36 | 0.48 | 1.68 |
| 4 | 0.36 | 1.46 | 0.46 | 1.81 |
| 5 | 0.45 | 1.80 | 0.55 | 2.50 |
| 6 | 0.36 | 1.08 | 0.55 | 2.50 |
| Mean | 0.37 | 1.30 | 0.57* | 1.95** |
| ±S.E. | 0.018 | 0.33 | 0.038 | 0.45 |

* $P < 0.005$; ** $P < 0.05$.

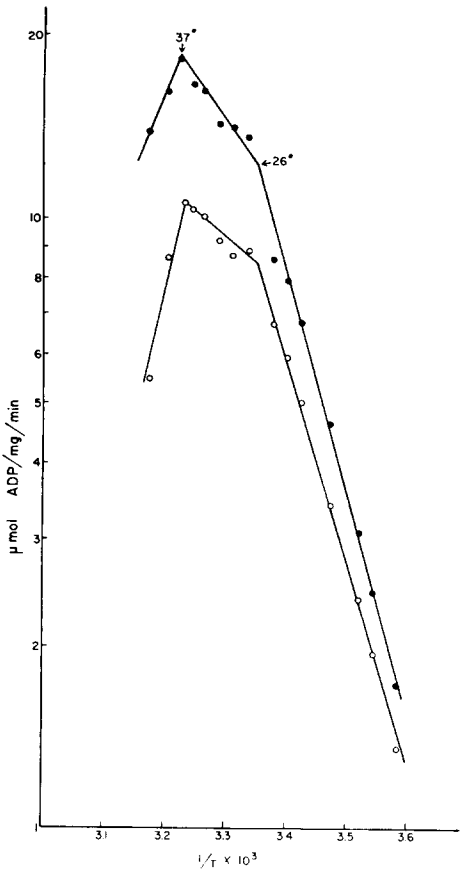


Fig. 2. Arrhenius plot for ATPase activity of liver plasma membranes from essential fatty acid-deficient and control rats. Units of activity are $\mu$moles of ADP formed per min per mg of membrane protein. ●–●, liver plasma membranes from deficient rats; ○–○, liver plasma membranes from control rats.

Arrhenius plots of ATPase activity from 6 to 43 °C were almost identical for the essential fatty acid-depleted and control membranes (Fig. 2). Both plots showed a break at about 26 °C, maximum activity at 37 °C, and decreased activity at higher temperatures. At all temperatures, the essential fatty acid-depleted membranes showed a higher activity. Maximum activity between 35–40 °C has been reported previously for ATPase of rat liver plasma membranes [46, 47].

*Adenylate cyclase.* Adenylate cyclase activity was measured after stimulation by $F^-$, glucagon or glucagon plus GTP in the presence of 4 mM $Mg^{2+}$ and 1.25 mM ATP (Table V). The specific activity with these activators remained lower in the liver plasma membranes from the essential fatty acid-deficient rats ($P < 0.001$ for $F^-$; $< 0.01$ for glucagon; $< 0.05$ for glucagon plus GTP). However, the relative activation (increase over basal) of adenylate cyclase by $F^-$, glucagon or glucagon plus GTP was similar in both deficient and control membranes. Glucagon plus GTP produced greater activation than glucagon alone with both deficient and control

TABLE V

ADENYLATE CYCLASE ACTIVITY IN LIVER PLASMA MEMBRANES FROM ESSENTIAL FATTY ACID-SUPPLEMENTED OR DEFICIENT RATS AFTER ACTIVATION BY $F^-$, GLUCAGON OR GLUCAGON PLUS GTP. MEAN AND S.E. SIX PREPARATIONS/GROUP.* EFA = ESSENTIAL FATTY ACID. RESULTS GIVEN IN PMOL CYCLIC AMP FORMED PER MIN/MG PROTEIN

| | Addition | | | |
|---|---|---|---|---|
| Treatment | Basal | +NaF | +glucagon | +GTP+glucagon |
| +EFA | 30±3 | 92±7 | 72±4 | 111±11 |
| −EFA | 13±2 | 46±5 | 40±6 | 57±7 |

* NaF, 10 mM; glucagon, $10^{-6}$ M; GTP, $10^{-4}$ M

TABLE VI

GLUCAGON CONCENTRATION M AND ACTIVATION OF ADENYLATE CYCLASE IN LIVER PLASMA MEMBRANES FROM ESSENTIAL FATTY ACID-SUPPLEMENTED OR ESSENTIAL FATTY ACID-DEFICIENT RATS. MEAN AND S.E. FIVE LPM PREPARATIONS PER GROUP. EFA = ESSENTIAL FATTY ACID. RESULTS GIVEN IN PMOL CYCLIC AMP FORMED PER MIN/MG PROTEIN

| Treatment | 0 | $10^{-9}$ | $10^{-8}$ | $10^{-7}$ | $5 \cdot 10^{-7}$ | $10^{-6}$ |
|---|---|---|---|---|---|---|
| +EFA | 36±7 | 57±8 | 66±9 | 75±9 | 84±8 | 96±12 |
| −EFA | 16±5 | 32±10 | 50±4 | 60±6 | 66±6 | 72±5 |

membranes. A level of 1.25 mM ATP was used in the assay system because preliminary experiments showed that maximum activity with both deficient and control membranes occurred at this ATP concentration in the presence of 10 mM $F^-$ and 4 mM $Mg^{2+}$, concentrations previously used [8, 29] for measurement of adenylate cyclase of liver plasma membranes. (Maximum activity with 10 mM $F^-$, 4 mM $Mg^{2+}$ and 1.25 mM ATP agrees with other results at similar concentrations [48]). In other experiments, $Mg^{2+}$ concentration was increased from 4 to 6 mM in the presence of 1.25 mM ATP and 10 mM $F^-$, but the activity of deficient membranes did not change and was still lower than that of controls. When $Mg^{2+}$ was increased to 8 mM, the activity of deficient membranes increased slightly, but the activity of controls decreased.

Dose-response curves to glucagon with both deficient and control membranes (Table VI) showed that activity increased as the glucagon concentration increased from $10^{-9}$ to $10^{-6}$ M. However, at all concentrations tested, the specific activity in the deficient membranes was lower than in controls. The glucagon concentration for one-half maximum stimulation of activity was 6.5±2.7 nM for membranes from deficient rats and 3.5±2.7 nM for controls (mean±S.E.)

## DISCUSSION

As Neville [49] has pointed out, each isolation procedure for plasma membranes is essentially a selection procedure. Therefore, similar degrees of purification

with two different preparation methods would be evidence against the possibility that one method was selecting preferentially for a certain population of membrane fragments. This problem is especially important in attempts to isolate membranes with altered structural components. Membranes with such alterations could respond differently to isolation procedures which were developed for "normal" membranes, and different populations of membrane fragments might then be isolated from the "altered" membranes. Our results show that the two-phase polymer method and the sucrose gradient method gave a comparable purification of marker enzymes for liver plasma membranes from both essential fatty acid-deficient rats and controls, as indicated by the ratio of specific activity in the membrane preparation to specific activity in the homogenate. The degree of purification is similar to that reported by others [38–44].

Although membrane purification, as indicated by marker enzymes, was similar for both methods of purification, the gel electrophoresis patterns show clearly that the membrane preparations made by the two methods were not identical. Nevertheless, with either method of purification, the gel patterns from deficient and control rats were very similar. This indicates that either method isolated corresponding populations of membrane fragments from both types of animal and strengthens the conclusion that the differences in enzyme activity indicate valid enzymatic differences as a result of essential fatty acid deficiency, rather than isolation of different populations of membrane fragments varying in enzyme complement.

The pattern of total membrane fatty acids in control rats agrees well with values reported by Gerson [50] for rats fed a purified diet supplying 4 % corn oil. The values for the membranes from the essential fatty acid-deficient rats are comparable to those reported for deficient rats fed a purified fat-free diet [50]. The more rapid depletion of arachidonate from phosphatidylcholine than from phosphatidylethanolamine is well established during essential fatty acid deficiency [36, 51]. The phospholipid fatty acid patterns in our control membranes are quite like those reported by Van Hoeven et al. [52] for liver plasma membranes from rats fed a chow-type diet.

The significance of the decreased 5′-nucleotidase activity in deficient membranes is not yet known, as the function of this enzyme in plasma membranes is still undefined [14, 53]. Decreased 5′-nucleotidase activity has been reported in brain homogenates from essential fatty acid-deficient rats [17] and in liver plasma membranes from hypophysectomized rats [54], but the degree of membrane purification was not reported for either the hypophysectomized rats or the normal rats. Alterations in pituitary function have been reported in essential fatty acid deficiency [55]. Consequently, the decreased 5′-nucleotidase activity in the deficient membranes might result from altered pituitary function, perhaps in response to altered prostaglandin production, as well as from changes in membrane fatty acid composition.

The Arrhenius plot of ATPase activity showed a discontinuity at $\approx 26\,^{\circ}C$ for both deficient and control membranes. This discontinuity is not an artifact produced by variation in pH of the medium at different temperatures since we adjusted the pH to 7.5 at each temperature. Such an adjustment may be necessary to avoid creation of artifacts in studying the temperature dependence of enzyme activity. Boldyrev and coworkers [56] detected no discontinuities in Arrhenius plots of $Mg^{2+}$-ATPase or $(Na^{+}+K^{-})$-ATPase of rabbit sarcolemma preparations when the pH was adjusted at each temperature. However, a break occurred in the $(Na^{+}+K^{+})$-ATPase activity when the pH was adjusted only at room temperature before the start of the

assays. Several inflections have been detected in Arrhenius plots of ($Na^+ + K^+ + Mg^{2+}$)-ATPase in plasma membrane preparations from mouse LM cells [57]. However, we were able to detect only one discontinuity with rat liver plasma membrane preparations under our conditons of measurement.

The ATPase activity of liver plasma membranes is chiefly $Mg^{2+}$-ATPase [3–5, 22, 23]. Quite wide variaton has been reported in the proportion of ($Na^+ + K^+$)-ATPase, i.e. from 10 to 33 %, as has been noted by Emmelot and Bos [3, 4]. We have not yet been able to determine whether essential fatty acid deficiency has altered the proportion of ($Na^+ + K^+$)-ATPase because of problems in estimating ($Na^+ + K^+$)-ATPase [58], especially in preparations of low activity such as liver plasma membranes. The major part of the ATPase activity in membranes from both deficient and control rats was ouabain-insensitive $Mg^{2+}$-ATPase, and the specific activity was higher in deficient rats. It remains to be established whether the increased ATPase activity in deficient membranes results from altered membrane fatty acid composition or whether altered pituitary function is involved [55]. Increased $Mg^{2+}$-ATPase activity has been reported for liver plasma membranes from hypophysectomized rats [54].

The lower adenylate cyclase activity in membranes from deficient rats is especially interesting because phospholipids are invclved in the hormonal activation of the enzyme system. The lower adenylate cyclase activity in the presence of $F^-$, glucagon, or glucagon plus GTP could result if (a) the deficient membranes consisted of a different population of membrane fragments containing adenylate cyclase systems not identical with the systems isolated in control membranes; (b) the amount of enzyme protein were reduced in deficient membranes; and/or (c) altered fatty acid composition produced structural changes which modified the concentration of cofactors required for adenylate cyclase activation, perhaps because of decreased binding affinity or changes in the number of receptor sites.

Determination of the amount of adenylate cyclase protein is not yet possible because purification of the enzyme complex is difficult [59, 60]. Additional work is needed to determine to what extent the activity in the deficient membranes might be increased by altering concentrations of cofactors required for adenylate cyclase activation. Consequently, one should interpret cautiously the lower activation by $F^-$, glucagon or glucagon plus GTP with membranes from the essential fatty acid-deficient rats. Conditions permitting maximum adenylate cyclase activation for control membranes may not be optimal for membranes from the essential fatty acid-deficient rats. However, we have observed [61] that the rise in liver cyclic AMP after glucagon injection is less in essential fatty acid-deficient rats. Thus, the decreased adenylate cyclase activity in liver plasma membranes can be related to evidence for decreased activity in vivo.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grant AM12024-07. We are indebted to Inese Hincenbergs for care of the rats. Hydrogenated coconut oil was kindly supplied by Mr. Sherman Lee of Glidden-Durkee. Vitamin A, D and E preparations were the gift of Mr. Rene LeStreto, Nopco Chemical Company. We especially thank Dr. J. Tinoco for many helpful suggestions throughout these experiments.

REFERENCES


1 Schwarz, A., Lindenmayer, G. E. and Allen, J. C. (1975) Pharmacol. Rev. 27, 3–134
2 Wheeler, K. P., Walker, J. A. and Barker, D. M. (1975) Biochem. J. 146, 713–722
3 Emmelot, P. and Bos, C. J. (1966) Biochim. Biophys. Acta 120, 369–382
4 Emmelot, P. and Bos, C. J. (1968) Biochim. Biophys. Acta 150, 341–353
5 Réthy, A., Tomasi, V. and Trevisani, A. (1971) Arch. Biochem. Biophys. 147, 36–40
6 Roelofsen, B. and van Deenen, L. L. M. (1973) Eur. J. Biochem. 40, 245–257
7 Pfeuffer, T. and Helmreich, E. J. M. (1975) J. Biol. Chem. 250, 867–876
8 Birnbaumer, L. (1973) Biochim. Biophys. Acta 300, 129–158
9 Rubalcava, B. and Rodbell, M. (1973) J. Biol. Chem. 248, 3831–3837
10 Levey, G. S. (1975) Metabolism 24, 301–310
11 Kimelberg, H. K. and Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 282, 277–292
12 Kimelberg, H. K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071–1080
13 Jarett, L. and Smith, R. M. (1974) J. Biol. Chem. 249, 5195–5199
14 Pommier, G., Ripert, G., Azoulay, E. and Depieds, R. (1975) Biochim. Biophys. Acta 389, 483–494
15 Guarnieri, M. and Johnson, R. M. (1970) Advances in Lipid Research (Paoletti, R. and Kritchevsky, D., eds.), Vol. 8, pp. 115–174, Academic Press, New York, N.Y.
16 Sun, G. Y. and Sun, A. Y. (1973) J. Neurochem. 22, 15–18
17 Bernsohn, J. and Spitz, F. J. (1973) Biochem. Biophys. Res. Commun. 57, 293–298
18 Farias, R. N. Goldemberg, A. L. and Trucco, R. E. (1970) Arch. Biochem. Biophys. 139, 38–44
19 Chandrasekhara, N. and Ananth Narayan, K. (1970) J. Nutr. 100, 477–480
20 Greenfield, H. and Briggs, G. M. (1971) Annu. Rev. Biochem. 40, 549–572
21 Williams, M. A., Tamai, K. T., Hincenbergs, I. and McIntosh, D. J. (1972) J. Nutr. 102, 847–856
22 Ray, T. (1970) Biochim. Biophys. Acta 196, 1–9
23 Lesko, L. Donlon, M., Marinetti, G. V. and Hare, J. (1973) Biochim. Biophys. Acta 311, 173–179
24 Ames, G. F. L. (1974) J. Biol. Chem. 249, 634–644
25 Laemmli, U. K. (1970) Nature 227, 680–685
26 Solyom, A. and Trams, E. G. (1972) Enzyme 13, 329–372
27 Harper, A. E. (1963) in Methods of Enzymatic Analysis, (Bergmeyer, H. U. ,ed.), p. 789, Academic Press, New York, N.Y.
28 Barnett, R. E. (1970) Biochemistry 9, 4644–4648
29 Maguire, M. and Gilman, A. G. (1974) Biochim. Biophys. Acta 358, 154–163
30 Oka, H., Kaneko, T., Yamashita, K., Susuki, S. and Oda, T. (1973) Endrocrinol. Japon 20, 263–270
31 Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305–312
32 Hübscher, G. and West, G. R. (1965) Nature 205, 800–801
33 Tisdale, H. D. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. 10, Academic Press, New York, N.Y.
34 Newkirk, J. D. and Waite, M. (1971) Biochim. Biophys. Acta 225, 224–233
35 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
36 Stancliff, R. C., Williams, M. A., Utsumi, K. and Packer, L. (1969) Arch. Biochem. Biophys. 131, 629–642
37 Skipski, V. P., Peterson, R. F. and Barclay, M. (1964) Biochem. J. 90, 374–377
38 Pohl, S. L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849–1856
39 Lauter, C. J., Solyom, A. and Trams, E. G. (1972) Biochim. Biophys. Acta 266, 511–523
40 Solyom, A., Lauter, C. J. and Trams, E. G. (1972) Biochim. Biophys. Acta 274, 631–637
41 Dorling, P. R. and LePage, R. N. (1973) Biochim. Biophys. Acta 318, 33–44
42 Wong, N. and Zull, J. E. (1974) Biochim. Biophys. Acta 352, 52–63
43 Nigam, V. N., Morai, R. and Karasaki, S. (1971) Biochim. Biophys. Acta 249, 34–40
44 Chandrasekhara, N. and Ananth Narayan, K. (1970) Life Sci. 9, pt. II, 1327–1334
45 Raftell, M. and Blomberg, F. (1973) Biochim. Biophys. Acta 291, 421–430
46 Emmelot, P. and Bos, C. J. (1968) Biochim. Biophys. Acta 150, 354–363
47 Lutz, F. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 615–616

48 Drummond, G. I., Severson, D. L. and Duncan, L. (1971) J. Biol. Chem. 246, 4166–4173
49 Neville, Jr., D. M. and Kahn, C. R. (1974) in Methods in Molecular Biology (Laskin, A. I. and Last, J. A., eds.), Vol. 5, pp. 57–88, Marcel Dekker, New York, N.Y.
50 Gerson, T. (1974) J. Nutr. 104, 701–709
51 Williams, M. A., Katyare, S. and Packer, L. (1975) Arch. Biochem. Biophys. 170, 353–359
52 Van Hoeven, R. P., Emmelot, P., Krol, J. H. and Oomen-Meulemans, E. P. M. (1975) Biochim. Biophys. Acta 380, 1–11
53 Evans, W. H. and Gurd, J. W. (1973) Biochem. J. 133, 189–199
54 Rubin, M. S., Swislocki, N. I. and Sonenberg, M. (1973) Arch. Biochem. Biophys. 157, 243–251
55 Roland, D. A., Sr. and Edwards, Jr. H. M. (1971) J. Nutrition 101, 1683–1694
56 Boldyrev, A. A., Tkachuk, V. A. and Titanji, P. V. K. (1974) Biochim. Biophys. Acta 357, 319–324
57 Wisnieski, B. J., Parkes, J. G., Huang, Y. O. and Fox, C. F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4381–4385
58 Charnock, J. S., Almeida, A. F. and To, R. (1975) Arch. Biochem. Biophys. 167, 480–487
59 Ryan, J. and Storm, D. R. (1974) Biochem. Biophys. Res. Commun. 60, 304–311
60 Neer, E. J. (1974) J. Biol. Chem. 249, 6527–6531
61 Louis, S. L., Brivio-Haugland, R. P. and Williams, M. A. (1975) J. Supramol. Struct., in the press