Movement of Apolipoprotein B into the Lumen of Microsomes from Hepatocytes Is Disrupted in Membranes Enriched in Phosphatidylmonomethyl ethanolamine*

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When monolayer cultures of rat hepatocytes are incubated with the ethanolamine/choline analogue, monomethyl ethanolamine, the secretion of apolipoproteins B<sub>100</sub> and B<sub>48</sub>, as well as the lipid constituents, of very low density lipoprotein (VLDL) is inhibited by approximately 50% (Vance, J. E. (1991) J. Lipid Res. 32, 1971–1982). In the present study we have investigated the mechanism by which monomethyl ethanolamine disrupts VLDL secretion. Hepatocytes were treated with 400 μM monomethyl ethanolamine overnight, which resulted in an increase in the cellular content of the derived phospholipid, phosphatidylmonomethyl ethanolamine, from 0.32 ± 0.15 to 2.92 ± 0.74 nmol/mg of cell protein. The biosynthesis of apoproteins B<sub>100</sub> and B<sub>48</sub> was not impaired by treatment of cells with monomethyl ethanolamine. However, monomethyl ethanolamine decreased by approximately 50% the amount of apoproteins B, but not of the typical secretory protein, albumin, present in the luminal content subfraction of microsomes. The intracellular degradation of apoproteins B was also increased in phosphatidylmonomethyl ethanolamine-enriched, compared with control, cells. Moreover, the pool of apoprotein B present in intact microsomes from hepatocytes incubated with monomethyl ethanolamine was more accessible to exogenously added trypsin, presumably because a larger pool of the apoprotein B was exposed on the cytosolic surface of these microsomes. The data strongly suggest that an increase in the microsomal content of phosphatidylmonomethyl ethanolamine diminishes the ability of apoprotein B to translocate across the endoplasmic reticulum membrane into the luminal compartment. Consequently, the association of apoprotein B with lipids and/or the normal assembly of mature VLDL particles is impaired.

The mechanisms that regulate the hepatic assembly and secretion of apolipoprotein B into plasma VLDL<sup>1</sup> are not yet completely understood. VLDL particles secreted from rat hepatocytes contain a single molecule of either apoB<sub>100</sub> or apoB<sub>48</sub> (Elovson et al., 1988). Under most, if not all, physiological conditions the amount of apoB secreted from hepatocytes is not regulated by the amount of apoB synthesized (Pullinger et al., 1989; Dashti et al., 1989; Sorci-Thomas et al., 1989). Instead, the apoB gene appears to be constitutively expressed and superfluous apoB that is not assembled into VLDL is degraded in the ER (Davis et al., 1990; Dixon et al., 1991; Furukawa et al., 1992; Sato et al., 1990). Consequently, it is generally believed that the availability of lipid components regulates the amount of apoB secreted as VLDLs. For example, secretion of VLDLs from hepatocytes has been shown to be contingent upon the active synthesis of phosphatidylinolylcholine (Yao and Vance, 1988) and perhaps also of cholesterol esters (Cianflone et al., 1990) and cholesterol (Khan et al., 1990) and is stimulated by addition of fatty acid to the culture medium (Dixon et al., 1991).

Definition of the intracellular site(s) of assembly of apoB with its lipid constituents (i.e. triacylglycerols, phospholipids, cholesterol, and cholesteryl esters) is an active area of investigation in several laboratories. The following three alternative models have been suggested for the formation of VLDL: (i) assembly of complete VLDL particles in the endoplasmic reticulum (Rusiñoľ et al., 1993; Borchardt and Davis, 1987; Alexander et al., 1976); (ii) association of apoB with membranes until the apoB reaches the Golgi apparatus, whereupon lipid is added to the particle (Higgins, 1988; Bamberger and Lane, 1988, 1990); and (iii) a sequential addition of lipid to apoB during its passage from the ER to the Golgi to secretion (Bostrom et al., 1986, 1988; Janero and Lane, 1983). We favor the first of these three possibilities. In a recent study we demonstrated that the apoB-containing particles in the lumina of a heavy (rough) and a light (smooth?) ER membrane fraction from rat liver, as well as from the Golgi, were of the same composition and density as those of newly secreted apoB-containing lipoproteins (Rusiñoľ et al., 1993). Experiments performed by Davis and co-workers (Borchardt and Davis, 1987), and by Olsson and colleagues (Börn et al., 1990), also suggest that apoB is assembled into VLDL particles in the endoplasmic reticulum, perhaps during translocation of the newly synthesized apoB molecule across the ER membrane into the lumen. The proposal that VLDL particles are completely assembled in the ER does not preclude a possible exchange of some of the lipid components, especially

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The abbreviations used are: VLDL, very low density lipoprotein; MME, monomethyl ethanolamine; PtdCho, phosphatidylcholine; PMME, phosphatidyl-N-monomethyl ethanolamine; ER, endoplasmic reticulum.
the surface components, phospholipids and unesterified cholesterol, between the VLDL particles and cellular or vesicle membranes during passage of the particles along the secretory route (Howell and Palade, 1982).

Of interest, with respect to the mechanism of VLDL assembly, is the finding that patients with the disease abetalipoproteinemia synthesize apoB normally but are unable to secrete apoB-containing lipoproteins from the liver or intestine (Lackner et al., 1986). Examination of intestinal biopsy samples has shown that a luminal microsomal protein, that has the ability to transfer triacylglycerol between membranes, is lacking in patients suffering from abetalipoproteinemia (Wetterau et al., 1992). This finding strongly implicates the involvement of this transfer protein in the assembly of apoB-containing lipoproteins.

In the present study we have identified a modification of the lipid composition of the hepatocyte microsomal membrane which reduces the amount of apoB that is present in the luminal space of microsomes as VLDL particles. We reported previously that when monolayer cultures of rat hepatocytes were incubated with monomethylethanolamine (an analogue of choline and ethanolamine) the cellular content of PMME, the phospholipid which is synthesized from MME and which is normally a quantitatively insignificant lipid, was increased (Vance, 1989). Consequently, the secretion by hepatocytes of triacylglycerols, and apoproteins B, B48, and E (but not albumin or apo A1) was decreased by approximately 50% compared with untreated cells (Vance, 1991).

Our original hypothesis for the mechanism by which MME inhibited VLDL secretion was that MME, by acting as an analogue of choline, blocked the biosynthesis of PtdCho and thereby inhibited VLDL secretion. The rationale for this proposal was that during choline deprivation, PtdCho synthesis and VLDL secretion are inhibited (Yao and Vance, 1988). However, the hypothesis was discounted when incubation of hepatocytes with MME did not affect PtdCho synthesis from either the CDP-choline pathway or from the methylation of phosphatidylethanolamine (Vance, 1991).

Nor did incubation of hepatocytes with MME decrease (i) the rate of phosphatidylethanolamine synthesis from either CDP-ethanolamine or from the decarboxylation of phosphatidylserine, (ii) the rate of phosphatidylserine biosynthesis, (iii) the rate of triacylglycerol synthesis, or (iv) the activity of acyl-CoA:cholesterol acyltransferase.

We have now demonstrated that apoB synthesis is not impaired in hepatocytes enriched in PMME. In contrast, in these cells the amount of apoB present in the microsomal lumen is reduced, as most likely because the movement of newly synthesized apoB from the microsomal membrane into the lumen is disrupted. In addition, an increased intracellular degradation of apoB is observed in hepatocytes in which the membranes are enriched in PMME, and in such cells microsomal apoB exposed to the cytosol is more readily degraded by extracellular protease. Apparently, incubation of hepatocytes with MME produces an abnormally high concentration of PMME in the microsomal membranes which interferes with the ability of apoB either to translocate across the membrane or to associate with lipid. Consequently, the number of VLDL particles secreted from the hepatocyte is reduced.

**EXPERIMENTAL PROCEDURES**

**Materials**—The radiochemical tracer [3H]methionine was purchased from ICN (Irvine, CA). The enhanced chemiluminescence detection reagents used for immunoblotting were obtained from Amersham Canada, Oakville, Ontario, Canada. Rabbit polyclonal antibodies directed against rat apoB were a generous gift from Dr. Roger Davis of San Diego State University. Rabbit polyclonal antibodies directed against rat albumin were a generous gift from Dr. Victor deGroot of San Diego State University. The enhanced chemiluminescence detection reagents used for immunoblotting were obtained from Bio-Rad. The polyvinylidene difluoride membranes were from Millipore. Monomethylethanolamine, trypsin, soybean trypsin inhibitor, and Triton X-100 were obtained from Sigma. The cell culture dishes were Primaria (60 mm) and were purchased from Goerz (Oxnard, CA). Eagle's minimum essential medium was from Life Technologies, Inc. and fetal bovine serum was from British Drug House Chemicals Ltd. (Carle Place, NY). All other reagents were from either Sigma or Fisher.

**Preparation and Culture of Rat Hepatocytes**—Primary rat hepatocytes were isolated from the livers of male Sprague-Dawley rats (150-200 g of body weight, fed standard laboratory diet) by the collagenase perfusion technique as described previously (Davis et al., 1979; Vance et al., 1984). The cells were plated at a density of 5 x 10^6 to 5 x 10^7 cells per dish in Eagle's minimum essential medium containing 17% fetal bovine serum. The cells were allowed to adhere to the dishes for 4 h, after which the serum-containing medium was removed. Fresh medium, without serum but containing either 0 or 400 μM MME, was added for overnight (16 h) incubation. Cell viability was confirmed by trypan blue exclusion and by measurement of leakage of lactate dehydrogenase into the medium (Vance, 1991). Culture medium was collected and centrifuged at 10,000 x g for 15 min to remove cell debris. The supernatant was washed with ice-cold phosphate-buffered saline (2 ml/dish) and scraped with a rubber policeman into 2 ml of phosphate-buffered saline. The cells were pelleted from the cell suspension by centrifugation for 5 min at 3,000 x g.

**Isolation of Microsomes and Separation of Microsomal Membranes and Luminal Contents**—Hepatocytes from 15-60 mm dishes combined were harvested and homogenized in 7 ml of buffer containing 10 mM Tris (pH 7.4), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin by hand using 35 up-and-down strokes (10 s each) in a motor-driven Dounce homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C. The supernatant was centrifuged at 100,000 x g for 1 h to remove nuclei and mitochondria.

**Immunoprecipitation, Electrophoresis, and Immunoblotting of ApoB and Albumin**—ApoB and albumin were immunoprecipitated from the cell homogenate, medium, and microsomal luminal contents and membranes by a procedure described previously (Borchardt and Davis, 1987). The proteins were solubilized from the immunoprecipitate by boiling for 5 min in a buffer containing 8 M urea and 2% sodium dodecyl sulfate and applied to a 5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. ApoB and apoA1 were identified by comparison with standard rat serum VLDL, prepared as described previously (Schumaker and Pippione, 1986). In some cases, the bands containing apoproteins B or albumin were excised from the gel, digested by heating in 0.5 ml of a mixture of 30% hydrogen peroxide and 60% perchloric acid 2:1 (v/v) for 1 h at 60 °C, and radioactivity was measured. For immunodetection of apoB and albumin, the proteins were separated on a 3-15% polyacrylamide mini-gel and transferred to a polyvinylidene difluoride membrane by modification of the method of Towbin et al. (1979). The transfer buffer used wasboric acid (92.5 mM pH 8.0), and the proteins were transferred for 2 h at 10 °C using 100 V. Nonspecific binding sites on the membrane were blocked by immersion of the membrane in a solution of Tris-buffered saline (20 mM Tris (pH 7.5) and 0.5 M NaCl) containing 5% dried milk and 0.05% Tween 20 for 12 h at 4 °C or for 1 h at room temperature. The primary antibodies used were rabbit anti-rat apoB and rabbit anti-rat albumin. The secondary antibody was anti-rabbit IgG linked to horseradish peroxidase. Bound antibodies were visualized with enhanced chemiluminescence reagents according to the manufacturer's instructions.

**Preparation of ApoB to Exogenously Added Trypsin**—A suspension of isolated microsomes (400 μg of protein) was incubated with 0.1 volume of a solution of 1-1-[[3-20s]leucine-labeled apoB, messenger RNA (tRNA) was obtained from Amersham Canada, Oakville, Ontario, Canada. Rabbit polyclonal antibodies directed against rat apoB were a generous gift from Dr. Roger Davis of San Diego State University. Rabbit polyclonal antibodies directed against rat albumin were a generous gift from Dr. Victor deGroot of San Diego State University. The enhanced chemiluminescence detection reagents used for immunoblotting were obtained from Bio-Rad. The polyvinylidene difluoride membranes were from Millipore. Monomethylethanolamine, trypsin, soybean trypsin inhibitor, and Triton X-100 were obtained from Sigma. The cell culture dishes were Primaria (60 mm) and were purchased from Goerz (Oxnard, CA). Eagle's minimum essential medium was from Life Technologies, Inc. and fetal bovine serum was from British Drug House Chemicals Ltd. (Carle Place, NY). All other reagents were from either Sigma or Fisher.
extract were subjected to polyacrylamide gel electrophoresis on a 3-15% gel containing 0.1% sodium dodecyl sulfate. Proteins were analyzed by immunoblotting as described above or, alternatively, radioactivity was measured in a scintillation counter.

Other Methods—The protein content of all samples was determined in the presence of 0.5% deoxycholate by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The phospholipid composition of microsomes was measured by phosphorus analysis (Rooser et al., 1960) of phosphatidylethanolamine, PtdCho, and PtdEtn that had been isolated by thin-layer chromatography on silica gel G plates (0.25 mm thickness) in the solvent system chloroform/propanolic acid/n-propanol/water, 3/2/6/1 (v/v).

RESULTS

Monomethylethanolamine Inhibits ApoB Secretion but Not ApoB Synthesis—Our previous studies demonstrated that when hepatocytes were incubated with MME, the secretion of apoB and the lipid constituents of VLDL was decreased by approximately 50% (Vance, 1991). The effect of MME treatment on apoB secretion was confirmed in the present study in which monolayer cultures of rat hepatocytes were incubated for 16 h with $[^{35}S]$methionine in the presence or absence of MME. ApoB and apoB secreted into the culture medium were separated by polyacrylamide gel electrophoresis, and their radioactivities were determined. In agreement with our previous observations (Vance, 1991), the secretion of apoB and apoB from cells incubated in the presence of MME was reduced by 51.5 ± 9.9% and 58.0 ± 18.0%, respectively, compared with that from cells incubated in the absence of MME. In response to MME treatment the cellular content of the phospholipid PMME increased from 0.32 ± 0.15 nmol/mg of cell protein to 0.92 ± 0.74 nmol/mg of cell protein. The concentration of PtdCho was unaffected by incubation of the cells with MME (420.8 ± 61.2 nmol/mg of cell protein in the presence of MME and 425.2 ± 61.7 nmol/mg of cell protein in the absence of MME), whereas the concentration of phosphatidylethanolamine was slightly increased (from 261.0 ± 15.7 to 300.2 ± 18.0 nmol/mg of cell protein) upon incubation of the cells with MME. No decrease in cell viability was observed during MME treatment under any of the conditions used in this study.

We previously demonstrated that diminished apoB secretion from hepatocytes incubated with MME was not the result of a detectable inhibition of lipid biosynthesis (Vance, 1991). We have now also eliminated the possibility that when hepatocytes are treated with MME, a decrease in the rate of apoB synthesis is responsible for the reduction in apoB secretion. Hepatocytes were incubated for 16 h in the presence or absence of 400 µM MME, and subsequently $[^{35}S]$methionine was added for various times from 10 min to 4 h. ApoB and apoB were immunoprecipitated from cells and culture medium. The proteins were separated by polyacrylamide gel electrophoresis and radioactivity was determined. The incorporation of $^{35}$S into cellular apoproteins B and B was unaffected by MME treatment (Fig. 1, A and C, respectively). As expected, however, secretion of radiolabeled apoB and apoB was reduced into the medium was reduced (Fig. 1, B and D, respectively). A decrease in the rate of apoB synthesis was, therefore, not responsible for the diminished secretion of apoB from MME-treated cells.

Intracellular Degradation of ApoB Is Increased in MME-Treated Cells—Intracellular degradation of apoB has been observed previously (Davis et al., 1990; Dixon et al., 1991; Sato et al., 1990) and has been suggested as an important mode of regulation of the amount of apoB secreted. Therefore, the rate of disappearance of radiolabel from labeled cellular apoB was examined in a pulse-chase experiment. Cells that had been incubated overnight in the presence or absence of MME were pulse-labeled for 15 min with $[^{35}S]$methionine, and radioactivity was chased with unlabeled methionine in the presence or absence of MME over a 4-h time period. ApoB was immunoprecipitated from cells and culture medium and electrophoresed on 3-15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The apoB-containing bands were excised from the gels for measurement of radioactivity. A and B, apoB; C and D, apoB. Open symbols = cells incubated without MME; closed symbols = cells incubated with MME. The experiment was repeated three times with essentially identical results.

MME were pulse-labeled for 15 min with $[^{35}S]$methionine, and radioactivity was chased with unlabeled methionine in the presence or absence of MME over a 4-h time period. ApoB was immunoprecipitated from cells and culture medium. ApoB and apoB were separated by polyacrylamide gel electrophoresis, and radioactivity was measured in these proteins. During the first 60 min after the pulse the amount of labeled intracellular apoB and apoB decreased by approximately 64 and 60%, respectively (Fig. 2, A and C), whereas during this time less than 15% of the labeled apoB had been secreted into the medium (Fig. 2, B and D). The loss of cellular apoB that was not recovered in the medium presumably corresponded to apoB that had been degraded intracellularly. Fig. 2A also demonstrates that during the first 40 min the rate of decay of intracellular apoB occurred more rapidly in MME-treated cells than in control cells. For example, 30 min after the start of the chase period MME-treated cells contained 91% less apoB than did control cells, whereas essentially no apoB had been secreted during this time. Only a small, if any, difference between MME-treated and untreated cells in the intracellular content of apoB up to 40 min of incubation was observed (Fig. 2, B and D).

The total amount of radiolabeled apoB at each time point (calculated from the data in Fig. 2) is represented in Fig. 3 by the amount of labeled cellular plus secreted apoB remaining at each time point, calculated as a percentage of total radiolabeled apoB present at the end of the pulse (i.e. at time = 0 min in Fig. 2). The majority of the degradation of apoB (Fig. 3A) and apoB (Fig. 3B) occurred within 60 min of the end of the pulse; in control cells very little additional apoB or apoB was degraded after this time. In contrast, in MME-treated cells the intracellular degradation of apoB and apoB continued for at least 4 h. Between 1 and 4 h after the start of the chase period, an additional 14.5% of apoB and 20.1% of apoB was degraded in the MME-treated cells, whereas in the untreated cells only 8.0% of apoB and 7.4% of apoB were degraded between 1 and 4 h. Approximately twice as much of the total apoB and apoB (intra-

![Fig. 1. MME does not inhibit apoB synthesis.](image-url)
cellular plus secreted) remained in the control cells after 4 h compared with the MME-treated cells. These data suggest that MME treatment may increase the susceptibility of apoB to intracellular proteolytic degradation, perhaps by causing the apoB to remain in the protease-sensitive compartment for a longer period of time in the MME-treated cells than in the control cells.

The Amount of ApoB in Luminal Contents Is Reduced in MME-treated Cells—Soon after, or during, its synthesis, apoB is believed to become associated with the endoplasmic reticulum membrane (Bamberger and Lane, 1988; Davis et al., 1990), and after a short lag time (~15 min) (Bostrom et al., 1988; Borén et al., 1990), apoB is translocated across the membrane and into the lumen of the endoplasmic reticulum. At some point during this process, apoB associates with its lipid components and nascent VLDL particles are formed.

In a pulse-chase experiment, we examined the time course of movement of newly synthesized apoB from the microsomal membrane into the luminal compartment and subsequently into the culture medium. Hepatocytes that had been incubated overnight in the presence or absence of MME were pulse-labeled for 15 min with [35S]methionine, and radioactivity in apoB was chased with unlabeled methionine in the presence or absence of MME. The cells were pulse-labeled for 15 min with [35S]methionine (300 µCi/dish), then washed and incubated with unlabeled methionine (1 mM) for the indicated times. apoB was immunoprecipitated from cells and medium, and radioactivity in apoB was determined as described in the legend to Fig. 1. Open symbols = cells incubated without MME; closed symbols = cells incubated with MME. The data have been normalized so that the counts/min in the MME-treated cells at the end of the pulse are equal to the counts/min in the control cells. Values are averages ± S.D. from three independent experiments. In some cases the error bars are too small to be visible.

In untreated cells, the radioactivity in membrane-associated apoB was maximal at the 20-min time point and subsequently declined, reaching a maximum in the luminal contents at the 40-min time point. Radioactive apoB was detected in the culture medium by 20 min and the amount increased steadily thereafter. In MME-treated cells, however, the pattern of incorporation of [35S]methionine into apoB in all compartments was distinctly different from that in the untreated cells. At 40 min the amount of radiolabeled apoB in the luminal contents of the microsomes from MME-treated cells was approximately one-half of that in the untreated cells. Moreover, in agreement with the experiments depicted in Figs. 1 and 2, the secretion of apoB into the culture medium was inhibited as a result of MME treatment. Clearly, the amount of apoB that traversed the secretory route was dramatically reduced in hepatocytes treated with MME.

The steady state level of apoB was assessed by immunoblotting of the proteins in the culture medium, microsomal membranes, and luminal contents from hepatocytes incubated overnight in the presence or absence of MME. As shown in Fig. 5, the mass of apoB was reduced in the culture medium and in the luminal contents of the MME-treated, compared with the untreated, cells. However, the intensity of the band corresponding to albumin (a "typical" secretory protein), in both luminal contents and in the culture medium, was not affected by MME treatment.

In a separate experiment, hepatocytes were incubated with [35S]methionine for 16 h in the presence or absence of MME. Microsomes were prepared from the cells and separated into luminal and membrane subfractions. ApoB was immunoprecipitated from the medium, the membranes, and the luminal contents. The proteins were separated by polyacrylamide gel electrophoresis and radioactivity in apoB was determined (Fig. 4).

In untreated cells, the radioactivity in membrane-associated apoB was maximal (Fig. 4A) and apoB was maximal at the 20-min time point and subsequently declined, reaching a maximum in the luminal contents at the 40-min time point. Radioactive apoB was detected in the culture medium by 20 min and the amount increased steadily thereafter. In MME-treated cells, however, the pattern of incorporation of [35S]methionine into apoB in all compartments was distinctly different from that in the untreated cells. At 40 min the amount of radiolabeled apoB in the luminal contents of the microsomes from MME-treated cells was approximately one-half of that in the untreated cells. Moreover, in agreement with the experiments depicted in Figs. 1 and 2, the secretion of apoB into the culture medium was inhibited as a result of MME treatment. Clearly, the amount of apoB that traversed the secretory route was dramatically reduced in hepatocytes treated with MME.

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measured (Table I). In MME-treated cells the amount of radiolabeled apoB
to and apoBm in the luminal contents was only 46.1 and 53.4%, respectively, of that in untreated cells. The quantity of 35S-labeled apoB that was secreted into the medium was reduced to a similar degree by MME treatment. In contrast, the amounts of radiolabeled albumin present in the luminal contents of microsomes and secreted into the medium from MME-treated cells were no different from those amounts in untreated cells (Table I).

The observed reduction in the amount of apoB protein in the luminal contents of microsomes was not a general phenomenon common to other secretory proteins, because the amount of albumin present in luminal contents was unaffected by MME treatment (Fig. 5 and Table I). Therefore, the defect in apoB secretion as a result of MME treatment appears to be due to a decreased ability of apoB, specifically, to move into the microsomal lumen.

ApoB Sensitivity to Exogenously Added Trypsin Is Increased in Microsomes from Hepatocytes Incubated with MME—Hepatic apoB is apparently synthesized constitutively (Pullinger et al., 1989). A pool of newly synthesized apoB that is not secreted, but is exposed to the cytosol, is proposed to be degraded by a protease that either is located on, or has access to, the cytosolic surface of the microsomal membrane (Davis et al., 1990). The data presented in Figs. 4 and 5 and Table I show that MME treatment decreased the amount of apoB in the microsomal lumen of hepatocytes and also increased the proportion of newly made apoB that was degraded intracellularly (Figs. 2 and 3). A possible explanation for these observations, that is consistent with the data, is that in MME-treated cells more apoB was exposed on the cytosolic surface of the microsomes than in untreated cells. The accessibility of microsomal apoB to an exogenously added protease (trypsin) was therefore examined. Intact microsomes were isolated from cells that had been incubated for 16 h in the presence or absence of MME. Microsomal samples were treated under one of the following conditions: (i) with trypsin, (ii) with trypsin inhibitors added first, then trypsin, and (iii) with trypsin in the presence of the detergent, Triton X-100. The action of trypsin was terminated by addition of soybean trypsin inhibitor. Microsomal proteins were subjected to polyacrylamide gel electrophoresis and immunoblotting and probed with a mixture of two antibodies, one directed against rat apoB, the other against rat albumin. A pool of apoB in both MME-treated and untreated microsomes was digested by trypsin (Fig. 6). Presumably, the undigested pool of apoB was protected from trypsin by being located either within the microsomal lumen or in a part of the membrane “hidden” from the exogenously added protease. In MME-treated cells, trypsin incubation decreased the mass of apoB in the microsomes to a greater extent than in the untreated cells (Fig. 6). When both types of microsomes were treated with trypsin in the presence of detergent (which permeabilizes the microsomes to exogenously added trypsin) nearly all of the microsomal apoB was degraded by trypsin. Parallel results were obtained for apoB100 and apoB48. As a control to confirm that the intact microsomes had indeed been impermeable to exogenously added trypsin (i.e. that luminal apoB in intact microsomes had not been digested by trypsin in the absence of detergent), we demonstrated that albumin (a protein that is almost completely luminal) was degraded by trypsin only when detergent was present (Fig. 6).

This experimental protocol was essentially repeated using 35S-methionine (added for 16 h) as a label for apoB and albumin. The bands corresponding to apoB100, apoB48, and

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**Fig. 4.** Pulse-chase analysis of apoB in microsomal membranes, luminal contents, and culture medium. Cultured rat hepatocytes were incubated for 16 h, and in all subsequent incubations, in the presence of 0 or 400 μM MME. [35S]methionine (200 μCi/dish) was added for 15 min, the cells were washed, and unlabeled methionine (1 mM) was added for the indicated times. Culture medium was collected. Microsomes were isolated from the cells and lysed by treatment with sodium carbonate at pH 11.3. Luminal and membrane subfractions of microsomes were separated by ultracentrifugation. apoB was immunoprecipitated from membranes, luminal contents, and culture medium and radioactivity was determined. A, apoB100; B, apoB48. Closed symbols = cells incubated without MME; open symbols = cells incubated with MME. The experiment was repeated twice with similar results.

**Fig. 5.** steady state levels of apoB and albumin in hepatocyte microsomes. Cultured rat hepatocytes were incubated for 16 h in the presence of 0 or 400 μM MME. The culture medium was collected. Microsomes were isolated from the cells, and luminal contents were released by sodium carbonate treatment. Proteins in the culture medium (lanes 1 and 2), in intact microsomes (lanes 3 and 4), in microsomal membranes (lanes 5 and 6), and in luminal contents (lanes 7 and 8) were subjected to polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with a mixture of two polyclonal antibodies (anti-rat apoB and anti-rat albumin) for detection of the proteins. Equal quantities of protein (250 μg) were applied to each lane of the gel. Samples from cells incubated without MME are in lanes 1, 3, 5, and 7; samples from cells incubated with MME are in lanes 2, 4, 6, and 8. The experiment was repeated three times with similar results. The locations of apoB100 (B100), apoB48 (B48), and albumin on the gel are indicated at the left-hand side.
Antibodies directed against rat apoB and rat albumin. The immunoprecipitated proteins were subjected to polyacrylamide gel electrophoresis at the left-hand side.

Radiolabeling of apoB and albumin in culture medium, intact microsomes, and membrane and luminal subfractions of microsomes from hepatocytes incubated in the presence or absence of MME

Hepatocytes were incubated for 16 h in medium containing 100 μCi/dish of [35S]methionine in the presence or absence of MME (400 μM). Culture medium and microsomes were isolated. Microsomes were separated into membrane and luminal subfractions by sodium carbonate treatment and subsequent ultracentrifugation. ApoB and albumin were immunoprecipitated from all samples with a mixture of polyclonal antibodies directed against rat apoB and rat albumin. The immunoprecipitated proteins were subjected to polyacrylamide gel electrophoresis on 5% polyacrylamide gradient gels containing 0.1% sodium dodecyl sulfate. The bands corresponding to apoB and albumin were detected when the gels and radioactivity were determined. The values given are averages of 10^3 × dpm/250 μg of microsomal protein ± S.D. from three independent experiments.

<table>
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<th></th>
<th>ApoB&lt;sub&gt;48&lt;/sub&gt;</th>
<th>ApoB&lt;sub&gt;100&lt;/sub&gt;</th>
<th>Albumin</th>
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<td></td>
<td>+MME</td>
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<td><strong>Medium</strong></td>
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<tr>
<td>Intact microsomes</td>
<td>5.46 ± 0.57</td>
<td>8.78 ± 1.23</td>
<td>2.36 ± 0.26</td>
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<tr>
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<td>8.93 ± 1.67</td>
<td>10.03 ± 1.20</td>
<td>4.91 ± 0.58</td>
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<tr>
<td>Microsome lumen</td>
<td>2.90 ± 0.26</td>
<td>4.05 ± 0.36</td>
<td>2.36 ± 0.21</td>
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Table I

Trypsin sensitivity of apoB and albumin in microsomes from cells incubated with or without MME

Microsomes were isolated from hepatocytes that had been incubated for 16 h in medium containing [35S]methionine (100 μCi/dish) in the presence or absence of MME (400 μM). Four separate aliquots of each type of microsomes were incubated for 40 min at 0 °C as follows. None = incubated without trypsin; trypsin + inhibitor = incubated with trypsin in the presence of soybean trypsin inhibitor; trypsin = incubated with trypsin; trypsin + Triton X-100 = incubated with trypsin in the presence of 0.5% Triton X-100. The proteolysis was terminated by addition of soybean trypsin inhibitor and the samples were immediately boiled for 5 min in the presence of 8 M urea and 2% sodium dodecyl sulfate. Microsomal proteins (250 μg) were separated on polyacrylamide gels and the bands corresponding to apoB<sub>48</sub>, apoB<sub>100</sub>, and albumin were excised from the gels and radioactivity was determined. The experiment was repeated twice with similar results. The values given are 10^3 × dpm/250 μg of microsomal protein.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ApoB&lt;sub&gt;48&lt;/sub&gt;</th>
<th>ApoB&lt;sub&gt;100&lt;/sub&gt;</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+MME</td>
<td>−MME</td>
<td>+MME</td>
</tr>
<tr>
<td><strong>None</strong></td>
<td>15.25</td>
<td>18.70</td>
<td>8.78</td>
</tr>
<tr>
<td><strong>Trypsin + inhibitor</strong></td>
<td>14.38</td>
<td>17.21</td>
<td>7.98</td>
</tr>
<tr>
<td><strong>Trypsin</strong></td>
<td>6.86</td>
<td>14.50</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Trypsin + Triton X-100</strong></td>
<td>0.16</td>
<td>0.43</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table II

Fig. 6. Digestion of microsomal apoB by exogenously added trypsin. Microsomes were prepared from rat hepatocytes that had been incubated overnight in the presence of 0 or 400 μM MME. Aliquots of each sample (400 μg of microsomal protein) were incubated with 0.1 volume of trypsin solution (0.5 mg/ml) for 40 min at 0 °C. Proteolysis was terminated by addition of 0.1 volume of soybean trypsin inhibitor (4 mg/ml) and microsomes were re-isolated by ultracentrifugation. Microsomal proteins (250 μg) were subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Apoproteins B and albumin were detected when the membrane was probed with a mixture of polyclonal antibodies directed against rat apoB and rat albumin. Lanes 1-4, microsomes from control cells; lanes 5-8, microsomes from MME-treated cells. Lanes 1 and 5, not treated with trypsin; lanes 2 and 6, trypsin inhibitor added before trypsin; lanes 3 and 7, incubated with trypsin; lanes 4 and 8, incubated with trypsin in the presence of 0.5% Triton X-100. The experiment was repeated twice with similar results. The locations of apoB<sub>48</sub> (B<sub>48</sub>), apoB<sub>100</sub> (B<sub>100</sub>), and albumin on the gel are indicated at the left-hand side.
for apoB, available data suggest that the movement of apoB out of the ER determines the overall rate of apoB secretion by rat hepatocytes (Borchardt and Davis, 1987) and HepG2 human hepatoma cells (Boström et al., 1988).

During synthesis, or shortly thereafter, apoB associates with the ER membrane (Boström et al., 1988; Borén et al., 1990; Chuck and Lingappa, 1992). The mechanism of apoB translocation across the ER membrane is not yet completely understood. Some available evidence, however, indicates that the translocation of apoB may occur by a unique process (Davis et al., 1990; Dixon et al., 1991; Sato et al., 1990; Furukawa et al., 1992). Evidence exists for proteases that degrade apoB both on the cytosolic (Davis et al., 1990; Dixon et al., 1991) and luminal sides of the microsomal membrane (Furukawa et al., 1992). The delayed movement of apoB across the ER membrane may provide an increased residence time in the membrane during which time the apoB could be susceptible to degradation.

In the experiments reported here we have investigated the mechanism by which apoB secretion from rat hepatocytes is inhibited by treatment of the cells with MME. When hepatocytes were incubated overnight with MME, the concentration of the normally quantitatively insignificant phospholipid, PMME, was increased approximately 10-fold. We have demonstrated previously that MME inhibited the secretion of apoB, triacylglycerols, and phospholipids from hepatocytes by approximately 50%, whereas the secretion of albumin and apoAI was not diminished (Vance, 1991). At that time, however, we were unable to define the underlying cause of this defect in VLDL secretion; in cells enriched in PMME the biosynthesis of the major lipid components of VLDL was not affected.

We have now shown that the rate of synthesis of apoB is not impaired by MME treatment. However, our experiments demonstrate that the amount of apoBapo and apoBap in the lumen of PMME-enriched microsomal membranes is decreased by approximately 50% compared with that in microsomes from untreated hepatocytes. Moreover, apoB in PMME-enriched microsomes has an increased susceptibility to degradation by the exogenously added protease, trypsin. We have also demonstrated that in PMME-enriched hepatocytes, compared with control hepatocytes, a higher percentage of newly synthesized apoB is degraded intracellularly, presumably by the action of the putative luminal and/or cytosolic protease(s) that have been reported to degrade apoB (Davis et al., 1990; Dixon et al., 1991; Furukawa et al., 1992; Sato et al., 1990). Most likely, therefore, MME treatment increases the susceptibility of apoB to degradation in the ER. The consequence of this is that PMME enrichment of hepatocytes decreases the ability of newly synthesized apoB to translocate across the ER membrane. As a result, less apoB is secreted in the form of VLDL, and the pool of apoB that is not secreted is degraded soon after synthesis. Significantly, enrichment of microsomal membranes with PMME does not affect the movement of albumin into the microsomal lumen. This finding supports our previous observation that the secretion of albumin and apoAI from hepatocytes were unaffected by PMME (Vance, 1991).

Two plausible hypotheses may explain the effect of increased levels of PMME on apoB movement into the ER lumen. First, the abnormally high concentration of PMME in the ER membrane might compromise the ability of the putative protein translocation channel in the ER membrane (Simon and Blobel, 1991) to transport apoB into the ER lumen. However, other proteins such as albumin and apoAI are secreted at normal rates in PMME-enriched cells. Therefore, either this explanation is not correct or a specific pathway, whose operation is affected by the change in lipid composition of the ER membrane, exists for translocation of apoB across the ER membrane. A second viable hypothesis is that the change in lipid composition of the ER membrane as a result of MME treatment might decrease the ability of the ER to supply lipids to nascent apoB molecules. In this case, one might visualize that only apoB molecules that had become associated with sufficient lipid would be permitted to enter the ER lumen. If the assembly of lipid with the apoB molecule were faulty, the apoB would be degraded before the defective VLDL could move into the ER lumen. Alternatively, enrichment of the microsomal membrane with PMME might increase the activity of the protease(s) responsible for degradation of apoB. We do not yet know whether the degradation of apoB in the ER controls the amount of apoB that is allowed to translocate into the ER lumen or whether all apoB traverses the membrane and the "redundant" apoB that does not associate with sufficient lipid in the form of a VLDL particle is degraded.

REFERENCES
Apolipoprotein B Movement into the Microsomal Lumen


