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Identification of the Thiol Ester Linked Lipids in Apolipoprotein B†

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ABSTRACT: Human plasma low-density lipoproteins of 1.032–1.043 g/mL density were totally delipidized. The reduced and carboxymethylated apolipoprotein B was incubated with 50 mM [^{14}C]methylamine at pH 8.5 at 30 °C. Covalent incorporation of [^{14}C]methylamine was observed with concomitant generation of new sulfhydryl groups, which could be blocked with [^{3}H]- or [^{14}C]iodoacetic acid. One type of the [^{14}C]methylamine-modified products was separated from the protein and was found to be lipid in nature. Its R_f on thin-layer chromatography (TLC) was similar to that of the synthetic *N*-methyl fatty acyl amides. After purification with TLC and transesterification in 3 N methanolic HCl, methyl esters of C_{16} and C_{18} fatty acids at 1:1 ratio were identified by gas–liquid chromatography. The transesterification method was verified with the known *N*-methyl fatty acyl amides. These results suggest the presence of labile thiol ester linked palmitate and stearate in apolipoprotein B. Under mild alkaline conditions, the thiol ester bonds are broken by methylamine and form *N*-methyl fatty acyl amides and release new –SH groups. Intramolecular thiol ester bonds linked between cysteine side chains and acidic amino acid residues were also found present, which will be reported separately.

Apolipoprotein B (ApoB)[1] is the major protein moiety of low-density (LDL) and very low density lipoproteins. It is an essential component of chylomicrons. ApoB is the major carrier for cholesterol and triglycerides in the circulation and the ligand of LDL receptor in many cells (Goldstein & Brown, 1977; Dashti et al., 1984). It plays a key role in the regulation of cholesterol metabolism (Goldstein & Brown, 1977) and in pathogenesis of atherosclerosis (Hoff et al., 1978).

Following delipidization of LDL, ApoB has a strong tendency to undergo aggregation and is insoluble in aqueous buffers directly. This nature has hampered the structural studies of ApoB. The reason for this aggregation tendency is not well understood.

Recently, the amino acid sequence of ApoB has been deduced from the sequence of its complement cDNA clone (Chen et al., 1986; Knott et al., 1986; Law et al., 1986; Cladaras et al., 1986). ApoB is a single polypeptide chain consisting of 4536 amino acids with a molecular weight of 512937. Twenty-five cysteine residues are present. However, the cDNA clone data cannot reveal the nature of the sulfur-containing linkages. We found ApoB extremely sensitive to alkaline and oxidative conditions. This has led us to suspect that some posttranslational labile linkages may be present in ApoB.

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[1] Abbreviations: ApoB, apolipoprotein B; LDL, low-density lipoproteins; LDL_2, LDL of 1.032–1.043 g/mL in this study; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; IAA, iodoacetic acid; MA, methylamine; εACA, ε-aminocaproic acid; buffer A, 0.02 M Tris/0.02% EDTA/0.13% εACA, pH 7.3; RCM, reduced and carboxymethylated; SDS, sodium dodecyl sulfate, sp act., specific activity; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; dpm, disintegrations per minute.

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Theoretically, the 25 cysteines could be present as –SH, –S–S–, and –C(=O)–S–. The purpose of this study was to determine the possible presence of thiol ester linkage in ApoB. This paper presents evidence for the presence of such a labile thiol ester linkage in ApoB and identification of the cleavage products as long chain fatty acids.

Experimental Procedures

Materials. Radioisotopes [^{14}C]methylamine (sp act. 56 mCi/mmol) and [^{3}H]- and [^{14}C]iodoacetic acid (90 and 58 mCi/mmol, respectively) were purchased from Amersham (Chicago, IL). Ultrogel AcA-202 was obtained from LKB (Sweden). Silica gel G-60 for TLC was from E. Merck (Darmstadt, West Germany). Methylamine hydrochloride and lipid standards for TLC including phosphatidylcholine, cholesterol, cholesteryl oleate, palmitic acid, palmitoleic acid, and linoleic acid were from Sigma (St. Louis, MO). Heptadecanoyl chloride and 9-*trans*-hexadecenoyl chloride were obtained from Nu Chek Prep., Inc. (Elysian, MN). Ultrapure urea was obtained from Schwarz/Mann, Orangeburg, NY, and was deionized before use (Huang & Lee, 1979).

Collection of Plasma. Plasma samples were collected by plasmapheresis from normolipidemic subjects after a 12-h overnight fast. Preservative mixtures as described previously (Lee et al., 1981, 1987) were added immediately.

Isolation of Low-Density Lipoproteins (LDL_2). The narrow density range of LDL_2 (apparent density 1.032–1.043 g/mL) was isolated immediately from the fresh plasma by a single-spin density gradient ultracentrifugation (Lee & Downs, 1982) and purified by reflotation (Lee et al., 1981).

Delipidization of LDL_2 and Solubilization of ApoB. The LDL_2 samples (2.5 mL in a 50-mL tube) containing 25 mg of protein were delipidized by five 1-h extractions with ethanol/ether (3:1 v/v) (Lee et al., 1981). One-tenth of the solvent from each extraction was concentrated for free fatty acid analysis by GLC until no free fatty acid was detected (see Table II). The protein residue was further extracted with 50 mL of ethanol for 1 h and washed with 0.05 M *N*-ethylmorpholine acetate, pH 7.3. ApoB was then dissolved in 6 M guanidine hydrochloride/0.02 M Tris containing preservatives (Lee et al., 1981) at pH 7.3. The reduced ApoB was carboxymethylated with iodoacetic acid (IAA) at pH 8.3 at room temperature for 1 h (Crestfield et al., 1963) and dialyzed extensively vs 6 M urea containing 0.02 M Tris/0.02% EDTA/0.13% ϵACA (designated buffer A), pH 7.3. Alternatively, the ApoB was dissolved in 2.5% SDS/0.05% DTT/buffer A, pH 7.3, followed with carboxymethylation. The reduced and carboxymethylated (RCM) ApoB was dialyzed against 0.1% SDS/buffer A. All procedures were carried out under N_2.

Methylamine Treatment. Methylamine (MA) was used for cleavage of thiol ester linkage (Tack et al., 1980). A total of 2 mCi of [^{14}C]MA was diluted with 1 M unlabeled MA to yield sp act. 7.0–16.7 mCi/mmol. The [^{14}C]MA was added to the RCM-ApoB (protein 3–4 mg/mL) in 6 M urea or in 2.5% SDS containing buffer A to make 50 mM MA. The mixture was adjusted to pH 8.3–8.5 with Tris and incubated under N_2 in a water bath at 30 °C with gentle shaking. Aliquots of 100–200 μL of the incubation mixture were taken out at time intervals and applied onto an Ultrogel AcA-202 disposable column (0.8 × 17 cm), which was equilibrated with 6 M urea or 0.1% SDS containing buffer A, pH 7.3, and eluted stepwise with the same buffer in 1-mL fractions. The eluted sample was stoppered under N_2 immediately after collection. Protein and radioactivity were determined for each fraction.

A 0.25-mL aliquot was taken from the eluted sample to incubate with 100 mM unlabeled MA for 1–3 h and rechromatographed to eliminate any nonspecific adsorption of the radioactive material.

Blocking with [^{3}H]- or [^{14}C]Iodoacetic Acid. A 0.25-mL aliquot was taken from the eluted sample containing [^{14}C]-MA-labeled RCM-ApoB to determine the presence of newly released sulfhydryl groups by interaction with [^{3}H]- or [^{14}C]IAA at 10 mM IAA (sp act. 90 mCi/mmol for [^{3}H]IAA and 7.0–13.3 mCi/mmol for [^{14}C]IAA) at pH 8.5 for 1 h at 30 °C. The incubation mixture was rechromatographed as above. Alternatively, the RCM-ApoB was incubated with 10 mM [^{14}C]IAA + 50 mM MA under the same condition as was used for [^{14}C]MA for time study. The eluted product was desorbed with 10-fold unlabeled IAA for 1–3 h and rechromatographed. Another alternative was to have two sets of RCM-ApoB: one incubated with 50 mM [^{14}C]MA + 10 mM [^{14}C]IAA (sp act. 7.0 mCi/mmol for each reagent) and one incubated with 50 mM [^{14}C]MA alone (same sp act.). The incorporation due to [^{14}C]IAA was calculated from the difference in the two sets.

Extraction and Analysis of the Released Lipids from the Incubation Mixture. After an extended length of incubation between [^{14}C]MA and RCM-ApoB samples in urea/buffer (usually 90–120 h) to ensure complete cleavage of any buried thiol ester bonds, a 0.2-mL incubation mixture was taken for three extractions each with 10 mL of hexane/chloroform (1:1 v/v) by vortexing for 1 h under N_2. The organic phase was concentrated to a small volume and applied to a TLC plate.

Separation by TLC. The unknown and the standard lipids and [^{14}C]MA at pH 8.5 were applied onto TLC and run in solvent system 1 (hexane/diethyl ether/glacial acetic acid, 113:35:3 v/v). The lipid spots were detected by iodine vapor in a closed chamber. Since the quantity of unknown was too low to be detected with iodine, the R_f of unknown and [^{14}C]MA were determined by radioactivity of silica gel scraped from TLC at 1-cm intervals. The remaining silica gel containing unknown was extracted with chloroform/methanol (2:1) and applied to a second TLC plate along with the standard lipids and run in solvent system 2 (hexane/ethyl acetate/methanol/glacial acetic acid, 90:20:20:2 v/v). A portion of the silica gel was scraped off at 1-cm intervals and extracted for measuring radioactivity.

Transesterification of Unknown. The labeled material after TLC purification in the second solvent system was scraped off with silica gel and extracted with chloroform/methanol (2:1 v/v). The solvent was dried and the material redissolved in methanol and transferred to a hydrolysis ampule; 2.0 μg of internal standard C_{15} fatty acid was added and dried. Then 3 N methanolic HCl was added and the ampule was sealed. Transesterification was carried out at 80 °C for 20 h. The solution was dried, redissolved in 0.2 mL of hexane, and analyzed with a Packard-Becker Model 421 gas chromatograph equipped with a flame ionization detector (McConathy et al., 1981).

Organic Syntheses of N-Methyl Fatty Acyl Amides. N-Methylheptadecanoyl (C_{17}) amide and *N*-methyl-9-*trans*-hexadecenoyl ($C_{16:1}$) amide were synthesized from the corresponding fatty acyl chlorides with methylamine hydrochloride. Methylamine hydrochloride was converted to free MA with 5 N NaOH and extracted into an ether phase. 9-*trans*-hexadecenoyl chloride (0.8 g) was dissolved in ether in a glass-stoppered reaction flask and mixed with excess MA by stirring for 2 h at room temperature, allowed to set overnight, and dried under N_2. The residue was suspended in 20 mL of hexane and shaken in a boiling water bath. The excess MA

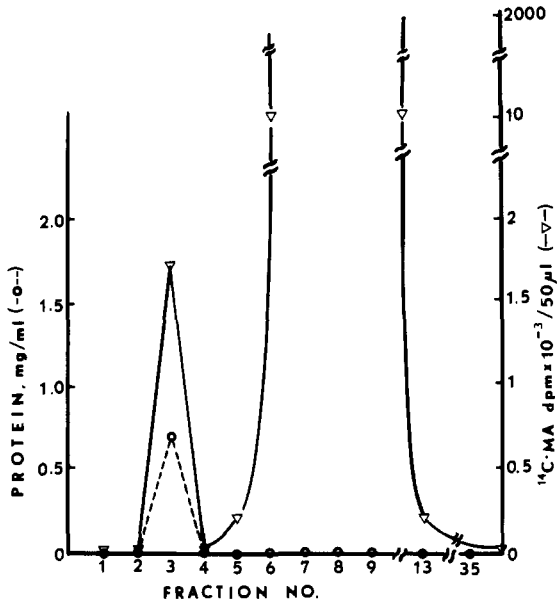

FIGURE 1: Typical elution profile of the incubation mixture of RCM-ApoB and [^{14}C]MA on an Ultrogel AcA-202 column. RCM-ApoB at 3.45 mg/mL in 6 M urea/0.075 M Tris/0.02% EDTA/0.13% ϵACA was incubated with 50 mM [^{14}C]MA (sp act. 7.0 mCi/mmol) at pH 8.3 and 30°C for 21 h, and 0.2 mL was applied to the disposable column (0.8 × 17 cm) and eluted at 1 mL/fraction. The incorporation of [^{14}C]MA into RCM-ApoB was 1.6 mol/mol.

turning into insoluble methylamine hydrochloride salt was discarded. The hexane solution was concentrated, and the white solid that appeared was the synthetic product *N*-methylhexadecenoyl amide. The product was redissolved in hexane, washed with water, and dried; the final yield was 81%. The melting point of the product was determined with MEL-TEMP (Laboratory Device, Cambridge, MA) and was 58–60°C. The same method was used to synthesize *N*-methylheptadecanoyl amide and yielded 76%; the melting point was 78–80 °C.

Analytical Methods. Protein determination was carried out according to the method of Lowry et al. (1951) using human serum albumin as standard.

^{14}C and ^{3}H radioactivities were measured in a liquid scintillation spectrometer system (Packard 2450 Tri-Carb). Column fractions or the silica gel was mixed with 5 mL of liquid scintillation cocktail (Ready Solv MP, Beckman, Fullerton, CA). The dpm values were converted to microcuries and then to nanomoles of [^{14}C]MA or [^{14}C]IAA on the basis of the specific activity of the isotope employed.

Results and Discussion

To determine whether thiol ester linkage was present, ApoB was first reduced and carboxymethylated. This eliminated further participation of sulfhydryls and disulfide linkages in the reaction. The RCM-ApoB was then treated with methylamine, a reagent specifically reactive with thiol ester and which forms an irreversible covalently modified product (Tack et al., 1980; Howard, 1981; Torchinsky, 1981). If no thiol ester linkage is present, no covalent incorporation of [^{14}C]MA should occur. This was tested with human serum albumin as control, and only 0.048 mol of [^{14}C]MA was found per mole of albumin after 52-h incubation with 50 mM [^{14}C]MA.

Incorporation of [^{14}C]MA and [^{3}H]IAA into RCM-ApoB. Figure 1 shows a typical chromatogram for RCM-ApoB after incubation with [^{14}C]MA. The protein and ^{14}C radioactivity coeluted in tube 3, which had clean separation from the small molecules eluted in tubes 6–12 where free [^{14}C]MA came off.

Table I: Covalent Incorporation of [^{14}C]Methylamine and [^{14}C]Iodoacetate in Reduced and Carboxymethylated Apolipoprotein B[a]

incubation time (h)	(A) incorpn of [^{14}C]methylamine (mol/mol of ApoB)	(B) incorpn of [^{14}C]iodoacetate (mol/mol of ApoB)	(B) − (A) (mol/mol of ApoB)
0.5	3.3	3.2	−0.1
2.5	2.9	8.2	5.3
6.0	2.9	10.0	7.1
24.0	2.9	12.5	9.6

[a] RCM-ApoB at 4.2 mg/mL in 0.1% SDS/0.075 M Tris/0.02% EDTA/0.13% ϵACA was incubated with (A) 50 mM [^{14}C]methylamine (sp act. 16.66 mCi/mmol) and (B) 50 mM methylamine (unlabeled) + 10 mM [^{14}C]iodoacetic acid (sp act. 13.33 mCi/mmol) at pH 8.5 and 30 °C. The excess radioactive reagents were separated by gel filtration. Fraction 3, containing the labeled RCM-ApoB, was determined for protein and radioactivity. An aliquot was desorbed with 100 mM unlabeled methylamine for (A) and 100 mM unlabeled iodoacetic acid for (B) at pH 8.5 and 25 °C for 1 h and rechromatographed. The eluted fraction 3 was reanalyzed, and the results are reported under columns A and B. Analyses of samples from (B) on SDS–polyacrylamide gel electrophoresis confirmed that the incorporation of [^{14}C]-iodoacetate at the ApoB-100 position increased with time (figure not shown).

The 1.6 mol of [^{14}C]MA associated with each mole of RCM-ApoB could not be removed after incubation with 100 mM unlabeled MA, suggesting covalent linkage occurred. An aliquot from the eluted [^{14}C]MA-RCM-ApoB was further incubated with [^{3}H]IAA and [^{14}C]IAA separately. Both ^{3}H and ^{14}C were incorporated into the [^{14}C]MA-RCM-ApoB sample. The ^{14}C radioactivity in the new product was 2.6-fold higher than that in the original [^{14}C]MA-RCM-ApoB sample. These results indicated the presence of newly released sulfhydryl groups in the MA-treated RCM-ApoB and suggest the possibility of the presence of thiol ester bond(s) in ApoB. A time study was carried out on RCM-ApoB incubated with 50 mM [^{14}C]MA and with 10 mM [^{14}C]IAA + 50 mM MA simultaneously. Table I shows that, initially, the incorporations of the *S*-[^{14}C]carboxymethyl (CM) group and the *N*-[^{14}C]MA group into RCM-ApoB were in equal molar ratio. However, as the incubation time increased, the incorporation of *S*-[^{14}C]CM increased, but it was not accompanied by the increased incorporation of [^{14}C]MA. These results suggest that both intra- and intermolecular thiol ester bonds may exist in ApoB. Three equal molar incorporations of *S*-[^{14}C]CM and *N*-[^{14}C]MA represent the possible presence of three intramolecular thiol ester bonds, whereas the extra 5–10 mol of *S*-[^{14}C]CM incorporated represent the possible presence of intermolecular thiol ester bonds. While the intramolecular thiol ester bonds found to be linking cysteine side chains and acidic amino acid residues of ApoB will be reported separately, this paper will address the intermolecular thiol ester linkages. As time increased, the molecules carrying the acyl group covalently linked to the [^{14}C]MA might be separately eluted from the main polypeptide chain of RCM-ApoB, whereas the newly exposed –SH groups are located at the main chain and give rise to the increased incorporation of [^{14}C]IAA with time. With this assumption, searching for the smaller molecule containing [^{14}C]MA-radioisotope label was initiated. We speculated on two possibilities: (1) the small molecule could be a small peptide or (2) it could be a lipid.

Presence of ^{14}C-Labeled Lipid Materials. After the excess [^{14}C]MA was eluted with urea/buffer from the Ultrogel column used in Figure 1, and the eluate reached background radioactivity level, the column was further washed with 45 mL of H_2O. The column was then cut at 1.5-cm intervals, and the Ultrogel was collected and extracted with 1 mL of H_2O and 10 mL of diethyl ether by vortexing for 1 h under N_2. The

Table II: Extraction Pattern of Unbound Fatty Acids from LDL_2 with Ethanol and Ether (3:1)[a]

extraction no.	fatty acid (μg)							
	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	total	% of total
1	42.22	8.85	20.06	74.80	179.32	47.89	373.14	75.06
2	44.01	0.79	7.35	13.79	35.08	7.29	108.31	21.79
3	4.18	0	0.30	0.73	0.63	0	5.84	1.17
4	5.72	0	0.86	0.85	2.41	0	9.84	1.98
5	0	0	0	0	0	0	0	0
total	96.13	9.64	28.57	90.17	217.44	55.18	497.13	100
% of total	19.34	1.94	5.75	18.14	43.74	11.10	100	
% extracted in all extractions	43.92	91.80	70.21	82.95	82.47	86.79		
	45.78	8.20	25.73	15.29	16.13	13.21		
	4.35	0	1.05	0.81	0.29	0		
	5.95	0	3.01	0.94	1.11	0		
	0	0	0	0	0	0		

[a] The fatty acids were methylated with BF_3 in methanol, analyzed by GLC (McConathy et al., 1981), and expressed as micrograms of fatty acids per 0.25 mL of LDL_2, which contained 2.45 mg of protein.

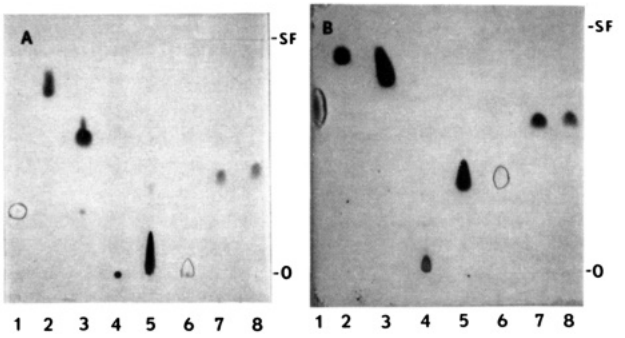

FIGURE 2: TLC separation of standard lipids in solvent system 1 (hexane/diethyl ether/glacial acetic acid, 113:35:3 v/v) (A) and in solvent system 2 (hexane/ethyl acetate/methanol/glacial acetic acid, 90:20:20:2 v/v) (B). The lipids and their R_f values in solvent systems 1 and 2 are (1) cholesterol, R_f 0.27 and 0.69; (2) cholesteryl oleate, R_f 0.77 and 0.91; (3) glyceryl trioleate, R_f 0.55 and 0.86; (4) L-α-phosphatidylcholine, R_f 0 and 0; (5) synthetic *N*-methylhexadecenoyl amide, R_f 0.1 and 0.43; (6) synthetic *N*-methylheptadecanoyl amide, R_f 0 and 0.41; (7) linoleic acid, R_f 0.39 and 0.63; and (8) palmitoleic acid, R_f 0.41 and 0.63. O = origin; SF = solvent front.

ether phase was dried to a small volume, and half of it was measured for ^{14}C radioactivity. Results showed that the top segment of the gel contained a significant amount of ^{14}C radioactivity (4410 dpm), whereas the remainder of the gel segments and the water phase contained only background level. This ruled out the possibility for the ^{14}C being free [^{14}C]MA or linked to a small peptide. The other half of the ether extract was applied onto a TLC along with common lipid standards and [^{14}C]MA and run in solvent system 2. The unknown moved with R_f 0.42 as monitored by ^{14}C radioactivity while [^{14}C]MA remained at the origin. These results suggest that the [^{14}C]MA-derivatized material recovered from the column top is lipid in nature. Its R_f is different from all the lipid standards tested, as seen in Figure 2B. Having established the lipid nature of the [^{14}C]MA-linked ligand, direct extraction of [^{14}C]MA-treated RCM-ApoB with organic solvent was employed to minimize loss due to being trapped inside the Ultrogel. Since bound fatty acids have been reported present in ApoB (Fisher, 1964; Fisher & Gurin, 1964; Hoeg et al., 1986a), and the possibility of these fatty acids being linked to ApoB via thiol ester bonds was not ruled out, precautions were taken to ensure complete delipidization of LDL, and the delipidization procedure was monitored with GLC. Table II shows a typical extraction pattern of unbound fatty acids from LDL_2. Though the extractability varied among fatty acids, the first two extractions removed 89–100% of the unbound fatty acids. The fifth extraction showed no detectable fatty acid, suggesting that all unbound fatty acids had been removed by this standard procedure.

Purification of Unknown by TLC in Two Solvent Systems. The hexane/chloroform extract of [^{14}C]MA-treated RCM-ApoB was concentrated and applied on TLC across the plate and run in solvent system 1. In this solvent system, cholesterol, cholesterol esters, triglycerides, and free fatty acids all moved up the plate, as shown in Figure 2A. While phospholipids remained at the origin, the unknown and free [^{14}C]MA also stayed at the origin, as monitored by ^{14}C radioactivity (figure not shown). The labeled unknown was extracted from the silica gel and concentrated and reapplied onto a second TLC plate and run in solvent system 2. In this solvent system, the unknown moved with R_f 0.42 while phospholipids and free [^{14}C]MA remained at the origin. All the other lipids had R_f values greater than 0.63 (see Figure 2B). Thus, all the lipids were well separated from the unknown after the second TLC purification.

Identification of Unknown. The radioisotope-labeled unknown with R_f 0.42 was recovered from TLC silica gel and hydrolyzed and transesterified with methanol. GLC analyses showed that the esterified products contained methyl palmitate and methyl stearate at equal molar concentrations. These results suggest that before hydrolysis and transesterification the [^{14}C]MA-labeled unknown contained *N*-[^{14}C]methylhexadecanoyl amide and *N*-[^{14}C]methyloctadecanoyl amide.

To confirm that the ^{14}C-labeled unknowns were *N*-methyl fatty acyl amides, *N*-methylheptadecanoyl ($C_{17:0}$) amide and *N*-methylhexadecenoyl ($C_{16:1}$) amide (the unsaturated fatty acid was used for better visual detection on TLC) were synthesized and characterized by TLC in both solvent systems. It was found that both *N*-methyl fatty acyl amides stayed at the origin in solvent system 1 (see Figure 2A) and moved with R_f 0.43 and 0.41, respectively, for $C_{16:1}$ and $C_{17:0}$ fatty acyl amide in solvent system 2 (see Figure 2B), consistent with the R_f values of unknown in both solvent systems. An aliquot of the hexane/chloroform extract of the [^{14}C]MA-treated RCM-ApoB was mixed with the synthetic *N*-methyl $C_{16:1}$ fatty acyl amide and run on TLC in solvent system 2. The peak of radioactivity of the ^{14}C-labeled lipid coincided with the *N*-methyl fatty acyl amides (Figure 3), confirming that the ^{14}C-labeled lipids are indeed *N*-[^{14}C]methyl fatty acyl amides.

To verify that the *N*-methyl fatty acyl amides isolated from the incubation mixture could be converted into *O*-methyl fatty acyl esters under the transesterification condition employed, the two synthetic $C_{16:1}$ and $C_{17:0}$ fatty acyl amides were tested

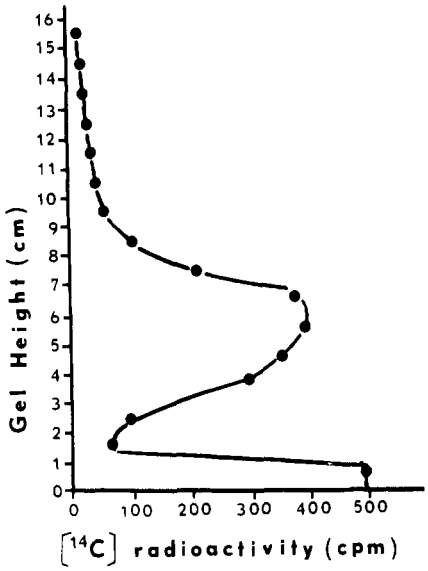

FIGURE 3: Radiogram of [^{14}C]methylamine-labeled unknown analyzed by TLC. An aliquot of the hexane/chloroform extract of the RCM-ApoB and [^{14}C]MA incubation mixture in urea/buffer was mixed with *N*-methylhexadecenoyl amide and run in duplicate on TLC in solvent system 2 as described in Figure 2. Silica gel from one spot was scraped off at 1-cm intervals and measured for ^{14}C radioactivity. The peak of the radiogram coincided with standard *N*-methylhexadecenoyl amide (spot not shown). Both unknown and standard retarded slightly (R_f 0.38) due to interference by urea in the sample. This was concluded by varying urea content in the organic phase. The radioactivity at the origin represents free [^{14}C]MA, which was present in the hexane/chloroform phase. The location of free [^{14}C]MA was identified separately with known [^{14}C]MA.

under the same transesterification condition. GLC analysis showed the transesterification products to be *O*-methyl $C_{16:1}$ fatty acid ester and *O*-methyl $C_{17:0}$ fatty acid ester. Thus, the methodology of transesterification was verified. To further ascertain that the ^{14}C-labeled fatty acyl amide was not a synthetic product arising from the incubation between free fatty acid and methylamine at pH 8.5 and 30 °C, palmitic acid was incubated with 50 mM MA in 85% ethanol at pH 8.5 and 30 °C for 41 h and was analyzed on TLC in solvent system 2. Results showed that no fatty acyl amide was produced from free fatty acid. These results suggest that $C_{16:0}$ and $C_{18:0}$ fatty acids are covalently linked to ApoB via thiol ester bonds to the side chain of cysteine residues. Under mild alkaline conditions, the thiol ester bonds are cleaved by [^{14}C]MA, producing new –SH groups and active acyl groups that are reactive with [^{14}C]MA and forming *N*-[^{14}C]methyl fatty acyl amides.

At present, the total number of fatty acids covalently linked to ApoB is uncertain. From the number of newly released –SH groups after MA treatment, at least 10 intermolecular thiol ester bonds may be present that can bind 10 mol of fatty acids. From the recovered radioactivity of [^{14}C]MA fatty acids, 5–11 mol of fatty acids can be accounted for covalently linked to ApoB. However, the exact number of bound fatty acids awaits the identification of the cysteine residues linked to fatty acids.

Bound palmitic and stearic acids were also observed earlier by Fisher (Fisher, 1964; Fisher & Gurin, 1964) after hydrolysis of chymotryptic peptides of ApoB. He estimated at least 8 mol of fatty acids per mole of ApoB (Fisher, 1964). Using $LiAlH_4$, Fisher (1964) reduced the fatty acids to alcohol and concluded that the linkages between fatty acids and ApoB were ester bonds. However, with a similar reducing agent ($NaBH_4$) on the thiol ester bond, the latter also is reduced to alcohol, at an even faster rate than that of the hydroxy ester bond (Torchinsky, 1981). Therefore, the data of Fisher (1964) cannot exclude the possibility of thiol ester linkage present in ApoB. In our experience, incubation of the intact LDL with [^{14}C]MA under the mild alkaline conditions employed did not result in cleavage of ester bonds of triglycerides, cholesterol esters, or phospholipids, suggesting it is most unlikely that the fatty acids are linked by ester bonds to ApoB.

Using HepG2 cells growing in medium containing [1-^{14}C]palmitate, Hoeg et al. (1986a) also found that [1-^{14}C]-palmitate incorporated into ApoB covalently. They speculated that palmitate could be linked to ApoB via ester or thiol ester.

Thus far the only other apolipoprotein known to be acylated with long-chain fatty acid is apolipoprotein A-I, by ester linkage (Hoeg et al., 1986b).

Thus, the thiol ester linkages in acylation of ApoB would be unique for plasma apolipoproteins. Thiol ester is a high-energy labile linkage; it is reportedly a better acyl donor than ester linkage (Torchinsky, 1981). This unusual linkage in ApoB may play an important functional role. Covalently linked fatty acids to protein lead to increased hydrophobicity of the protein. It may facilitate the interaction between ApoB and membrane. The presence of bound fatty acids in ApoB held by labile linkages may be one of the reasons for its strong tendency to undergo aggregation. The presence of readily releasable –SH groups explains why ApoB is sensitive to autoxidation (Lee et al., 1981).

Among non-plasma lipoproteins, myelin proteolipid was the first acylated protein described, more than 35 years ago (Folch-Pi & Lees, 1951). However, the wide occurrence of acylation as a posttranslational modification of membrane proteins has been revealed only during the last decade (Schmidt et al., 1979; Magee & Schlesinger, 1982; Schmidt, 1983). Amide, ester, and thiol ester are the three types of linkages found between fatty acids and proteins directly and thiol ether indirectly. The proteins involved are mostly those of enveloped viruses (Magee & Schlesinger, 1982) and of bacterial membrane (Nielson et al., 1981) and a few of normal membrane. The latter include the transferrin receptor (Omary & Trowbridge, 1981), histocompatibility antigen (Kaufman et al., 1984), and cAMP-dependent protein kinase and calcineurin B (Carr et al., 1982; Aitken et al., 1982). The thiol ester linkage found in histocompatability antigen and eukaryotic cell membrane is hydroxylamine-sensitive (Kaufman et al., 1984; Magee et al., 1984), whereas the amide linkage is hydroxylamine-insensitive (Carr et al., 1982; Aitken et al., 1982). The thiol ester linkage has been found to involve the most abundant cellular acyl chains, i.e., palmitate, stearate, and oleate (Schmidt et al., 1979), while the amide linkage is exclusively to myristate, a low-abundance acyl chain (Magee & Courtneidge, 1985). Our findings on palmitate and stearate for thiol ester linkages are in accord with the literature data.

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