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PHOSPHOLIPID COMPOSITION OF DYSTROPHIC CHICKEN ERYTHROCYTE PLASMALEMMAE

II. CHARACTERIZATION OF A UNIQUE LIPID FROM DYSTROPHIC ERYTHROCYTE MEMBRANES AS ETHANOLAMINE PLASMALOGEN

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The phospholipid content of normal (line 412) and dystrophic (line 413) chicken erythrocyte plasmalemmae has been quantified on a developmental basis using sex matched controls. A specific minor phospholipid component, ethanolamine plasmalogen, is identified from dystrophic erythrocyte membrane preparations. To arrive at this identification, data from studies utilizing gas-liquid chromatography, thin-layer chromatography, [^{14}C]ethanolamine incorporation, and biochemical assay for specific organic moieties were correlated. This phospholipid has the potential to alter and regulate membrane fluidity and thus membrane function. The possible presence of significant concentrations of plasmalogen in human dystrophic tissues may serve as a marker for dystrophy and thus be of clinical importance.

Introduction

In the ccompanying article [21], significant differences in phospholipid content are noted between normal and dystrophic chicken erythrocyte membrane preparations. A unique phospholipid adjacent to phosphatidylethanolamine is observed exclusively on thin-layer chromatography (TLC) plates spotted with dystrophic membrane preparations. In this study, utilizing biochemical, biophysical and physiological techniques, this unknown polar lipid is identified as ethanolamine plasmalogen.

Plasmalogens (1-alkyl,1′-enyl,2-acyl lipids) are a class of phospholipids that have a fatty acid bonded to the α carbon of the glycerol moiety through a vinyl ether bond instead of the usual ester bond [1]. The β carbon of the plasmalogen glycerol moiety has an ester bond linking it to the fatty acid. The ether bonded group is relatively saturated predominating in 16 : 0, 18 : 0, and 18 : 1 fatty acyl chains [2]. The second fatty acid position is filled with acyl compounds that are predominantly unsaturated, particularly those with a double bond at the 12, 13 carbon positions [2]. Plasmalogens may have either choline or ethanolamine as headgroups.

Plasmalogens are found in the membranes of mammals, marine fish, molluscs, protozoans, and possibly in higher plants [2]. Also, plasmalogens are identified in several avian tissues, including chicken sciatic nerve, brain, liver and muscle [3,4]. High percentages of plasmalogens are found in the myelin sheaths of the central nervous system of mammals [2]. A decreased plasmalogen content is noted in two animal models for demyelinating diseases, the jimpy mouse and the quaking mouse [5].

Membrane fluidity can influence the enzymatic

activity of proteins embedded in the membrane as well as ion transport through the membrane [6]. Ether phospholipids are postulated to participate in the regulation of membrane fluidity and therefore an altered plasmalogen concentration may lend to anomalies in membrane function or integrity [7].

Methods and Materials

Source and care of chickens (Gallus domesticus). One-day-old dystrophic line 413 (early onset, genetically homozygous) and normal line 412 chickens were obtained from the Department of Avian Sciences at the University of California, Davis. All chickens were males. They were maintained in our university facilities at an ambient temperature of 25.5°C under a 12/12 hour light cycle. The chickens were provided with food and water ad libitum.

Isolation of phospholipids from erythrocyte plasmalemmae. Plasmalemmae were prepared and the phospholipids were isolated in the presence of butylated hydroxytoluene according to the procedures in the accompanying article. Phospholipids were prepared for further analyses by transferring the silica gel from those areas of the TLC plate corresponding to visualized phospholipids to separatory funnels containing chloroform/methanol (10 : 10, v/v). 9 ml of distilled water were then added. After vigorous shaking, the lower layer was separated and the procedure was repeated two more times. The phospholipid extract was stored under nitrogen at −70°C.

Plasmalogen quantitation [8]. When an ether bond is cleaved under severe hydrolytic conditions, a long chain aldehyde will be formed. Ester bonds will liberate a carboxyl moiety upon hydrolysis. Aldehydes will react with 2,4-dinitrophenylhydrazine to form a hydrazone complex which can be detected at 390 nm. This reaction is specific for aldehydes of chain length greater than C equals 6 [9].

1.6 ml of 95% ethanol were added to dried lipid samples. 0.2 ml of freshly prepared 0.02 M *p*-nitrophenylhydrazine in 95% ethanol and 0.2 ml 0.5 M sulfuric acid were added to the test tubes. The mixture was heated at 70°C for 20 min and then cooled on ice. Then, 1.0 ml of double distilled water and 2.0 ml of *n*-hexane were added to the mixture and shaken. The hexane layer was removed and washed two times with 2.0-ml aliquots of water. The extract was centrifuged at $500 \times g$ for 5 min. The hexane solution was evaporated almost to dryness under N_2. To the sample, 3.5 ml of 95% ethanol were added. The solution was analyzed spectrophotometrically for long chain aldehyde dinitrophenylhydrazone complexes at 390 nm. For determination of the endogenous aldehyde concentration in each sample, the H_2SO_4 was added after the solution was cooled following the 70°C incubation [10]. The absence of the acid during the reaction prevented degradation of the phospholipids and their subsequent release of long chain fatty aldehydes into the solution. A calibration curve was generated using plasmalogen obtained from Applied Science Corporation as a standard.

[^{14}C]Ethanolamine incorporation into whole cells [11]. The plasma was removed from blood samples of normal and dystrophic chickens by centrifugation at $500 \times g$ for 5 min. The packed cells were resuspended in an equal volume of isotonic sample buffer consisting of 0.113 M dextrose, 0.016 M $MgCl_2$, 0.027 M sodium citrate, 0.003 M citric acid, 0.050 M NaCl, 0.010 M cysteine and 0.002 M adenosine triphosphate (ATP), pH 7.4. The buffer medium was oxygenated prior to use. 25 μl of [^{14}C]ethanolamine (95 mCi/mM) and 100 μl of cold ethanolamine (10 mg/ml) were added to the mixture. The cells were incubated in a constant temperature shaking bath at 39.5°C for 120 min under a constantly perfused oxygen atmosphere. The cells were lysed, and the plasma membranes were prepared on ice. Lipids were separated by two-dimensional TLC and added to scintillation fluor (Bioscint, Amersham). The isolated phospholipid samples were counted in a Beckman liquid scintillation counter. The data were corrected for background and quench.

The TLC system consisted of chloroform/methanol/acetic acid (40 : 20 : 4, v/v) in the first dimension and chloroform/acetone/methanol/acetic acid/water (20 : 8 : 4 : 4 : 2, v/v) in the second dimension. Membrane samples also were analyzed for individual phospholipid content utilizing the reduction of 1-amine-2-naphthol-4-sulfonic acid.

Gas-liquid chromatography. 1 ml of 0.5 M HCl in anhydrous methanol was added to dried lipid samples under nitrogen. The methanol was stored over anhydrous sodium sulfate. The sealed tubes were heated at 75°C for 1 h under nitrogen and subsequently cooled. Then, 2.0 ml of petroleum ether were added and the mixture was shaken. The esters were extracted twice with petroleum ether and the methyl ester extracts were combined. The solvent of the extracts was evaporated under nitrogen, and a few drops of benzene were added. The vials were stored under nitrogen.

A preparative procedure resulted in the preparation of methyl esters only from phospholipids containing a glycerol moiety. Methyl ester moieties from sphingosine derivatives were not produced by this procedure. To dried lipid extracts, 5.0 ml of 10% (w/v) KOH in an 80% aqueous methanol solution were added. The tubes were flushed with nitrogen and heated at 37°C for 1 h. The solutions were transferred to separatory funnels, and two volumes of diethyl ether were added to the funnels. The lower phase was removed and then re-extracted with two volumes of diethyl ether. The lower phase was acidified by addition of 1 M HCl and extracted twice more with diethyl ether. Subsequently, the upper (ether) phase was kept. The combined ether extracts were dried over anhydrous Na_2SO_4 in a rotary evaporator. The dried extracts were weighed. The samples were dissolved in HCl/methanol and methyl esters were prepared as above.

The prepared methyl esters were spotted (2 μl) and eluted on a Varian Aerograph Gas Chromatograph (series 2100) using a H1-EFF-1-BP 15% diethyl glycol succinate column obtained from Applied Sciences. A calibration curve of relative retention times of various fatty acid methyl esters was prepared. The weights of eluted methyl ester peaks were obtained by cutting out observed peaks and weighing on a Perkin-Elmer AD_2 Automicrobalance. The amount of methyl ester applied to the column was calculated using a measured amount of methyl stearate as a standard. The polar glycerol moieties that were extracted and stored in the preparation of fatty acyl methyl esters were utilized in subsequent experiments. These glycerol moieties were frozen at −70°C under nitrogen. They were later spotted and run on two-dimensional TLC plates.

Solvent system 1 consisted of chloroform/methanol/acetic acid (40 : 20 : 4, v/v) in the first dimension and chloroform/acetone/methanol/acetic acid/water, (20 : 8 : 4 : 4 : 2, v/v) in the second dimension. Solvent system 2 consisted of 30 ml of a stock solution containing 0.0125 M EDTA and 1.65 M NH_4HCO_3 adjusted to pH 9.0 with concentrated NH_4OH and 70 ml of 95% ethanol in the first dimensoin and isobutyric acid/water/NH_4OH (66 : 33 : 1, v/v) in the second dimension [12].

Results

Studies to identify and characterize a minor phospholipid membrane component of dystrophic erythrocyte plasmalemmae

Standard. The following standards were run on the two-dimensional thin-layer chromatography system: lysophosphatidyl compounds, cerebrosides, gangliosides, and glycerol esters of serine and ethanolamine. These compounds did not co-chromatograph with the unknown lipid, isolated from TLC plates spotted with dystrophic erythrocyte membrane preparations.

Gas-liquid chromatography. Two different techniques for the preparation of methyl esters of fatty acyl chains were utilized. Similar results were noted for a technique that prepared methyl esters from all types of phospholipids including sphingolipids and another procedure that prepared methyl esters only from phospholipids that contained a glycerol backbone. After methyl ester preparation, it was estimated that only 67% of the fatty acyl chains from the unknown phospholipid were recovered. This value of 67% was determined by comparing the concentration of phospholipid subjected to methyl ester formation and the concentration of fatty methyl esters produced by that process (i.e., as determined by the summation of peak areas recorded from the GLC column). Known concentrations of phosphatidylethanolamine samples subjected to methyl ester formation were used as controls. It was assumed that 100% of all esters applied to the column was recovered from that column since the column ran well past the point at which long chain unsaturated fatty methyl esters elute from the column.

The gas-liquid chromatography data also revealed that the fatty acid profile for the unknown compound resembled a C_2 or beta carbon pattern (rich in polyunsaturates) (Fig. 1a). This was compared to the fatty acid profile for phosphatidylserine or phosphatidylethanolamine (Figs. 1b and 1c) which showed typical diacyl phospholipid composition (almost 50% saturated fatty acyl groups).

After the preparation of methyl esters for GLC, the remaining polar glycerol moieties were recovered and chromatographed on the polar (solvent system 1) two-dimensional TLC system. The unknown compound's glycerol moiety migrated to the position for lysophosphatidylethanolamine (Fig. 2a). The completely deacylated forms of phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine remained at the origin. Furthermore, on this TLC system, lysophosphatidylserine and lysophosphatidylcholine standards migrated to different positions from lysophosphatidylethanolamine. Also, when these deacylated glycerol moieties were run in the nonpolar solvent system (No. 2), the unknown's glycerol moiety migrated to the far upper right position of the plate (Fig. 2b). Glycerolphosphorylserine and glycerolphosphorylethanolamine migrated in this solvent system to other position, similar to those in the literature [12].

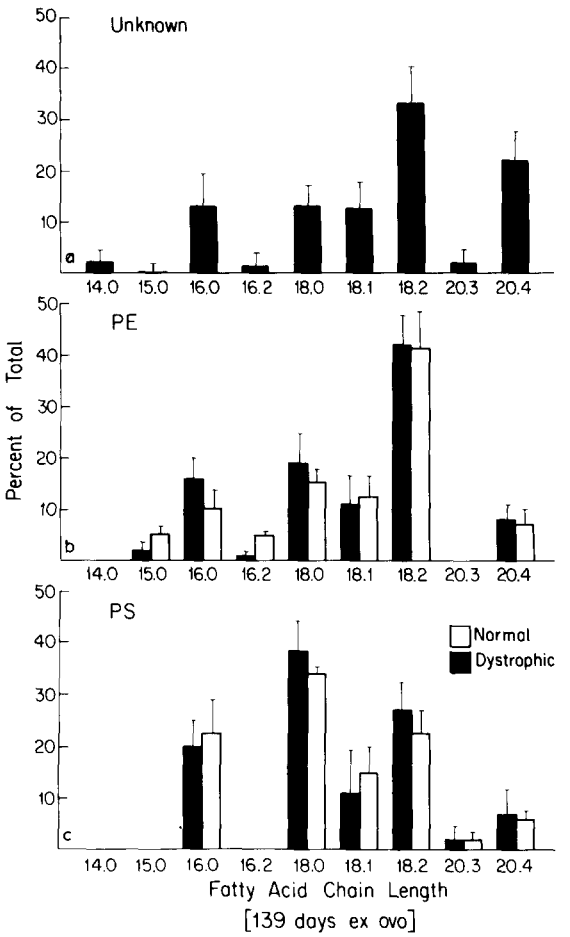

Fig. 1. Fatty acid composition. The fatty acyl profiles of the unknown lipid, phosphatidylserine and phosphatidylethanolamine from erythrocyte preparations are represented. $N = 7$ for these studies on 139 day ex ovo normal and dystrophic chickens. The data bars represent the mean ± S.D.

2,4-Dinitrophenylhydrazine reaction. A standard curve was generated relating plasmalogen content to the absorbance at 390 nm. The regression coefficient, r, for this curve was 0.97. Using this assay, it was determined that the unknown compound was rich in plasmalogen ether bonds while relatively little plasmalogen activity was evident in the TLC spots corresponding to phosphatidylethanolamine or phosphatidylserine.

[^{14}C]Ethanolamine incorporation studies. To determine the possible existence of ethanolamine in

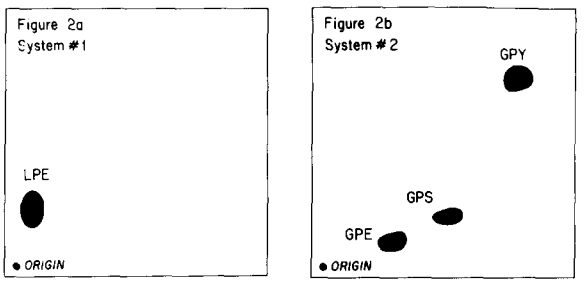

Fig. 2. Thin-layer chromatography of deacylated phospholipids. The upper chromatograph run in solvent solution 1 represents the relative position of the unknown compound's glycerol moiety after it had been deacylated through the GLC methyl ester procedure. This compound co-chromatographed with lysophosphatidylethanolamine. The lower chromatograph run in solvent system 2 represents the relative position of glycerol phosphoryl ethanolamine, glycerol phosphoryl serine and the deacylated unknown compound. LPE, lysophosphatidylethanolamine; GPE, glycerol phosphoryl ethanolamine; GPS, glycerol phosphoryl serine; GPY, deacylated unknown compound.

the polar head group of the unknown phospholipids, [^{14}C]ethanolamine-HCl was incorporated into membrane phospholipids under an atmosphere saturated with oxygen. For incorporation to occur, ethanolamine has to permeate the plasmalemmae, enter the microsomes, react with cytidine triphosphate to form cytidine diphosphate ethanolamine which could react with 1,2-diacylglycerol to form phosphatidylethanolamine. Phosphatidylethanolamine could then be transferred and incorporated into the plasmalemmae. Table Ia shows percent incorporation of radioactive label into membrane phospholipid. Most of the ^{14}C label was incorporated into phosphatidylethanolamine. Yet in the dystrophic erythrocyte, 3.4% of the total label was found within the unknown phospholipid. These data were also reported as specific activity measurements, μmole [^{14}C]ethanolamine/μmole phospholipid recovered (Table 1b). Approximately 10-times as much radioactivity is evident in the dystrophic phosphatidylethanolamine TLC spot compared with the unknown TLC spot. This corresponds with the ratio for phospholipid phosphorus content between these lipids (approx. 30% to 3%). Also, a decrease of at least two orders of magnitude for radioactive incorporation values was noted in the region on normal plates corresponding to the unknown, ninhydrin-positive lipid on dystrophic plates. The values for the [^{14}C]ethanolamine incorporation into the unknown compound between normal and dystrophic samples were significantly different from each other ($P < 0.005$).

Small amounts of radioactive label were noted in both normal and dystrophic phosphatidylcholine. This result was not unexpected, since phosphatidylethanolamine can be enzymatically methylated to form phosphatidylcholine. Almost no radioactivity was shown to reside in phosphatidylinositol or sphingomyelin. Values for the phosphatidylcholine/phosphatidylethanolamine ratios were 0.0086 in normal erythrocytes and 0.0081 in dystrophic erythrocytes.

Thin-layer co-chromatography. R_F values (X and Y axis) were determined from the co-chromatography on thin-layer plates of samples and standards of ethanolamine plasmalogen and phosphatidylethanolamine. Aliquots of ethanolamine plasmalogen from dystrophic chicken erythrocyte plasmalemmae (day 250 ex ovo), ethanolamine plasmalogen standard, phosphatidylethanolamine from normal and dystrophic chicken erythrocyte plasmalemmae (day 250 ex ovo) and phosphatidylethanolamine standard were run on two-dimensional thin-layer chromatography plates. The plasmalogen preparation had a greater X-axis R_F value than the phosphatidylethanolamine preparation or standard. Furthermore, the ethanolamine plasmalogen standard co-chromatographed with the plasmalogen sample preparation, and were not significantly different from each other.

TABLE I

[^{14}C]ETHANOLAMINE-HCl INCORPORATION INTO WHOLE CELLS

The cells were incubated in isotonic buffer under O_2 for 2 h at 39.5°C. $N = 7$ for these experiments on 150-day ex ovo birds (mean ± S.D.). These data were corrected for background radiation and quench.

	(a) % of [^{14}C] radioactivity recovered in membrane		(b) μmol [^{14}C]ethanolamine/μmol phospholipid recovered ($\times 10^{13}$)	
	Dystrophic	Normal	Dystrophic	Normal
Phosphatidylethanolamine	95.3	96.7	1040.0 ± 191.0	783.0 ± 71
Unknown	3.40	0.12	116.0 ± 45.5	3.42 ± 1.8
Phosphatidylcholine	0.80	0.90	7.96 ± 4.74	6.35 ± 1.18
Phosphatidylserine	0.50	0.90	3.75 ± 1.8	11.30 ± 5.69
Sphingomyelin	0.15	0.10	1.14 ± 0.64	2.91 ± 1.92
Phosphatidylinositol	0.12	0.10	1.27 ± 0.52	0.93 ± 0.85
Phosphatidic acid	0.15	0.07	1.14 ± 1.23	1.91 ± 1.88
Neutral lipid	0.37	0.70	1.74 ± 0.71	4.17 ± 1.71

Discussion

An unknown distinct polar lipid exclusively visualized on TLC plates spotted with dystrophic chicken erythrocyte plasmalemmae preparations has been identified as ethanolamine plasmalogen. Initially, the identification of the structure of ethanolamine plasmalogen was obtained by the preparations of methyl esters for gas-liquid chromatography. Since similar fatty acid profiles have been obtained from (i) a method that prepared methyl esters from all types of phospholipids, and (ii) a method that characterized phospholipids that contained only a glycerol backbone, it has been determined that the unknown compound is not a sphingolipid derivative. Furthermore, the unknown compound contains cleaved fatty acids which are rich in polyunsaturates. Fatty acid profiles for phosphatidylserine and phosphatidylethanolamine contain higher percentages of saturated chains which are typical of diacyl phospholipids. This observation suggests that for the unknown compound the fatty acids of the alpha position (predominantly saturated) are still quite possibly attached to the moelcule after methyl ester preparation and that there might be an altered bond linking the alpha fatty acid to the glycerol moiety in the unknown compound. Furthermore, the polar glycerol moiety derived from methyl ester preparation migrates to the lyso position for phosphatidylethanolamine in the polar two dimensional TLC eluent system. A totally deacylated glycerol moiety would remain at the origin. In a nonpolar TLC system, this polar glycerol moiety migrates to the far upper right of the plate. In this system, deacylated lipids move relatively slowly. These results lend support to the notion that the alpha substituent of the known compound is still attached to the glycerol moiety after methyl ester preparation. To account for these findings, it is probable that this altered alpha bond is an ether bond of a plasmalogen rather than the expected ester bond of a diacyl phospholipid. The ether bond of a plasmalogen remains stable on mild acid hydrolysis [13]. Therefore, upon mild methylation of the unknown compound, a lysophospholipid and a methyl ester result. A definitive test for the presence of an ether bond in the fatty acid moiety of a phospholipid is the 2,4-dinitrophenylhydrazine-long chain aldehyde assay [8]. The increased activity of the unknown compound in this assay compared with phosphatidylethanolamine and phosphatidylserine samples is direct evidence for the plasmalogen ether bond.

The polar glycerol moiety, derived as a by-product from methyl ester formation for the GLC procedures, migrates to the position for lysophosphatidylethanolamine on the polar two dimentional TLC system. Lysophosphatidylcholine and lysophosphatidylserine migrate to different positions in this TLC system. Moreover, the unknown compound runs adjacent to phosphatidylethanolamine on TLC plates and is also ninhydrin positive. It is, therefore, possible that the polar head group of the unknown compound is ethanolamine. Evidence for the presence of ethanolamine in the polar head group of the unknown lipid is obtained from the relatively high incorporation of [^{14}C]ethanolamine into the dystrophic phospholipid. These data suggest that there is an equimolar chance of one mole of [^{14}C]ethanolamine labeling either phosphatidylethanolamine or the unknown compound.

The co-chromatographic TLC data support the contention that the unknown lipid is in fact ethanolamine plasmalogen. The co-chromatography of plasmalogen standard and plasmalogen sample, obtained from the membrane preparation, yield one distinct spot when these compounds are run together on the TLC system. The identification of ethanolamine in the polar head group of the phospholipid, the presence of an ether bond, and the co-chromatography data taken together strongly suggest that the unknown compound from TLC plates spotted with preparations of dystrophic membranes is ethanolamine plasmalogen.

The presence of ethanolamine plasmalogen in dystrophic membranes may be due to abnormal plasmalogen metabolic turnover. A decreased plasmalogen turnover rate would explain the elevated concentration of plasmalogen in dystrophic membranes. Specifically, the altered kinetic properties of plasmalogenase, the CDP-ethanolamine phosphotransferase system or enzymes of ether bond formation in plasmalogens (alkyl dihydroxyacetone phosphate synthetase, dihydroxyacetone phosphate acyl transferase) might lead to the

presence of plasmalogen in dystrophic membranes. Also, increased concentrations of ethanolamine plasmalogen in plasma and/or erythrocyte cytosol of dystrophic chickens might partially account for the accumulation of this polar lipid in dystrophic membranes.

Assuming similar [^{14}C]ethanolamine incorporation rates into the phospholipids of dystrophic and normal chicken erythrocyte plasmalemmae, the increased ^{14}C specific activity value that is observed for phosphatidylethanolamine from dystrophic preparations ($1040 \cdot 10^{-13}$ μmol [^{14}C]ethanolamine/μmol phospholipid recovered) compared with normal preparations ($783 \cdot 10^{-13}$ μmol [^{14}C]ethanolamine/μmol phospholipid recovered) may be a reflection of the decreased concentration of phosphatidylethanolamine observed in dystrophic erythrocyte plasmalemmae. Alternatively, this might be a reflection of a decreased phospholipid turnover time in dystrophic preparations.

Utilizing nuclear magnetic resonance spectroscopy, Chalovich and Barany [14] have identified serine-ethanolamine phosphodiester in dystrophic chicken muscle membranes. The possible correlation of the studies of Chalovich and Barany with this study may be that the enzymatic pathways of serine-ethanolamine phosphodiester and ethanolamine plasmalogen are similar. They suggest that the phosphodiester is the result of the reaction between serine and CDP-ethanolamine [16]. CDP-ethanolamine through a phosphotransferase transfers the phosphoethanolamine moiety to diacylglycerol, serine and plasmalogen. The reaction product is determined by the specific substrate, either diacylglycerol-3-phosphate, 1-alkyl-1′-enyl-2-acyl glycerol-3-phosphate, or serine [14]. High levels of the substrate CDP-ethanolamine could explain, in part, the existence of both serine-ethanolamine phosphodiester and ethanolamine plasmalogen in dystrophic preparations. Also, the phosphotransferase that transfers CDP-ethanolamine to either phospholipids, plasmalogens or phosphodiesters could be a branch point enzyme which determines the final concentration of these compounds in dystrophic membranes. It is postulated that different isozymes of this phosphotransferase enzyme can catalyze these separate reactions [15].

Any possible physiological or structural role for the plasmalogen form of phospholipids in membranes is still unresolved. Indirect evidence supports the contention that plasmalogens may have an influence on membrane fluidity in a manner similar to the effect of fatty acyl chains on membrane fluidity. Roots [16] observes that goldfish vary their plasmalemma plasmalogen content in response to the acclimating temperature of the water medium. It is inferred that plasmalogens influence membranes by decreasing the fluidity of the plasmalemma. This correlates with the observation of decreased plasmalemma fluidity in dystrophic chicken erythrocytes [2]. Butterfield [17] suggests that plasmalogens influence membrane properties (viz., fluidity) due to their unique dielectric properties and steric configuration in the membrane. It is open to question whether this change in plasmalogen content is a primary abnormality or a possible mechanism of the cell to regulate an already altered membrane fluidity. Other possible functions for plasmalogens include uses as prostaglandin or platelet activating factor precursor storage sites [18,19].

A distinct polar lipid, observed exclusively on TLC plates spotted with extracted dystrophic erythrocyte membrane preparations has been identified as ethanolamine plasmalogen. The possible correlation between elevated membrane plasmalogens in chicken dystrophy and altered membrane fluidity and function is still to be conclusively demonstrated. The use of artificial bilayers and liposomes may be beneficial in determining these relationships.

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