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TIGHTLY (COVALENTLY) BOUND FATTY ACIDS IN CELL MEMBRANE PROTEINS

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We find tightly (covalently) bound fatty acids in membrane proteins from human red cells and polymorphonuclear cells and several tissues of the rat. These fatty acids are not removed by exhaustive extraction with organic solvents, by phospholipase A_2 treatment, or by sodium dodecyl sulfate (SDS). The fatty acids are released by refluxing with hot methanolic-HCl. They account for 0.2–6.8% of the total fatty acids. 50–60% of the tightly bound fatty acids are removed by mild alkaline hydrolysis. [^{3}H]Palmitic acid is incorporated into lipids and proteins of the red cell membrane. In red cell ghosts incorporation is stimulated by ATP and CoA but maximal incorporation is achieved with [^{14}C]palmityl-CoA. The labeling of lipids and proteins by [^{14}C]palmityl-CoA is greater in ghosts than in cells, however basal incorporation of [^{3}H]palmitic acid into lipids and proteins is greater in cells than in ghosts. [^{3}H]Palmitic acid is also incorporated into proteins of polymorphonuclear cells. The incorporation in polymorphonuclear cell proteins is inhibited by cycloheximide but not by actinomycin D. Labeled stearic, linoleic, linoleic, and palmitic acid are incorporated into red cell membrane proteins. These incorporated fatty acids are not removed by exhaustive solvent extraction, by prolonged dialysis or by SDS. On SDS-polyacrylamide gel electrophoresis several protein bands are labeled by all the fatty acids although each fatty acid gives a different pattern of labeling. In all cases, the major protein band which is labeled stains very weakly with Coomassie blue and runs between band 4.2 and 5. Other proteins which have appreciable label are bands 1, 2 and 3. We postulate that fatty acids are covalently attached to certain proteins as a post-translational event and act to direct, insert, and anchor the proteins in cell membranes. A similar postulate has been enunciated by Schmidt and Schlessinger (Cell 17, 813–819 (1979)).

Introduction

Membrane proteins interact with membrane lipids through several types of non-covalent forces [1,2]. The degree of specificity of lipid-protein interactions and whether lipids are covalently bound to membrane proteins remain provocative problems to be solved in membrane biology. The requirement for a lipid environment for membrane enzymes has been discussed in reviews by DePierre and Ernster [3] and Sanderman [4].

Fatty acids are known to bind to certain proteins such as albumin [5] and α-fetoprotein [6]. Albumin also binds phospholipids such as lysophosphatidylcholine [7]. Covalent binding of lipid to protein has been reported by Hantke and Braun [8]. The relatively low polarity of membrane proteins [9] accounts in part for their affinity for lipids and for their incorporation into artificial liposomes [10]. How certain proteins are directed and inserted into specific membranes is a current unresolved problem of great interest. The peptide signal hypothesis offers one mechanism for secretory protein recognition, insertion, and passage through membranes [11].

In order to test whether membrane proteins

contain covalently bound lipids we examined the fatty acids which remain in membrane proteins from rat tissues and human polymorphonuclear cells and red cells after exhaustive extraction with organic solvents. We find that membrane proteins contain tightly (covalently) bound fatty acids and ^{3}H-labeled fatty acids are incorporated into this fraction in the human red cell and human polymorphonuclear cell. The labeling of the membrane proteins of the red cell by [^{3}H]palmitate, [^{3}H]stearate, [^{3}H]linoleate and [^{3}H]linolenate and the labeling of polymorphonuclear cell protein by [^{3}H]palmitate are also given in this report.

Methods and Reagents

White Sprague-Dawley male rats (150–180 g) were sacrificed by decapitation and the organs were removed. One gram of tissue from heart, kidney, spleen, brain, and liver were minced and homogenized in 20 ml of Krebs-Ringer bicarbonate buffer pH 7.4 at 0°C using a polytron homogenizer for all tissues except liver which was homogenized in a Dounce homogenizer. The homogenates were centrifuged at 2000 rev./min for 10 min at 4°C. The supernatants were removed and centrifuged at 30000 rev./min for 1 h at 4°C in a type 40 rotor on a Beckman ultracentrifuge. The pellets containing mitochondria, microsomes and plasma membranes were extracted by the procedure of Folch et al. [12]. This is Extract I. The delipidized protein pellets were extracted twice with 3 ml chloroform/methanol (1:1, v/v) and three times with 3 ml of methanol. Analysis showed the last two methanol extracts had no detectable fatty acids. These extracts were combined with Extract I and are designated loosely bound lipid. The solvents contained 0.01% butylated hydroxtoluene to prevent lipid peroxidation.

The delipidized protein pellets were suspended in 4 ml of 3 M HCl in methanol, sonicated, and heated at 80°C for 2 h. The tubes were cooled and extracted with hexane to obtain the fatty acid methyl esters. The hexane phase was washed with 0.1% aqueous Na_2CO_3 and then with water to remove any non-esterified fatty acids. The hexane phase was dried under N_2 and the fatty acid methyl esters were dissolved in 200 μl of hexane. These represent the tightly bound fatty acids. The loosely bound lipids given above were esterified in methanol-HCl and the fatty acid methyl esters were dissolved in 2 ml of hexane.

The supernatant of the liver sample obtained by centrifuging the homogenate at 30000 rev./min for 1 h represents the cytosol proteins. The proteins were precipitated by addition of trichloroacetic acid to give a final concentration of 5% trichloroacetic acid. The protein pellets were washed with distilled water and extracted as given above for the membrane pellets to obtain tightly and loosely bound fatty acids.

Liver (2 g) was used to obtain mitochondria and microsomes using the method of Schnaitman and Greenawalt [13]. The tightly and loosely bound lipids were isolated as described above.

Red cell ghosts were prepared by the method of Dodge et al. [14]. Polymorphonuclear leukocytes were prepared by the method of Böyum [15]. The tightly and loosely bound fatty acids were obtained as described above for the rat tissues. Red cells were obtained from the Red Cross Blood Bank or from volunteers. SDS-polyacrylamide gel electrophoresis was carried out by the procedure of Laemmli [16]. Protein was analyzed by the method of Lowry et al. [17].

The fatty acids methyl esters of the tightly and loosely bound lipids were analyzed by gas chromatography using a Hewlett-Packard 5830A chromatograph equipped with a flame detector and a microprocessor. Separation of the fatty acids was done on a 6 ft $\times$ 0.25 inch (diameter) glass column packed with 10% diethyleneglycol succinate on 100/120 mesh Supelcoport (Supelco, Inc.). The analysis was carried out at 190°C using a nitrogen flow rate of 3 ml/min. Standard fatty acid methyl esters (PUFA-1, PUFA-2, even chain $C_{14} - C_{24}$, odd chain $C_{13} - C_{21}$) were obtained from Supelco, Inc. The results are expressed as area % of each fatty acid relative to the total area of all fatty acids. By use of a quantitative mixture of fatty acids, the average response of the flame detector was determined per μg of fatty acid mixture. This was used to convert the total fatty acid area of the tissue samples to μg of fatty acid.

The following labeled fatty acids were obtained from Amersham: [1-^{14}C]stearic acid, (56.5 mCi/mmol); [1-^{14}C]linoleic acid, (52 mCi/mmol); [1-^{14}C]linolenic acid, (56.2 mCi/mmol); [1-^{14}C]-

palmityl-CoA, (59 mCi/mmol); [9,10-^{3}H]-palmitic acid, (500 mCi/mmol). Cycloheximide, and actinomycin D were obtained from Sigma Chemical Co.

Incubation of cells or ghosts with labeled fatty acids. Unless stated otherwise, red cells, polymorphonuclear cells or red cell ghosts were incubated at 37°C in a total volume of 2.0 ml of Krebs-Ringer phosphate buffer pH 7.4 containing 1% bovine serum albumin and 5 mM glucose. In most experiments 0.5–1 ml of packed red cells or ghosts from 0.5 to 1 ml of packed red cells or $(1–2) \cdot 10^7$ polymorphonuclear cells were used. The red cells were washed three times in 10 ml of isotonic NaCl prior to use in order to remove white cells and platelets.

Results

The fatty acid methyl esters representing loosely and tightly bound lipids are given in Table I. The tightly bound fatty acids represent a small fraction (0.2–6.8%) of the total fatty acids. Heart and liver contained the smallest amount and red cells contained the largest amount of tightly bound fatty acid.

The loosely and tightly bound fatty acids were analyzed by gas chromatography. The results are given in Table II. There were specific differences in each tissue. Thus, the tightly bound lipids of heart were richer in 18:0 and 18:1 but had less 18:2 fatty acids than the loosely bound lipids. The content of 18:1 fatty acid which was tightly bound was higher in heart, spleen, total liver, liver mitochondria and red cells. The content of 18:2 fatty acid which was tightly bound was lower in spleen, kidney, liver microsomes and red cells, but higher in liver mitochondria. The polyunsaturated 20:4 acid which was tightly bound was considerably enriched in brain and red cells but lower in liver mitochondria. Therefore, each tissue had a pattern of tightly bound fatty acid which was different. It remains to be determined whether these fatty acids are directly covalently bound to membrane proteins or to other lipids such as phospholipids and glycolipids which are covalently bound to membrane proteins. In the case of the red cell we find that the fatty acids are not removed by extensive dialysis or by phospholipase A_2 treatment of delipidized ghosts but 50–60% are removed by treatment with 0.2 M NaOH at 37°C for 1 h. These latter fatty acids may be linked to proteins by carboxylic ester linkages which are labile to mild alkaline hydrolysis.

TABLE I

LOOSELY AND TIGHTLY BOUND FATTY ACIDS OF PARTICULATE AND CYTOSOL FRACTIONS OF DIFFERENT TISSUES

The results represent μg of fatty acid per g wet weight of rat tissue or per ml of packed red cells. The results on isolated liver mitochondria, microsomes and cytosol are from 2 g of wet weight liver. The experimental details are given in the text. L, loosely bound; T, tightly bound.

Tissue (rat)	Fatty acids (μg)		
	L	T	%T
Heart	4743	23	0.48
Spleen	2415	94	3.6
Liver	5148	34	0.66
Brain	2146	59	2.7
Kidney	2709	80	2.9
Liver mitochondria	4723	11	0.23
Liver microsomes	6488	134	2.0
Liver cytosol	785	16	2.0
Red cells (human)	1925	140	6.8

We next investigated the incorporation of labeled fatty acids into red cell and polymorphonuclear cell membrane proteins and lipids. The data in Table III show that ATP stimulates the incorporation of [^{3}H]palmitate into the lipids and proteins of red cell ghosts. 0.5, 1.0 and 2.0 mM ATP are equally effective in enhancing the incorporation into lipids whereas 4 mM ATP inhibits this incorporation. However, the incorporation into proteins is steadily increased by 0.5 to 4.0 mM ATP. A 4-fold increase in incorporation was produced by ATP into lipids whereas a 20-fold increase was produced into protein.

Since fatty acid activation generally requires conversion to the acyl-CoA ester, we examined the effect of ATP and CoA on the incorporation of [^{3}H]palmitate into red cell ghost lipids and proteins. As seen in Table IV, the greatest incorporation was obtained by the combined addition of ATP and CoA. These produced a 14-fold increase in the incorporation into lipid and a 20-fold increase into protein.

TABLE II

LOOSELY AND TIGHTLY BOUND MEMBRANE FATTY ACIDS OF RAT TISSUES AND HUMAN RED CELLS

Fatty acid values (average of 2 experiments) represent the % of total area of all fatty acids. The designation 16:0 signifies 16 C atoms and 0 double bonds. Loosely and tightly bound fatty acids and the details of the fatty acid analysis are given in the text. For sake of brevity not all fatty acids are shown. For this reason the values given may not add up to 100%.

	Loosely bound						Tightly bound					
	16:0	18:0	18:1	18:2	20:4	22:6	16:0	18:0	18:1	18:2	20:4	22:6
Heart	12	18	10	24	15	9	13	26	18	12	16	10
Spleen	22	13	11	11	18	3	34	20	15	6	21	3
Brain	21	18	16	4	12	17	29	20	13	1	24	12
Kidney	19	16	8	14	21	3	33	22	8	8	20	3
Liver (total)	20	16	8	15	19	10	18	17	11	15	19	9
Liver mito.	17	17	8	21	20	11	9	18	16	27	12	7
Liver micro.	19	18	8	14	19	10	15	30	8	9	20	7
Red cells	18	12	10	8	12	6	18	29	14	Tr	24	Tr

TABLE III

EFFECT OF ATP CONCENTRATION OF THE LABELING OF RED CELL GHOST LIPIDS AND PROTEINS BY [^{3}H]PALMITATE

Ghosts from 1.0 ml of packed red cells were incubated for 1 h at 37°C in 2 ml of Krebs-Ringer phosphate buffer containing 10 μCi of [^{3}H]palmitate. The experimental details are given in the text. Values are the mean ± S.D. for $N = 6$.

	pmol/μmol lipid	pmol/mg protein
Control	12.6 ± 0.8	0.26 ± 0.03
0.5 mM ATP	49 ± 1.3	2.83 ± 0.4
1 mM ATP	50 ± 2.4	3.49 ± 0.17
2 mM ATP	51 ± 6.9	3.71 ± 0.11
4 mM ATP	32 ± 4.2	4.71 ± 0.32

TABLE IV

EFFECT OF ATP AND CoA ON THE INCORPORATION OF [^{3}H]PALMITATE INTO MEMBRANE LIPIDS AND PROTEINS OF RED CELL GHOSTS

Ghosts from 1 ml of packed red cell were incubated with 10 μCi of [^{3}H]palmitate with and without 1 μM CoA and 1 mM ATP in 2 ml of Krebs-Ringer phosphate buffer for 1 h at 37°C. Analysis of the lipids and proteins are given in the text. Values are the mean ± S.D. for $N = 6$.

	pmol/μmol lipid	pmol/mg protein
Control	12.4 ± 0.4	0.14 ± 0.02
CoA	13.6 ± 0.6	1.17 ± 1.2
ATP	36 ± 2.2	0.97 ± 0.4
CoA + ATP	163 ± 13	2.8 ± 0.4

Since ATP + CoA gave the greatest enhancement of incorporation of [^{3}H]palmitate into ghosts, we studied the incorporation of [^{14}C]palmityl-CoA into ghost proteins and lipids. The results in Table V show the labeling of lipids and proteins from this precursor. The labeling in ghosts was much greater than in cells. Most of the radioactivity in the membrane protein fraction was non-

TABLE V

LABELING OF MEMBRANE LIPIDS AND PROTEINS IN INTACT RED CELLS AND GHOSTS BY [^{14}C]PALMITYL-CoA

0.5 ml of packed cells or ghosts from 0.5 ml of packed cells were incubated for 1 h at 37°C in 2 ml of Krebs-Ringer phosphate buffer containing 2 μCi of [^{14}C]palmityl-CoA. Analysis of the lipids and proteins are given in the text. Values are the mean ± S.D. for $N = 3$. Dialysis was carried out at 4°C for 48 h against 1% SDS containing 1% bovine serum albumin.

	pmol/μmol lipid	pmol/mg protein
Cells	25 ± 1.2	35 ± 0.8
Ghosts	1193 ± 7	81 ± 3.5

	Total pmol incorporated into proteins [a]	
	Before dialysis	After dialysis
Cells	0.17 ± 0.05	0.13 ± 0.05
Ghosts	22.3 ± 0.7	16.5 ± 0.3

[a] This represents 10% of the total samples.

TABLE VI

LABELING OF LOOSELY AND TIGHTLY BOUND LIPIDS OF RED CELLS WITH FATTY ACIDS

Fresh red cells (0.5 ml packed cells) were incubated in 2 ml of Krebs-Ringer phosphate buffer containing labeled fatty acid as follows: (20 μCi [^{14}C]stearic; 20 μCi [^{14}C[linoleic acid; 20 μCi [^{14}C]linolenic; and 50 μCi of [^{3}H]palmitic acid. The cells were incubated for 2 h at 37°C, then centrifuged, washed three times with Krebs-Ringer phosphate buffer and ghosts were prepared. Aliquots of the ghost pellets were counted for radioactivity. The remaining ghost pellets were delipidized by extraction witgh chloroform/methanol by the procedure of Folch et al. [12] and then textracted with chloroform/methanol (1 : 1, v/v) twice and two times with methanol. The pellets were suspended in water and aliquots taken for counting. Values are the mean ±S.D. for $N=3$. The nmol of labeled fatty acid added were as follows: stearate, 354; linoleate, 384; linolenate, 356; palmitate, 200.

Fatty acid	nmol/mg protein	
	Loosely bound	Tightly bound
[^{14}C]Linoleic	44 ±0.5	0.4 ±0.04
[^{14}C]Linolenic	22 ±0.14	0.39 ±0.18
[^{14}]Stearic	78 ±1.1	0.16 ±0.08
[^{3}H]Palmitic	8.4±0.1	0.083±0.03

dialyzable at 4°C after 48 h.The time course of incorporation of [^{14}C]palmityl-CoA into lipids and proteins of red cell ghosts shows that a plateau is not reached by 3 h for the lipids but is attained by one hour for the proteins (data not provided for sake of brevity).

We examined the incorporation of labeled palmitate, stearate, linoleate, linolenate into the lipids and proteins of intact red cells. The results in Table VI show that the order of incorporation into lipids is stearate > linoleate > linolenate > palmitate but the incorporation into protein is stearate > linoleate = linolenate > palmitate. The lower incorporation by palmitate may be due to its being used at a smaller concentration (200 nmol) as compared to 354–384 nmol for the other three fatty acids. When compared to red cell ghosts (Table III) it can be seen that [^{3}H]palmitate was incorporated to a much greater extent into lipids and proteins of intact cells.

Since human red cells contained an appreciable amount of tightly bound fatty acids we examined in more detail the incorporation of labeled palmitic

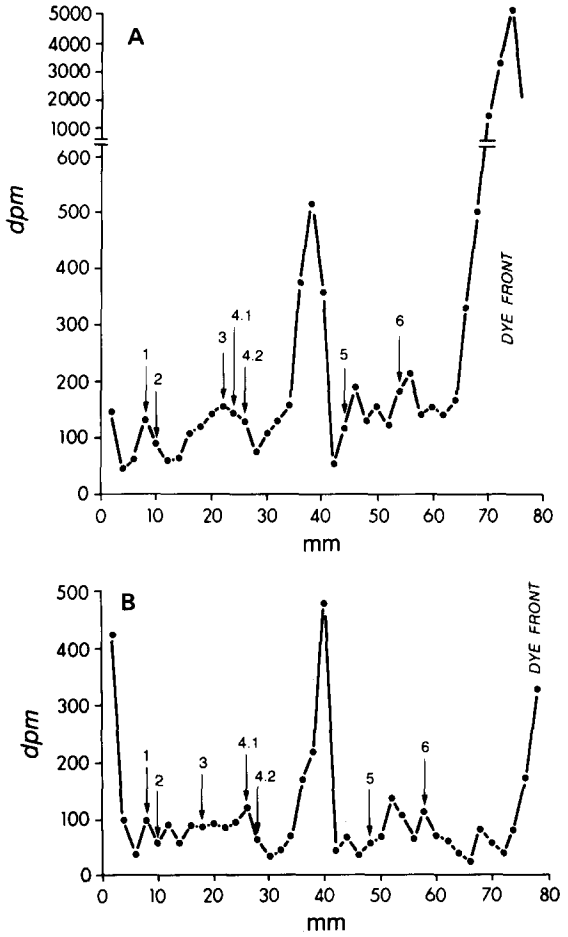

Fig. 1. SDS-gel electrophoresis of [^{3}H]palmitate-labeled red cell membrane proteins before and after delipidation. The incorporation of palmitic acid into the red cell membrane was carried out by incubating 1 ml of packed human red cells for 2 h at 37°C in 2 ml of Krebs-Ringer phosphate buffer containing 50 μCi of [^{3}H]palmitic acid. Ghosts were prepared by hyptonic lysis in 10 mM Tris buffer pH 7.4 containing 1 mM EDTA [14]. The ghost sample was divided into two equal parts. One part was dissolved in 1% SDS and aliquots containing 40 μg of protein were used for electrophoresis (Fig. 1A). The other part was extracted by the method of Folch [12] and further extracted with chloroform/methanol (1 : 2, v/v) and methanol (Fig. 1B). The protein pellets were dissolved in 1% SDS at 90°C for 1 h. SDS polyacrylamide gel electrophoresis was carried out by the method of Laemmli [16] using 40 μg of protein per tube. The gels were stained with Coomassie blue and destained in 7.5% acetic Acid in a Bio-Rad diffusion destainer. The gels were cut into 2 mm slices, dissolved in 0.2 ml of NCS/water (9 : 1), heated 2 h, at 50°C, cooled, neutralized with 80 μl of glacial acetic acid and counted in 15 ml of Amersham ACS cocktail. Three tubes were combined representing 120 μg of protein. Protein was assayed by the method of Lowry et al. [17]. Counting was done on a Searle Delta 300 scintillation counter.

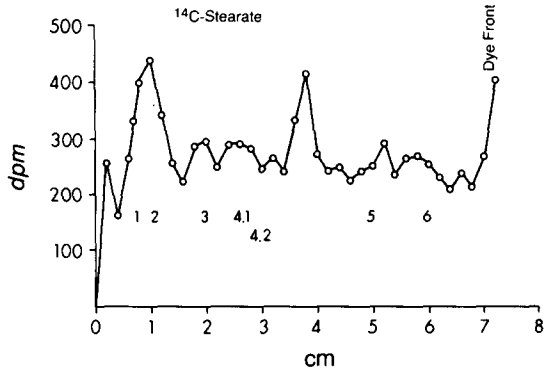

Fig. 2. SDS-gel electrophoresis of [^{14}C]stearate-labeled delipidized red cell membrane proteins. Red cells were labeled with 20 μCi of [^{14}C]stearic acid, ghosts were prepared, delipidized and run on SDS-polyacrylamide gels as described in Fig. 1.

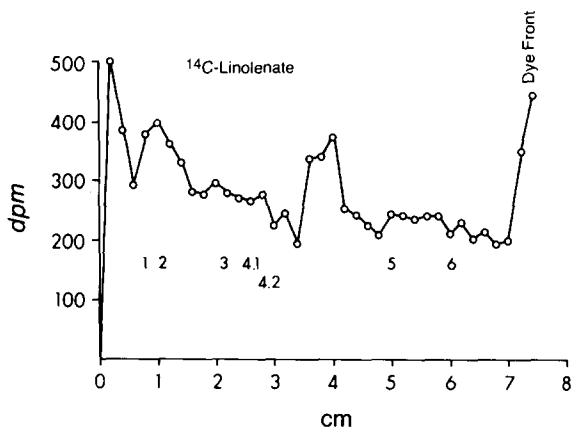

Fig. 4. SDS-gel electrophoresis of [^{14}C]linoleate-labeled delipidized red cell membrane proteins. Red cells were labeled with 20 μCi of [^{14}C]linolenic acid, ghosts were prepared, delipidized and run on SDS-gel as described in Fig. 1.

acid into this tightly bound fraction. Intact red cells were incubated with [^{3}H]palmitate, ghosts were prepared and extracted to obtain the loosely and tightly bound lipids. The fatty acid methyl esters were separated by thin-layer chromatography on silver nitrate-treated silicic acid using chloroform/isopropanol (98.5:1.5, v/v) as solvent. 2.2% of the total incorporated radioactivity was present as tightly bound fatty acid. Of this, 98% migrated as palmitic acid. This experiment showed that the [^{3}H]palmitate was incorporated as an intact molecule and was not extensively metabolized.

When the fatty acid-labeled red cell ghosts and delipidized ghosts were analyzed by SDS-gel electrophoresis, one major labeled band and several minor labeled bands were observed (Figs. 1A and 1B). The major labeled band moved between bands 4.2 and 5.0 and by Coomassie blue staining was seen as a weak band. The radioactivity in this band was not removed by SDS treatment or by delipidizing with organic solvents. This represents very tightly bound or covalently bound fatty acid in this membrane protein. The radioactivity in some of the other bands was decreased to some extent by delipidation. Thus several red cell membrane proteins contain fatty acids which are tightly bound to different degrees.

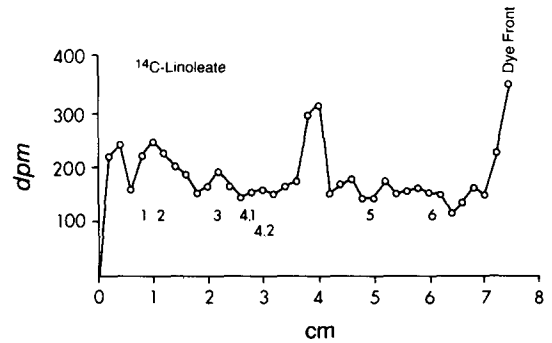

Fig. 3. SDS-gel electrophoresis of [^{14}C]linoleate-labeled delipidized red cell membrane proteins. Red cells were labeled with 20 μCi of [^{14}C]linoleic acid, ghosts were prepared, delipidized and run on SDS-gels as described in Fig. 1.

In order to see whether different fatty acids would yield different labeled proteins we treated red cells with [^{14}C]stearic acid, [^{14}C]linoleic acid, and [^{14}C]linolenic acid. The delipidized membrane proteins were dissolved in SDS and separated by gel electrophoresis. The patterns of the labeled red cell proteins are shown in Figs. 2–4. The patterns are similar but not identical. In all samples a major labeled protein band was seen which migrated between bands 4.2 and 5. This band was the major protein labeled with [^{3}H]palmitic acid. Label also found in bands 1, 2, 3, 4.1, 4.2, 5, and 6 in varying amounts. Bands 1 and 2 were labeled most heavily by [^{14}C]stearic acid. In all samples, very high molecular weight proteins which barely migrated into the gel had incorporated fatty acids. This band was most pronounced with [^{14}C]lino-

TABLE VII

EFFECT OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON THE LABELING OF POLYMORPHONUCLEAR CELL LIPIDS AND PROTEINS FROM [^{3}H]PALMITIC ACID

$1.2 \cdot 10^7$ Polymorphonuclear cells were incubated in 2 ml of Krebs-Ringer phosphate buffer containing 5 mM glucose, 1% bovine serum albumin, 50 μCi of [^{3}H]palmitic acid and with and without 100 μg of cycloheximide, or 100 μg of actinomycin D. The cells were incubated for 1 h at 37°C, washed, and extracted by the procedure of Folch et al. [12] to obtain the total lipids. The protein residues were delipidized further as explained in the text. The protein residues were sonicated in 0.5 ml of 0.1 M NaOH and 100 μl aliquots were removed for counting and for protein analysis. The lipid extract was made to 0.5 ml volume in chloroform/methanol (1 : 1, v/v) and 100 μl aliquots were removed for analysis of lipid P and for counting. Values are the mean ± S.D. for $N = 6$.

	Lipids (pmol incorporated per nmol P_i)	Proteins (pmol incorporated per mg protein)
Control	89.2 ± 2.4	73.2 ± 5
Cycloheximide	71.8 ± 1.6	69.4 ± 1.5
Actinomycin D	53.0 ± 3.9	72.4 ± 2

lenic acid. Since the free fatty acids moved with the dye front, the labeled proteins appear to contain covalently bound fatty acids since they are not removed by exhaustive solvent extraction or by extensive dialysis.

The labeling of lipids and proteins of polymorphonuclear cells was also carried out in order to compare a cell which actively synthesizes protein with the red cell which does not synthesize protein. A time study showed that by 45 min the incorporation of [^{3}H]palmitate into delipidized proteins reached a plateau. In contrast, the labeling of lipids continues to increase up to 3 h (data not shown for sake of brevity). The labeling of proteins by [^{3}H]palmitate is not influenced by actinomycin D but is inhibited to a small degree by cycloheximide (Table VII). The labeling of lipids is inhibited by both cycloheximide and actinomycin D.

Discussion

Our results provide new data on the occurrence of tightly (covalently) bound fatty acids in cell membrane proteins and the incorporation of fatty acids into red cell membrane proteins and into polymorphonuclear cell proteins. We have shown that the intact red cell can incorporate four different labeled fatty acids into proteins and that some of these fatty acids appear to be covalently bound since they are not removed by SDS-treatment, by delipidizing the ghosts with organic solvents or by extensive dialysis. We also find that the tightly protein bound palmitate in the red cell membrane is not removed by phospholipase A_2 treatment. However, 50–60% is removed by 0.2 M NaOH treatment at 37°C for 1 h. Thus some of the palmitate appears to be bound as a carboxylic ester, possibly to serine and threonine hydroxyl groups on the proteins. The alkali stable palmitate may be covalently linked as an amide bond to proteins. Since the mature human red cell has no capacity to synthesize proteins, we studied the polymorphonuclear cell to see whether the palmitic acid is attached to the proteins in the membrane. We find that the labeling of polymorphonuclear proteins is not influenced by actinomycin D but is inhibited by cycloheximide. The cycloheximide inhibition is consistent with the fatty acid labeling being a post-translational event.

Post-translational modification of proteins can occur through methylation, phosphorylation and glycosylation. Recently, post-translational modification by fatty acid acylation has been reported [18,19]. This latter modification by fatty acids may play a role in inserting proteins into membranes. How specific membrane proteins are directed to an inserted into specific membranes is a current unresolved problem in membrane biology. The nature of the linkage of the fatty acid to proteins remains to be elucidated. We plan to determine whether they are directly linked to protein or whether they are linked to phospholipids, glycolipids acylglycerols which in turn are linked to protein.

The covalent attachment of lipids to protein has been found in the outer membrane of *E. coli* [8]. Lipid A of *E. coli* anchors the lipopolysaccharide to the outher membrane [20]. Brain proteolipids and myelin proteins also contain bound fatty acids [21–23]. These cases offer evidence that lipids can be covalently attached to membrane proteins in prokaryote and eukaryote cells.

The Blobel signal hypothesis [11] offers a pro-

vocative mechanism for insertion of proteins into the membranes but suffers the drawback that the signal peptide is relatively small and in some cases is removed after insertion in the membrane. Therefore, it does not explain adequately how the major part of the protein is inserted and kept in the membrane nor does it explain how certain proteins are selectively found in specific membranes. We postulate as do Schmidt and Schlessinger [18,19] that post-translational attachment of fatty acids (or other lipids) at specific sites on the protein may act to direct, insert, and anchor these proteins in the membrane. It is also possible that specific fatty acids or specific phospholipids or glycolipids may direct specific proteins to specific membranes.

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