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Use of a Fluorescent Probe to Determine the Viscosity of LM Cell Membranes with Altered Phospholipid Compositions†

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ABSTRACT: The phospholipid composition of LM cells grown in tissue culture was altered by substituting ethanolamine for choline in the growth medium. The plasma membrane isolated from cells grown in medium containing ethanolamine for 83 h had a sixfold increase in the ratio of phosphatidylethanolamine to phosphatidylcholine, the two major phospholipid classes. This was accompanied by small changes in other lipid components of the membrane. There was also a sixfold increase in the amount of triacylglycerols and alkyldiacylglycerols which were not associated with the membrane fraction of the cell. No significant changes occurred in the lipid composition of cells during growth in choline containing medium. The viscosity of plasma membranes was studied in whole cells and isolated membranes using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. Plasma membranes isolated from ethanolamine-supplemented cells had greater viscosities than membranes isolated from choline-supplemented cells. When whole cells were labeled with the fluorescent probe, the opposite trend in the apparent membrane viscosity was observed. This was due primarily to the probe penetrating into nonmembranous neutral lipids rather than remaining localized in the surface membrane of the cells. Since the enthanolamine-supplemented cells contained more low viscosity neutral lipids, the whole cells gave an apparently lower viscosity as compared with choline-supplemented cells. Thus, measurements carried out on whole cells gave an inaccurate determination of the viscosity of the surface membrane.

Many methods have been used to study the role of lipids in problems of membrane structure and function. One approach has been to manipulate the lipid composition of cells growing under defined conditions (for a review, see Silbert, 1975). This approach, which has largely involved alterations in the fatty acid composition of *E. coli* and *A. laidlawii,* has recently been extended to animal cells in tissue culture (Wisnieski et al., 1973; Williams et al., 1974; Horwitz et al., 1974; Ferguson et al., 1975). Changes in the fatty acid composition in these cells have been shown to alter lectin binding and agglutination (Horwitz et al., 1974; Rittenhouse et al., 1974) and virus infection (Li et al., 1975).

In comparison with the studies on the properties of fatty acids, relatively little has been done with respect to the properties of phospholipid polar head groups in membranes. In order to study the effect of the phospholipid head group in mammalian cell membranes, techniques have been developed

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received October 1, 1976.* This work was supported by National Institutes of Health Grant GM 21953 from the National Institutes of General Medical Sciences. M.G. was supported by National Institutes of Health Research Career Development Award GM 00193 from the Institute of General Medical Sciences. The fluorescence instrumentation of the Weber research group was supported through National Institutes of Health Grant GM 11223.

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to manipulate the phospholipid composition of LM cells grown in tissue culture (Glaser et al., 1974). When the growth medium was supplemented with choline analogues such as *N*-methylethanolamine or 3-amino-1-propanol, up to 50% of the membrane phospholipids contained the analogue supplied. The analogues were incorporated into all membrane fractions of the cell (Schroeder et al., 1976). The polar head group composition can be altered independently or in conjunction with the fatty acid composition (Glaser et al., 1974; Ferguson et al., 1975). Blank et al. (1975) have reported the incorporation of *N*-isopropylethanolamine into LM cell phospholipids and have shown that it stimulated the incorporation of [1-^{14}C]palmitic acid and [1-^{14}C]hexadecanol into triacylglycerols and alkyldiacylglycerols. In *Neurospora crassa* mutants, where the phospholipid composition can also be altered, Hubbard and Brody (1975) have shown that the overall contribution by phospholipids to the membrane charge was maintained despite changes in some phospholipid species.

One property that should be affected by alterations in the phospholipid composition is the viscosity of the membrane. For example, synthetic phosphatidylethanolamine has more than a 25 °C higher gel to liquid-crystalline phase transition temperature than phosphatidylcholine with the same acyl groups (Ladbrooke and Chapman, 1969). Changes produced in the polar head group by changing the pH or the type of cation can also dramatically shift the transition temperature of synthetic phospholipids (Träuble and Eibl, 1974; Jacobson and Papahadjopoulos, 1975; Michaelson et al., 1974).

In this study the viscosity of membranes in whole cells and isolated membranes was measured using the technique of fluorescence polarization (Shinitzky et al., 1971; Cogan et al., 1973). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH[1]), appears to have unusually good spectral characteristics for these studies (Shinitzky and Barenholz, 1974). It has been extensively utilized in studies on synthetic phospholipids (Andrich and Vanderkooi, 1976; Suurkuusk et al., 1976), isolated membranes (Aloni et al., 1974; Stubbs et al., 1976; Kutchai et al., 1976), and whole cells (Fuchs et al., 1975; Toyoshima and Osawa, 1975; Shinitzky and Inbar, 1976). Recently, Barenholz et al. (1976) have used DPH to compare the properties of LM cell plasma membranes and vesicular stomatitis virus grown on these cells. In experiments on DPH-labeled whole cells, it has been assumed that DPH was predominantly located in the surface membrane, and, consequently, the measurements reflected the viscosity in the surface membrane (Shinitzky and Inbar, 1974). The results presented in this paper show that alterations in the phospholipid composition of LM cells produced by supplementation with ethanolamine caused an increase in the viscosity of isolated membranes. The results also demonstrate that DPH can give an inaccurate measurement of surface membrane viscosity in whole cells primarily due to the partitioning of DPH into neutral lipid droplets.

Materials and Methods

Cell Culture. Mouse LM cells were obtained from the American Type Culture Collection and grown as described previously (Glaser et al., 1974; Schroeder et al., 1976). For the past 2 years they have been continuously maintained in suspension culture in Higuchi's medium (1970) with 20 mM Hepes, pH 7.4, 1 g/L methylcellulose and 0.02 g/L sodium dextran sulfate (Sigma Chemical Co.). Initial cell densities were 0.8×10^6 and 1.3×10^6 cells/mL for control cells (with 40 μg/mL choline) and ethanolamine-supplemented cells (40 μg/mL), respectively.

Plasma Membrane Isolation. The procedure for the isolation of plasma membranes was based on the methods used by Schimmel et al. (1973) and Atkison and Summers (1971). Suspension culture (200–400 mL) at densities of 3 to 4×10^6 cells per mL was centrifuged at 1100*g* for 5 min and was washed with an equal volume of PBS, pH 7.2 (Dulbecco and Vogt, 1954, without magnesium or calcium). All procedures were carried out at 4 °C. The final cell pellet was resuspended in approximately 10 mL of PBS and slowly dripped into 1 mM Tris, pH 7.2, with gentle stirring. The volume of buffer was adjusted so that the final density was 5×10^6 cells/mL. Cell lysis was monitored by phase microscopy. The hypotonically lysed cells were centrifuged at 3000*g* for 15 min and the pellet was resuspended in 15 mL of 1 mM Tris, pH 7.2. The nuclei were separated from the cell membranes by forcibly pipetting the material 15 times through a Kimax 10-mL serological pipet. After diluting the sheared cells to the original lysing volume with 1 mM Tris, pH 7.2, they were centrifuged for 10 min at 3000*g*. The nuclear pellet was removed from the centrifuge bottle with a pipet and the supernatant was centrifuged at 48 500*g* for 1 h. The resulting membrane pellet was resuspended in 2 to 3 mL of 1 mM Tris, pH 7.2, with a 29-gauge hypodermic needle. This was layered onto two to three discontinuous sucrose gradients containing 0.5 mL of 55% (w/w) sucrose, then 2.0 mL each of 40, 35, 32, and 29% sucrose, and finally 1.7 mL of 20% sucrose. The gradients were centrifuged at 41 000 rpm in a SW-41 rotor for 2.5 h. This resulted in five membrane bands at the interfaces which were collected, diluted to 9 mL with Tris buffer, and centrifuged at 105 000*g* for 1 h. Samples from each step in the preparation were diluted similarly and centrifuged at 105 000*g* for 1 h so that only particulate protein was later assayed for enzyme activity.

Enzyme Assays. Oubain sensitive (Na^+,K^+)ATPase was assayed as described by Schimmel et al. (1973) except the reaction was terminated after 20 min with 100 μL of 1.5% sodium dodecyl sulfate. This avoided the centrifugation prior to the phosphate determination. TPNH-dependent and succinate-dependent cytochrome *c* reductase activities were assayed spectrophotometrically according to Sottocasa et al. (1967). Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Extraction and Isolation of Lipids. Materials for lipid determination were extracted by the method of Bligh and Dyer (1959) as described by Ames (1968). The phospholipid composition was determined on cells that were uniformly labeled with [^{32}P]phosphate (Glaser et al., 1974). [^{32}P]Phosphate (0.5 μCi/mL) was added several generations before the start of the experiment and maintained at the same specific activity throughout the experiment. Individual phospholipids were separated by two-dimensional thin-layer chromatography on Silica Gel G (250 μm, Analtech, Inc.). The first dimension consisted of chloroform–methanol–water (65:25:4) and the second dimension consisted of 1-butanol–glacial acetic acid–water (6:2:2). The spots were visualized by autoradiography, scraped, and counted.

Neutral lipids were separated from polar lipids by passing the crude extract over methanol–chloroform washed Unisil (100–200 mesh, Clarkson Chemical Co.). Neutral lipids were

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; DMPC, dimyristoylphosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TPNH, reduced triphosphopyridine nucleotide.

eluted with 5 mL of chloroform and polar lipids with 5 mL of methanol (Ferguson et al., 1975). The various neutral lipids were further resolved by one-dimensional thin-layer chromatography using hexane–diethyl ether–glacial acetic acid (60:20:1). Desmosterol, triacylglycerols, and alkyldiacylglycerols were identified by comparison with standards (Supelco, Inc., and Applied Science Laboratories). The amount of desmosterol in isolated membranes and in whole cells was measured by the method of Watson (1960), except the assay volume was reduced to 0.3 mL and the absorbance read at 610 nm. Total phospholipid phosphate was measured by the ashing procedure of Ames and Dubin (1960). The amount of triacylglycerols and alkyldiacylglycerols obtained after the Unisil column was measured together by the procedure of Snyder and Stephens (1959). This method determines the amount of fatty acid ester equivalents in the sample. LM cells contained negligible amounts of sterol esters that would interfere with the assay.

DPH Distribution in Prelabeled Cells. DPH in each fraction obtained during the membrane isolation was extracted by the chloroform–methanol procedure used for the phospholipid extraction. This procedure extracts greater than 98% of the phospholipids and DPH. The concentration of the DPH in chloroform was determined by measuring its fluorescence intensity on a Hitachi Perkin-Elmer spectrofluorometer Model MPF-2A and comparing it with standard DPH solutions of known concentrations.

Fatty Acid Analysis. Fatty acid methyl esters were prepared from phospholipid or neutral lipid samples by the HCl–CH_3OH (1:20) method described previously (Ferguson et al., 1975). Fatty acid methyl esters were separated isothermally at 180 °C on a 1.5-m column of cyanosilicon SP-2340 on 80–100 mesh Chromosorb W AW (Supelco, Inc.). The gas–liquid chromatographic analysis was conducted on a Hewlett Packard 5830a chromatograph equipped with a flame ionization detector and peak integrator.

Lipid Dispersions. Lipids were dispersed for fluorescence measurements using the method of Bangham et al. (1965) as a guide. DPH was added to 0.1 to 1.0 μmol of lipid phosphate in chloroform so that a molar ratio of 2000:1 (phospholipid–DPH) was obtained. Total lipid dispersions from DPH prelabeled cells required no additional DPH since the dye was extracted along with the lipids, and comparable ratios of phospholipid to DPH were obtained. The lipid–DPH mixture was dried under a stream of nitrogen and 1 to 2 mL of PBS was added. The tube was incubated at 55 °C under argon for 45 min and vortexed periodically.

Fluorescence Techniques. All fluorescence measurements were made on samples containing the fluorophore 1,6-diphenyl-1,3,5-hexatriene based on the methods of Shinitzky and Barenholz (1974) and Shinitzky and Inbar (1974). Whole cells in growth medium were counted and diluted to 2×10^6 cells per mL with PBS at 37 °C which contained DPH at a concentration such that the final concentration of DPH was 10^{-15} mol per cell. The DPH was initially dissolved in tetrahydrofuran at 2×10^{-2} M and the final concentration of tetrahydrofuran never exceeded 0.02% in the incubation. The cells were allowed to take up the dye for 7 min at 37 °C with shaking after which the suspension was immediately centrifuged at 1100*g* for 5 min. The cells were washed one time with PBS and used for the preparation of plasma membranes or used directly for fluorescence measurements. The dye in the aqueous incubation buffer has practically no fluorescence and as it was incorporated into membranes there was a rapid increase in fluorescence intensity. The uptake of the dye into cells appeared to saturate after approximately 20 min and no change in polarization was observed during this labeling period. Similar polarization values were obtained with the cells when the dye incubations were carried out at 25 °C, or different concentrations of dye were used.

Instrumentation. Fluorescence polarization (or anisotropy) was measured on an instrument with the T-format design similar to that described by Weber and Bablouzian (1966). The instrument was equipped with both voltage and ratiometric modes and an averaging circuit designed by Mitchell and Spencer (1973). Exciting light was obtained from a 200-W mercury arc lamp using a 366-nm interference filter (22% transmission at 366 nm, Baird Atomic) and polarized with a Glan-Thompson polarizer. The fluorescence was observed through two independently cross-polarized channels (Glan polarizers) after passing through aqueous 2 M sodium nitrite and Corning 3-72 or 3-73 cut-off filters which reduced corrections due to highly polarized scattered light to less than 3%. Instrumental design allowed the simultaneous measurement of $I_\parallel/I_\perp$ and $I_\parallel$ and $I_\perp$ where the latter quantities are the intensities polarized parallel and perpendicular to the exciting light. These parameters are related to the fluorescence polarization and anisotropy (Shinitzky et al., 1971).

Lifetimes were measured directly on a cross-correlation phase fluorometer previously described by Spencer and Weber (1969), with updated electronics by SLM Instruments, Inc. A 35° polarizer was placed in the excitation path to compensate for rotational motion (Spencer and Weber, 1970). Light scattered by the samples did not significantly change the observed fluorescence lifetimes and thus the correction for light scattering was not required.

Lifetimes were the average values calculated from phase (τ_{phase}) and modulation (τ_{mod}) measurements at 30 MHz. The difference between τ_{phase} and τ_{mod} is an indication of the degree of heterogeneity of the probe environment (Weber et al., 1976). Cells and membranes gave a difference between τ_{phase} and τ_{mod} of approximately 1 ns. Differences of this magnitude do not indicate a very great heterogeneity in the probe environment, and, consequently, the data were treated as if the probe was in a single environment.

The temperature at which the fluorescent measurements were made was monitored by a thermocouple (YSI Telethermometer) in a duplicate curvette. Temperature studies were carried out by incubating the sample at each temperature for 10 min starting at 2 °C before the fluorescent measurement was made. Exposure to exciting light was limited to 10 s to prevent photoisomerization (Shinitzky and Barenholz, 1974). When the samples were cooled after the experiment similar polarization values were obtained.

Viscosity (or microviscosity) was calculated from the measured anisotropy and lifetime as described by Shinitzky and Inbar (1974). This technique is based on the Perrin equation for the depolarization of fluorescence due to rotation of a nonspherical fluorophore (Weber, 1953).

Results

A growth curve for choline- and ethanolamine-supplemented cells is given in Figure 1. Choline-supplemented cells grew logarithmically for over 80 h, while ethanolamine-supplemented cells grew logarithmically for approximately 60 h and then stopped growing.

The viscosity of whole cells grown on a variety of fatty acid supplements and choline analogues was determined using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene. All alterations of the phospholipid composition showed a decrease in

TABLE I: Distribution and Activities of Protein and Membrane Marker Enzymes in the Isolation of Plasma Membranes from Cells Supplemented with Choline.[a]

Fraction	Protein[b]	(Na^+,K^+)ATPase	TPNH Cytochrome *c* Reductase	Succinate Cytochrome *c* Reductase	μmol of Phospholipid/ mg of Protein
Lysate, particulate	54.0 (100%)	11.8 (100%) 637[c]	20.6 (100%) 1110[c]	35.2 (100%) 1900[c]	0.27[d]
3000*g* supernatant after lysis	10.6 (19.6)	3.5 (5.9)	13.1 (12.4)	27.8 (15.5)	0.15
3000*g* supernatant after shearing	21.0 (38.8)	21.6 (70.9)	32.2 (58.8)	70.8 (78.2)	0.58
3000*g* pellet after shearing	17.9 (33.1)	7.6 (21.5)	5.9 (9.5)	12.6 (11.8)	0.12
48 500*g* supernatant	2.9 (5.4)	0.9 (0.4)	8.4 (2.2)	[e]	0.10
48 500*g* pellet	14.9 (27.6)	27.1 (63.1)	34.7 (46.2)	102 (79.7)	0.60
Band I (20–29% interface)	3.3 (6.1)	33.8 (17.4)	38.0 (11.1)	1.0 (0.2)	[f]
Band II (29–32% interface)	1.0 (1.9)	66.1 (9.9)	46.8 (4.0)	1.9 (0.1)	0.94
Band III (32–35% interface)	1.5 (2.8)	86.4 (20.2)	40.8 (5.5)	14.0 (2.6)	0.91
Band IV (35–40% interface)	2.8 (5.2)	27.5 (12.2)	31.0 (7.9)	209 (30.7)	0.50
Band V (40–55% interface)	2.5 (4.6)	9.0 (3.5)	44.0 (9.9)	24.5 (3.2)	0.45
Total gradient recovery	11.1 (20.6)	(63.2)	(38.4)	(36.8)	

[a] Cells grown for 83 h with choline were fractionated as described in Materials and Methods. Enzyme activities are expressed as nmol min^{-1} (mg of protein)$^{-1}$. Assays were done on the particulate part of each fraction after centrifugation for 1 h at 105 000*g*. Numbers in parentheses are percent recoveries of the particulate lysate. [b] Milligrams of particulate protein after centrifugation for 1 h at 105 000*g*. [c] Total activities expressed as nmol min^{-1}. [d] The amount of phosphate was determined after phospholipid extraction. [e] None detectable. [f] Not determined.

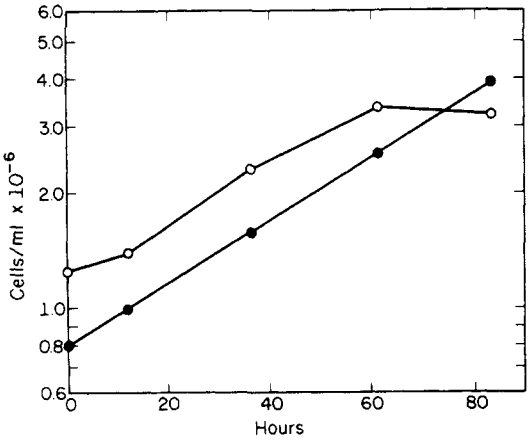

FIGURE 1: The growth of LM cells supplemented with choline (●—●) or ethanolamine (○—○). The initial cell density for cells supplemented with choline or ethanolamine was 0.8 × 10^6 and 1.3 × 10^6 cells/mL, respectively.

the viscosity of whole cells with ethanolamine supplementation causing the largest change. Ethanolamine supplementation for 83 h shifted the viscosity from 1.64 P in control cells to 0.86 P in the supplemented cells at 37 °C. Since this was opposite to the effect predicted from studies on synthetic phospholipids, plasma membranes were prepared from these cells in order to carefully evaluate the changes in viscosity with changes in the lipid composition.

The results of a typical plasma membrane purification are given in Table I. The procedure involved hypotonic lysis and shearing which resulted in virtually 100% breakage of the cells. The method was very reproducible and gave much better results than the use of Dounce homogenizers or other mechanical methods.

Approximately 50% of the total cellular protein was associated with the membrane fraction when the crude lysate was centrifuged for 1 h at 105 000*g*. There was also an approximate twofold increase in membrane marker enzyme specific activities and the phospholipid-to-protein ratio in the particulate lysate relative to the total lysate (data not shown). When the cells were centrifuged at low speed (3000*g*) after lysis in the hypotonic buffer, approximately 50% of the total protein was found in the supernatant (data not shown). Only 5.9% of the ATPase activity and slightly more of the other membrane marker enzymes were found in the particulate part of this supernatant. This indicated that mainly soluble protein was released from the cells while the membranes were still associated with the nuclei in the pellet. When the pellet was resuspended in Tris buffer and sheared, 70.9% of the ATPase along with most of the other marker enzyme activities were now found in the low speed supernatant.

A 5- to 8-fold purification of plasma membranes relative to the particulate lysate was routinely observed as measured by the (Na^+,K^+)ATPase. The highest purification of this marker, 7.3-fold, was observed in band III at the 32–35% sucrose interface. Pooling bands II and III resulted in 30.1% yield of the ATPase with an average purification of 6.5-fold relative to the particulate lysate. In these bands a 2.1-fold purification of the microsomal marker TPNH-dependent cytochrome *c* reductase was observed, but the yield was only 9.5%. These bands will be referred to as a plasma membrane fraction even though they may have contained a substantial amount of microsomes. The amount of the mitochondrial marker, succinate-dependent cytochrome *c* reductase, was very small in these bands. If the purification in bands II and III were expressed on the basis of phospholipid rather than protein, the ATPase specific activity increased 1.9-fold while the TPNH-dependent cytochrome *c* reductase decreased 1.6-fold. A similar purification was obtained with ethanolamine-supplemented cells (Table II). Overall, this purification procedure gave final ATPase specific activities comparable to other procedures and was relatively quick and reproducible. It also yielded a plasma membrane fraction that contained no detectable triacylglycerols or alkyldiacylglycerols which was particularly important for this study.

If DPH was only located in the plasma membrane in labeled whole cells, it should copurify with the (Na^+,K^+)ATPase and give constant polarization values during purification. This was not observed with either choline- or ethanolamine-supplemented cells (Table III). There was a large amount of DPH found in the 3000*g* pellet and DPH was spread out in the sucrose gradient in a manner that did not coincide with the

TABLE II: Distribution and Activities of Protein and Membrane Marker Enzymes in the Isolation of Plasma Membranes from Cells Supplemented with Ethanolamine.[a]

Fraction	Protein[b]	(Na^+,K^+)ATPase	TPNH Cytochrome *c* Reductase	Succinate Cytochrome *c* Reductase	μmol Phospholipids/ mg of Protein
Lysate, particulate	36.3 (100%)	18.1 (100%) 656[c]	21.0 (100%) 761[c]	45.2 (100%) 1640.4[c]	0.26[d]
3000*g* supernatant after lysis	7.3 (20.0)	5.5 (6.1)	8.4 (8.4)	*f*	0.15
3000*g* supernatant after shearing	10.5 (29.0)	37.7 (60.5)	39.2 (54.3)	*f*	0.52
3000*g* pellet after shearing	15.2 (42.0)	9.3 (21.7)	9.4 (18.9)	*f*	0.11
48 500*g* supernatant	2.0 (5.5)	1.7 (0.5)	14.0 (3.7)	*f*	0.12
48 500*g* pellet	7.8 (21.5)	49.8 (59.2)	45.4 (46.3)	77.9 (37.0)	0.46
Band I (20–29% interface)	0.95 (2.6)	29.9 (4.3)	32.0 (4.0)	*f*	1.1
Band II (29–32% interface)	0.46 (1.3)	101.8 (7.1)	44.0 (2.7)	*e*	0.79
Band III (32–35% interface)	0.81 (2.2)	150.2 (18.5)	43.2 (4.6)	3.5 (0.2)	0.70
Band IV (35–40% interface)	1.6 (4.4)	51.9 (12.6)	54.1 (11.3)	*f*	0.55
Band V (40–55% interface)	2.2 (6.2)	8.4 (2.8)	46.1 (13.3)	*f*	0.45
Total gradient recovery	6.0 (16.6)	(45.4)	35.9		

[a] Cells grown for 83 h with ethanolamine were fractionated as described in Materials and Methods. Enzyme activities are expressed as nmol min^{-1} (mg of protein)$^{-1}$. Assays were done on the particulate part of each fraction after centrifugation for 1 h at 105 000*g*. Numbers in parentheses are percent recoveries of the particulate lysate. [b] Milligrams of particulate protein after centrifugation for 1 h at 105 000*g*. [c] Total activities expressed as nmol/min. [d] The amount of phosphate was determined after phospholipid extraction. [e] None detectable. [f] Not determined.

TABLE III: Distribution of DPH and Fluorescent Polarization for Fractions Obtained in the Isolation of Plasma Membranes from Cells Supplemented with Choline or Ethanolamine.[a]

Fraction	Choline			Ethanolamine		
	DPH[b]	Yield[c]	Polarization[d]	DPH[b]	Yield[c]	Polarization[d]
Whole cells[e]			0.210			0.147
Lysate[e]	0.24	100.0	0.207	0.22	100.0	0.159
3000*g* pellet after shearing[e]	0.64	54.8	0.210	0.31	43.5	0.161
48 500*g* supernatant[e]	0.12	4.1	0.184	0.83	31.4	0.093
48 500*g* pellet[e]	0.56	34.4	0.195	0.47	30.0	0.201
Band I (20–29% interface)	0.67	17.4	0.228	0.59	13.3	0.231
Band II (29–32% interface)	1.11	3.7	0.210	0.84	3.5	0.228
Band III (32–35% interface)	0.88	4.0	0.199	0.72	2.0	0.222
Band IV (35–40% interface)	0.54	6.3	0.177	0.75	4.0	0.193
Band V (40–55% interface)	0.83	4.4	0.187	0.28	3.3	0.203
Total gradient recovery		35.8			26.1	

[a] Cells grown for 84 h with choline or ethanolamine were labeled with DPH and fractionated as described in Materials and Methods. No additional dye was added for the fluorescence measurements. [b] Nanomoles of DPH (milligrams of protein)$^{-1}$. [c] Percentage yield of lysate. [d] Fluorescent polarization was measured at 37 °C. [e] Assays were done on total fractions (i.e., not centrifuged at 105 000*g* as in Tables I and II).

ATPase. In particular, ethanolamine-supplemented cells showed 31.4% of the DPH in the 48 500*g* supernatant as compared with 4.1% for the choline-supplemented cells. The 48 500*g* supernatant contained very small amounts of ATPase activity (<1%) in either cell type. It is possible that DPH redistributed among the cellular fractions during cell lysis or in subsequent steps. The redistribution on lysis could not have been extensive, however, because no reproducible changes in polarization were observed when either cell type was lysed.

The 48 500*g* supernatant as well as high-speed supernatants (105 000*g*) of other fractions from ethanolamine-supplemented cells exhibited a substantial amount of turbidity especially near the top of the centrifuge tube that suggested the presence of low density lipid droplets. Lipid analysis showed that the supernatant contained approximately 90 mol % triacylglycerols and alkyldiacylglycerols, 10 mol % desmosterol, and only a trace of phospholipid or free fatty acids. The 48 500*g* supernatant gave very low polarization and lifetime values. Since the amount of DPH in the 48 500*g* supernatant was small in choline-supplemented cells, the contribution to the polarization of whole cells should also be small. In ethanolamine-supplemented cells, however, where the amount of DPH in the 48 500*g* supernatant was much larger, the contribution to the whole cell polarization should also be larger.

The amount of DPH found in a given fraction of the prelabeled cells (Table III) may not necessarily reflect the contribution of that fraction to the whole cell fluorescent intensity. For example, the DPH in the nuclei appeared to be partially quenched (data not shown). Also, the whole cell polarization value would not be changed unless DPH penetrated into a region with a different polarization than the plasma membrane. The 48 500*g* supernatant had the lowest polarization value in ethanolamine-supplemented cells. In order to determine if the

TABLE IV: Fluorescence Polarization, Lifetime, and Viscosity Values at 10, 25, and 37 °C for Plasma Membranes Isolated from Cells Supplemented with Choline and Ethanolamine.[a]

Supplement	Hours	Polarization			Lifetime (ns)			Viscosity (cP)		
		10 °C	25 °C	37 °C	10 °C	25 °C	37 °C	10 °C	25 °C	37 °C
Choline		0.342 ± 0.009	0.275 ± 0.011	0.219 ± 0.010	9.8 ± 0.4	9.6 ± 0.4	9.1 ± 0.3	592 ± 80	314 ± 43	189 ± 19
Ethanolamine	12	0.340	0.268	0.198	10.3	9.7	9.4	605	294	160
Ethanolamine	36	0.368	0.288	0.228	10.2	10.0	9.6	841	365	215
Ethanolamine	61	0.369	0.293	0.239	10.3	9.6	9.3	856	368	277
Ethanolamine	83	0.372	0.299	0.236	10.6	10.6	9.8	926	426	234

[a] Plasma membranes were isolated as described in Materials and Methods from cells supplemented with choline and ethanolamine at 12, 36, 61, and 83 h. No trends were observed in membranes from choline-supplemented cells, so the values were averaged.

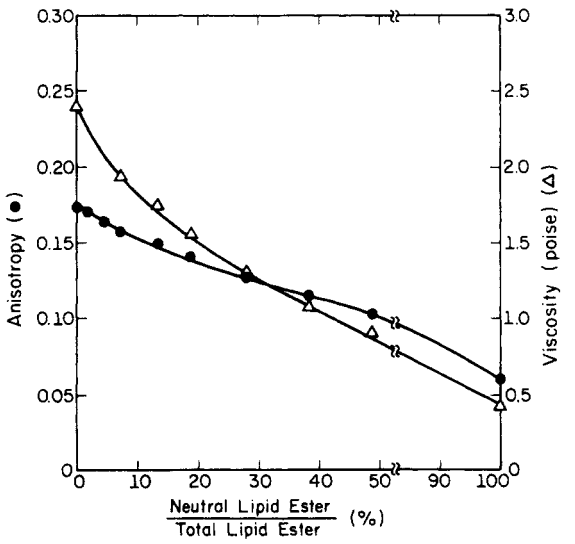

FIGURE 2: Titration of plasma membranes with the neutral lipid containing 48 500*g* supernatant isolated from cells supplemented with ethanolamine for 83 h. Aliquots of the supernatant were added to a fixed amount of plasma membranes and the anisotropy (●—●) and viscosity (Δ—Δ) were measured. Whole cells were prelabeled with DPH before isolation of the plasma membranes and 48 500*g* supernatant.

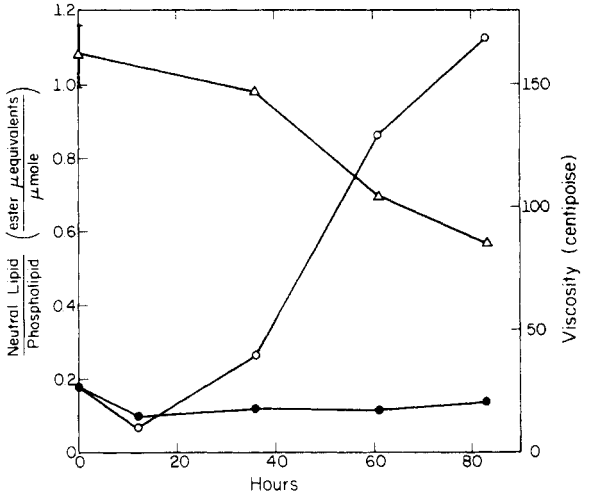

FIGURE 3: The amount of ester equivalents measured in triacylglycerols and alkyldiacylglycerols, and the viscosity of whole cells. The amount of neutral lipid was determined in cells grown with choline (●—●) or ethanolamine (O—O) for 12, 36, 61, and 83 h as described in Materials and Methods. The viscosities of cells supplemented with ethanolamine (Δ—Δ) for the indicates times were determined on aliquots of whole cells immediately after incubation with DPH. No trend in the viscosity of whole cells supplemented with choline was observed. The range of observed values for the viscosity of these cells is given as a vertical bar at zero time.

DPH in this fraction could account for the low whole cell polarization value, the plasma membrane was titrated with the 48 500*g* supernatant (Figure 2). When the supernatant was added to the plasma membranes, the anisotropy and viscosity decreased continuously. Lipid analysis of the whole cells used for this experiment showed that there was 36% neutral lipid esters in the cell (i.e., 36% of the fatty acid esters were in triacylglycerols and alkyldiacylglycerols out of the total amount of fatty acid esters). This corresponded very well to the value of 31.4% for the amount of DPH found in the 48 500*g* supernatant. Thirty-one to thirty-six percent on the curve in Figure 2 gives a viscosity of approximately 1.2 P which is close to the viscosity of 0.86 P observed in the whole cells. Better agreement would probably have been obtained if the entire membrane fraction of the cell (i.e., the particulate lysate) was used for the experiment shown in Figure 2 instead of the plasma membrane fraction. This was not done, however, because only the plasma membrane fraction was free of triacylglycerols and alkyldiacylglycerols. The 3000*g* pellet and the 48 500*g* pellet (as well as some of the other bands on the sucrose gradient) contained small amounts of triacylglycerols and alkyldiacylglycerols which could account for their lower polarization values compared with the plasma membranes (Table III).

The correlation between the increase in the amount of triacylglycerols and alkyldiacylglycerols after ethanolamine supplementation, and the decrease in viscosity of whole cells is shown in Figure 3. The major increase in the amount of these neutral lipids occurred after 36 h of supplementation. The amount continued to increase after 60 h of supplementation even though the cell number had stopped increasing. After 83 h there was a sixfold overall increase in these neutral lipids. The decrease in viscosity measured in whole cells correlated very well with the increase in neutral lipids. Cells supplemented with choline, on the other hand, did not show an increase in neutral lipid or a change in viscosity.

Polarization, lifetimes, and viscosity values at 10, 25, and 37 °C were determined for plasma membranes isolated from DPH labeled cells after different times of supplementation (Table IV). In plasma membranes isolated from choline-supplemented cells at various stages of growth, these values remained fairly constant. In ethanolamine-supplemented cells,

TABLE V: Phospholipid Composition of Plasma Membranes Isolated from Cells Supplemented with Choline or Ethanolamine.[a]

Supplement	Hours	Phospholipid Composition (%)					
		PC	PE	PI & PS[b]	Sph	Other[c]	PE/PC
Choline		58.1 ± 2.9	20.1 ± 1.0	9.3 ± 1.5	7.6 ± 0.6	4.9 ± 0.2	0.35
Ethanolamine	12	45.5	33.4	6.5	7.4	7.2	0.73
Ethanolamine	36	27.1	43.4	8.6	15.2	5.7	1.60
Ethanolamine	61	21.1	44.9	11.1	17.5	5.4	2.13
Ethanolamine	83	20.5	43.1	13.9	15.0	7.5	2.10

[a] Plasma membranes were isolated as described in Materials and Methods from cells labeled with [^{32}P]phosphate and supplemented with choline and ethanolamine for 12, 36, 61, and 83 h. No trends were observed in membranes phospholipid compositions from choline-supplemented cells so the values were averaged. [b] PI and PS are shown together because the thin-layer chromatography system did not separate these two phospholipids completely. [c] This category consists of several minor phospholipids including cardiolipin, phosphatidylglycerol, phosphatidylmethylethanolamine, lysophosphatidylcholine, and lysophosphatidylethanolamine.

TABLE VI: Fatty Acid Composition of Plasma Membrane Phospholipids and Whole Cell Neutral Lipids from Cells Supplemented with Choline or Ethanolamine.[a]

Supplement	Hours	Fatty Acid Composition (%)							
		14:0	16:0	16:1	18:0	18:1	Odd Chain[b]	>C-18[c]	% Saturated Fatty Acids
					Plasma Membrane Phospholipids				
Choline		1.0 ± 0.4	19.6 ± 0.6	7.8 ± 1.0	11.6 ± 1.1	45.4 ± 1.2	6.0 ± 0.5	9.5 ± 1.2	35.2 ± 1.2
Ethanolamine	12	1.5	18.6	10.8	10.5	45.6	5.7	7.3	34.8
Ethanolamine	36	1.1	17.2	10.5	12.8	42.2	9.0	6.7	38.9
Ethanolamine	61	1.0	16.0	10.0	14.9	39.1	12.9	8.6	40.7
Ethanolamine	83	1.0	15.0	10.0	15.1	38.6	11.9	10.0	37.5
					Whole Cell Neutral Lipids				
Choline		4.4 ± 0.9	26.2 ± 0.5	8.5 ± 0.9	10.5 ± 0.9	43.0 ± 0.6	2.0 ± 0.6	5.6 ± 0.6	42.1 ± 1.6
Ethanolamine		5.0 ± 1.0	25.4 ± 1.6	10.7 ± 0.6	9.3 ± 0.4	43.5 ± 1.4	1.6 ± 0.2	4.5 ± 0.6	40.6 ± 1.7

[a] Plasma membranes were isolated as described in Materials and Methods from cells supplemented with choline or ethanolamine for 12, 36, 61, and 83 h. Neutral lipids were isolated from whole cells as described in Materials and Methods. No trends were observed for the fatty acid composition of plasma membranes from choline-supplemented cells, or in the fatty acid composition of whole cell neutral lipids, so the values obtained were averaged. [b] Includes 15:0, 17:0, 17:1, and 19:0. [c] Includes 19:0, 20:1, 22:0, 22:1, 24:0, and 24:1.

however, the polarization and lifetimes of DPH, and consequently the viscosity, increased with time of supplementation. By 36 h of supplementation most of the change in polarization had occurred. After 83 h, the overall viscosity measured at 37 °C increased approximately 24% compared with the average value for choline-supplemented cells, while at lower temperatures the difference became greater. The viscosity measured at 10 °C increased approximately 56%. If instead of prelabeling cells with DPH, the plasma membranes were first isolated and then labeled with DPH, similar viscosity values were obtained. Absolute viscosity values varied between different experiments, but similar viscosity differences were always observed between choline- and ethanolamine-supplemented cells. In each experiment the growth, membrane isolation, and measurements were carried out simultaneously on parallel cultures of cells supplemented with choline or ethanolamine to ensure comparable conditions as much as possible.

After 12 h the ratio of phosphatidylethanolamine to phosphatidylcholine approximately doubled and by 83 h this ratio went up sixfold (Table V). Small increases were observed in sphingomyelin and phosphatidylserine plus phosphatidylinositol. There were no significant changes in the other minor phospholipids which included lysophospholipids and phosphatidylglycerol. The major changes in phospholipid composition occurred by 36 h which did not correlate with the rate of change in the viscosity of whole cells or the increase in neutral lipids. The fatty acid composition of membrane phospholipids showed no significant variations for choline-supplemented cells with time (Table VI). In ethanolamine-supplemented cells the fatty acid composition underwent small, but continuous, changes. The amount of odd chain fatty acids and 18:0 increased, while 16:0 and 18:1 decreased. The fatty acid composition of the neutral lipids did not change significantly in both cell types, even though the amount of neutral lipid increased sixfold in ethanolamine-supplemented cells.

Arrhenius plots of the viscosity values for membranes isolated from choline- and ethanolamine-supplemented cells after 83 h are given in Figure 4. The temperature behavior of synthetic dimyristoylphosphatidylcholine is given for comparison. This phospholipid showed a sharp gel to liquid-crystalline transition at 24 °C (see Andrich and Vanderkooi, 1976). The viscosity behavior of the membranes, on the other hand, gave straight lines with no indication of a break or discontinuity. The apparent activation energies for membranes from choline- and

TABLE VII: Fluorescence Polarization and Viscosity Values for Membranes, Total Lipid Dispersions, and Phospholipid Dispersions at 10, 25, and 37 °C from Cells Supplemented with Choline or Ethanolamine for 84 Hours.[a]

	Polarization			Lifetime (ns)			Viscosity (cP)		
	10 °C	25 °C	37 °C	10 °C	25 °C	37 °C	10 °C	25 °C	37 °C
Choline									
Membranes	0.318	0.251	0.191	9.6	9.4	9.2	445	246	148
Total lipids[b]	0.299	0.219	0.163	9.4	9.3	8.5	356	184	105
Phospholipid[c]	0.257	0.165	0.118	9.2	8.2	7.9	242	100	62
Ethanolamine									
Membranes	0.347	0.287	0.224	10.0	9.8	9.4	636	354	203
Total lipids[b]	0.347	0.268	0.208	9.8	9.5	9.3	618	288	173
Phospholipid[c]	0.316	0.194	0.142	9.7	9.4	8.9	423	149	90

[a] Plasma membranes and lipid dispersions were prepared from cells labeled with DPH as described in Materials and Methods. These fractions were completely free of triacylglycerols and alkyldiacylglycerols. [b] Total lipids consisted of phospholipids and desmosterol. [c] Additional dye was added to the phospholipid so that the ratio of phospholipid to dye was 2000:1.

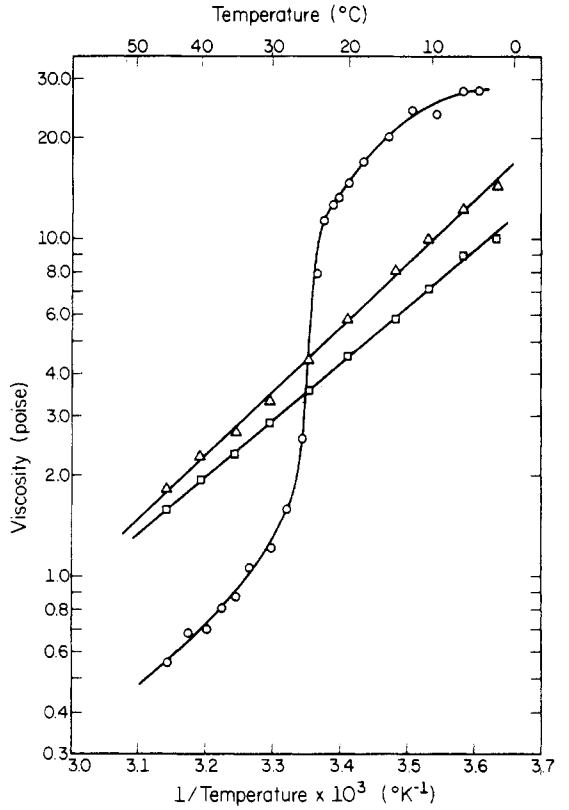

FIGURE 4: The temperature dependence of viscosity for plasma membranes isolated from cells supplemented with choline (□—□) or ethanolamine (△—△), and for DMPC vesicles (○—○). The procedure for vesicle dispersion and fluorescent measurements were carried out as described in Materials and Methods.

ethanolamine-supplemented cells were 7.7 and 8.7 kcal/mol, respectively. Arrhenius plots of polarization values for the membranes which were taken at closer temperature intervals had a gradual change in slope, but also showed no breaks or discontinuities. When membranes or lipid dispersion were heated to 55 °C and cooled, little or no hysteresis was observed.

Fluorescent lifetimes that were determined directly, did not always compare with lifetimes obtained through fluorescent intensity measurements. In particular, large deviations were observed when the temperature of the membrane samples were raised. The fluorescent intensity often increased at 20 to 25 °C while the direct lifetime measurements always decreased. The increase in fluorescent intensity may have been due to further incorporation of the dye which was not washed away or was in aggregates on the surface of the membrane (Shinitzky and Inbar, 1974). Consequently, lifetimes were always measured directly.

Polarization and viscosity values at 10, 25, and 37 °C for membranes were much higher than for dispersion made from the extracted lipids (Table VII). The lipids from both choline- and ethanolamine-supplemented cells behaved similarly. When the lipid extract was passed over a Unisil column to remove desmosterol, the polarization and viscosity values decreased further, indicating the effect of sterols on viscosity (Shinitzky and Inbar, 1976). Sonication of the lipid dispersion caused little or no change in the viscosity values (data not shown). The viscosity of the phospholipid dispersion from ethanolamine-supplemented cells was higher than those for choline-supplemented cells, indicating that the changes in phospholipid composition were responsible for the changes in viscosity observed in the membranes.

Discussion

Most tissue culture cells are grown in complex media containing serum. It has been known for some time that cells are capable of taking up fatty acids from the serum in the medium and using them for phospholipid and triglyceride synthesis (see Rothblat and Kritchevsky, 1967). As the concentration of exogenous fatty acid increased, progressively more triglycerides are formed and stored as droplets within the cell. Cells are also capable of endogenous triglyceride synthesis. An extreme example of triglyceride accumulation in a tissue culture cell is the conversion of 3T3 cells into adipocytes (Green and Kehinde, 1976).

The LM cells used in this study are different from most tissue culture cells in that they are grown in a chemically defined medium with no serum and no protein or lipid. Control cells grown on choline-containing medium have very small amounts of triglyceride. When the polarization was measured in DPH-labeled whole cells, the values obtained were close to those from the plasma membranes isolated from the cells (Table III). In contrast, cells grown on ethanolamine-containing medium accumulated substantial amounts of triacylglycerols and alkyldiacylglycerols and the polarization values for whole cells were very different from those for isolated plasma membranes. DPH was not localized in the plasma

membrane but penetrated into neutral lipids which were not membrane associated. The low polarization and viscosity values for DPH in the neutral lipids caused the whole cell values to decrease substantially. This may be a general problem whenever a hydrophobic probe is used to measure membrane properties where variable amounts of neutral lipid droplets are present. The data also indicate that DPH penetrated into other membranes of the cell but this could not be accurately evaluated.

During supplementation with ethanolamine there were changes in the lipid composition of the plasma membrane that paralleled changes in the viscosity. The largest change in the lipid composition of cells supplemented with ethanolamine was in the two major phospholipid classes, phosphatidylethanolamine and phosphatidylcholine. After 83 h of supplementation the ratio of phosphatidylethanolamine to phosphatidylcholine increased sixfold (Table V). There were small changes in the other phospholipids, desmosterol content, and the fatty acid composition of the individual phospholipids (Tables VI and VII; Ferguson et al., 1975; Schroeder et al., 1976). Some of these changes may reflect the cells' attempt to compensate for the effects of an altered polar head group composition. The difference in viscosity between the two types of membranes was less at 37 °C, the growth temperature, than at lower temperatures.

The other changes in the lipid composition during ethanolamine supplementation make the interpretation of differences in membrane properties more complex. It should be possible, however, to evaluate the contribution from these different changes by also supplementing the cells with other polar head group analogues, fatty acids or sterols. For example, both fatty acid and ethanolamine supplementation altered the activity of adenylate cyclase in these cells, but they had distinct effects (Engelhard, V. H., Esko, J. D., Storm, D. R., and Glaser, M., manuscript submitted). It should also be possible to produce larger or smaller changes in the viscosity on altering the polar head groups by additionally altering the fatty acid or sterol composition.

The viscosity of the isolated membranes had a pronounced temperature dependence from 0 to 50 °C (Figure 4). The change in viscosity in this temperature range was only approximately twofold less than a homogeneous synthetic phospholipid with no sterol, DMPC, which underwent a gel-like to liquid-crystalline phase transition in this temperature range. The membranes were more viscous than DMPC at high temperatures and less viscous than DMPC at low temperature. The viscosity of DMPC continued to change with temperature outside the immediate transition region in a manner that was not very different from the behavior of the membranes. Arrhenius plots of membrane polarization or viscosity did not give an indication of a distinct transition or a discrete region of lateral phase separation as has been found with electron spin resonance studies of membranes from LM cells (Wisnieski et al., 1974). The electron spin resonance results also showed that isolated lipids behaved differently from the membranes which agree with the results found in this study (Table VII). Barenholz et al. (1976), on the other hand, found no evidence for a region of lateral phase separation in LM cell membranes using DPH, but found that extracted lipids had similar or slightly higher viscosity values than the membranes. There are a number of examples in other systems where membrane proteins have been shown to increase the viscosity of the bilayer (for a review, see Gennis and Jonas, 1977). Also, artifacts generated in the lipid extraction or the gross structural organization of a particular lipid dispersion (i.e., vesicle, hexagonal phase, aggregate, loss of asymmetry, etc.) could cause the dispersion to have different viscosities when compared with the lipid bilayer in the membrane. If this were the case, then a comparison of the viscosity of the lipid dispersion with the membrane would not be valid. In any event, the results illustrate the problem in comparing the properties of a lipid dispersion with a membrane whether the study involves structural aspects or the specificity involved in the reconstitution of an enzyme activity. The in vivo modification of the lipid composition of LM cells appears to offer a good approach toward understanding the role of lipids and viscosity in animal cell membranes.

Acknowledgments

We thank Nina Cohn and Dee Stahl for their skillful technical assistance and Drs. Gregorio Weber, William Mantulin, and the other members of the Weber research group for use of their excellent fluorescence instrumentation and helpful suggestions. We also thank Victor Engelhard and Arthur Hale for their assistance during the course of this study.

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Fluorescent Derivatives of the Pyruvate Dehydrogenase Component of the *Escherichia coli* Pyruvate Dehydrogenase Complex†

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ABSTRACT: One sulfhydryl group per polypeptide chain of the pyruvate dehydrogenase component of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* was selectively labeled with *N*-[*p*-(2-benzoxazolyl)phenyl]-maleimide (NBM), 4-dimethylamino-4′-maleimidostilbene (NSM), and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) in 0.05 M potassium phosphate (pH 7). Modification of the sulfhydryl group did not alter the enzymatic activity or the binding of 8-anilino-1-naphthalenesulfonate (ANS) or thiochrome diphosphate to the enzyme. The fluorescence of the NBM or NSM coupled to the sulfhydryl group on the enzyme was quenched by binding to the enzyme of the substrate pyruvate the coenzyme thiamine diphosphate, the coenzyme analogue thiochrome diphosphate, the regulatory ligands acetyl-CoA, GTP, and phosphoenolpyruvate, and the acetyl-CoA analogue, ANS. Fluorescence energy transfer measurements were carried out for the enzyme-bound donor-acceptor pairs NBM-ANS, NBM-thiochrome diphosphate, ANS-DDPM, and thiochrome diphosphate-DDPM. The results indicate that the modified sulfhydryl group is more than 40 Å from the active site and ~49 Å from the acetyl-CoA regulatory site. Thus, a conformational change must accompany the binding of ligands to the regulatory and catalytic sites. Anisotropy depolarization measurements with ANS bound on the isolated pyruvate dehydrogenase in 0.05 M potassium phosphate (pH 7.0) suggest that under these conditions the enzyme is dimeric.

The *Escherichia coli* pyruvate dehydrogenase complex which catalyzes the overall reaction

$$CH_3COCOOH + CoA + DPN^+ \rightarrow \text{Acetyl-CoA} + CO_2 + DPNH + H^+ \quad (1)$$

has been separated into three component enzymes (Koike et al., 1963; Eley et al., 1972). The pyruvate dehydrogenase component, E_1,[1] which utilizes thiamine diphosphate as a co-

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received December 13, 1976.* This work was supported by grants from the National Institutes of Health (GM 13292) and the National Science Foundation (BMS 73-012179).

‡ National Institutes of Health Postdoctoral Fellow (GM 05410).

[1] Abbreviations used are: E_1, pyruvate dehydrogenase; ANS, 8-anilino-1-naphthalenesulfonate; NBM, *N*-[*p*-(2-benzoxazolyl)phenyl]-maleimide; NSM, 4-dimethylamino-4′-maleimidostilbene; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; E_1-NBM, E_1-NSM, and E_1-DDPM, pyruvate dehydrogenase with a sulfhydryl group modified with NBM, NSM, and DDPM, respectively; CoA, coenzyme A; DPN, diphosphopyridine nucleotide.