

## A sensitive method to analyze in vitro secretion of lipoproteins: distribution of apolipoproteins is modulated by oleic acid in HepG2 cells

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**Abstract** Lipoprotein metabolism can be studied by the analysis of lipoprotein production in cell culture. An inherent problem in such an analysis is the low concentration of lipoproteins in culture supernatants. The difficulty comes from the fact that the samples must be concentrated prior to any analysis. The concentrating methods (e.g., dialysis or ultrafiltration) induce a heterogeneous loss of components. In order to minimize these losses, we have developed a sensitive three-step method to analyze the distribution and the amount of apolipoproteins in the different classes of lipoproteins secreted by the human hepatoma cell line HepG2. Cells were labeled with [<sup>14</sup>C]acetate and [<sup>35</sup>S]methionine for 4 h in the presence of 0.08 mM BSA, complexed or not, with 0.75 mM oleic acid. The <sup>14</sup>C-radiolabeled cellular lipids were extracted and analyzed by thin-layer chromatography and the secreted lipoproteins were analyzed by the following three-step method. First, the lipoproteins were isolated by flotation ultracentrifugation. Second, total lipoproteins were directly applied to native agarose-acrylamide gel electrophoresis in order to separate lipoproteins with respect to their diameter. After migration, the gel was sliced and each fragment was eluted in a buffer containing sodium dodecyl sulfate and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This allowed evaluation of the proportion of apolipoproteins in lipoproteins. Oleic acid (0.75 mM) increased the rate of triglyceride biosynthesis and apoB-100 secretion by 1.7-fold and 2.4-fold, respectively. Moreover, oleic acid treatment modified the profile of secreted lipoproteins. Oleic acid-treated cells secreted more apoB-100 within VLDL than control cells.—**Benoist, F., and T. Grand-Perret.** A sensitive method to analyze in vitro secretion of lipoproteins: distribution of apolipoproteins is modulated by oleic acid in HepG2 cells. *J. Lipid Res.* 1995. **36**: 2243–2250.

**Supplementary key words** apolipoproteins • electrophoresis • lipoproteins • oleic acid

Lipoproteins secreted by the liver are classified into groups of increasing density depending on the protein/lipid ratio (1): very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoprotein (HDL)

(2). ApoB-100 is present exclusively in VLDL, IDL, and LDL particles. Increased concentrations of plasma LDL-cholesterol and apoB-100 are risk factors for the development of atherosclerosis (3). By contrast, apoA-I is associated with HDL particles that are involved in the reverse (anti-atherogenic) cholesterol transport (4). Other apolipoproteins such as apoC-III, C-II, C-I, and E are present in both HDL and VLDL (5). The human hepatoblastoma-derived cell line, HepG2, expresses many functions of normal human hepatocytes. This cell line synthesizes and secretes lipoprotein fractions within the density ranges of VLDL, LDL, and HDL (6, 7). It secretes the apolipoproteins B-100, A-I, A-II, A-IV, E, C-I, C-II, and C-III (8), and HepG2 has been used as a model system to study lipoprotein synthesis and catabolism (7, 9). The assembly and secretion of apoB-containing lipoproteins is still poorly understood. A key step in the modulation of the rate of secretion of apoB-containing lipoproteins seems to be the delivery of fatty acids to hepatocytes: several groups have reported that the addition of oleic acid to the culture medium of HepG2 cells stimulates the secretion of apoB (10–13). Epidemiological studies have shown that the cardiovascular risk depends on the lipid and apolipoprotein content of lipoproteins (14). The modulation of this composition by pharmacological agents could thus be of therapeutic interest. Therefore, it would be of interest to use cell models to study the effects

Abbreviations: VLDL, very low density lipoproteins; ELISA, enzyme-linked immunosorbent assay; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; BME, basal medium Eagle; apo, apolipoprotein; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HMG-CoA reductase, hydroxymethylglutaryl coenzyme A reductase; EDTA, ethylenediaminetetraacetate; ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography.

of potential drugs on the relative distribution and amount of apolipoproteins in lipoproteins. So far, very few studies have analyzed the composition of lipoproteins secreted by cells in culture. This is mainly due to technical problems related to the low amount of lipoproteins secreted in the culture medium. Likewise, there is no simple method to analyze the modulation of apolipoprotein secretion by various compounds or to process several samples at once. Methods used to concentrate the lipoproteins secreted into the culture medium, such as dialysis, ultrafiltration, and lyophilization often induce heterogeneous loss of lipoproteins or apolipoproteins. For example, published data concerning the proportion of the apolipoproteins secreted by HepG2 differ according to the method used (15, 16). Proportions of apoA-I, A-II, B, and E were 62%, 9%, 27%, and 1%, respectively, (15) using dialysis and 25%, 6%, 52%, and 17%, respectively, using an ultrafiltration step (16). Moreover, epitope accessibility could be different after pharmacological treatment of cells, thus making inaccurate the immunological characterization of the lipoproteins. In order to avoid these artefacts, a sensitive three-step method for the characterization of the newly synthesized lipoprotein particles secreted by cells seeded into microwell plates is described. The lipoprotein particles present in culture medium may represent the end product of several postsecretory processes. Therefore, in our experiment, we analyzed the lipoproteins secreted after a short 4-h incubation to minimize the effect of these processes.

In order to validate our novel method for lipoprotein analysis, we studied the effect of oleic acid, a well-known modulator of apoB-100 production by HepG2 cells (10–13).

## MATERIALS AND METHODS

### Cell culture and labeling

HepG2 cells (obtained from ATCC, Rockville, MD) were seeded into 48-well plates ( $8 \cdot 10^4/1 \text{ cm}^2$  well) and

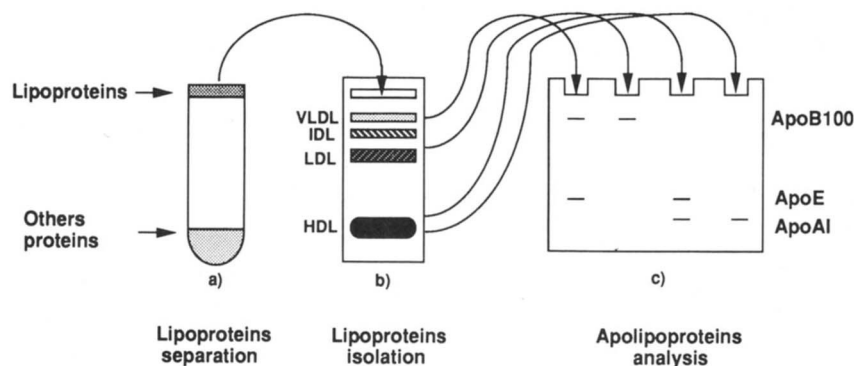
grown in BME (Gibco, Grand Island, NY) containing 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), penicillin/streptomycin (100 units/ml each) (Gibco), and 10% fetal bovine serum (Gibco). The cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator. After 4 days, the cells reached 70–90% confluency. Then HepG2 cells were labeled for 4 h in methionine-free RPMI1640 medium (Gibco) containing 2  $\mu\text{Ci}$  [<sup>14</sup>C]acetic acid (56 mCi/mmol, Amersham Bucks, UK) and 48  $\mu\text{Ci}$  [<sup>35</sup>S] (protein labeling mix 1000 Ci/mmol, NEN) or L-[<sup>35</sup>S]methionine (1000 Ci/mmol, Amersham) per 100- $\mu\text{l}$  well. Either 0.08 mM BSA (Sigma, St. Louis, MO) or 0.75 mM oleic acid (Sigma) complexed to 0.08 mM BSA (molar ratio = 9:1) (10) was added. For 24-h labeling, RPMI 1640 (Gibco) is used.

### Lipid analysis

At the end of the 4-h labeling, conditioned medium was harvested and centrifuged 10 min at 550 *g*, to eliminate cell debris, and subsequently analyzed. Cells were washed twice with phosphate-buffered saline (without Ca<sup>2+</sup> and Mg<sup>2+</sup>), scraped, and the cellular lipids were extracted as described by Bligh and Dyer (17). The organic solvent was evaporated under a N<sub>2</sub> stream. Cellular lipids were resuspended in 30  $\mu\text{l}$  chloroform. An aliquot (5  $\mu\text{l}$ ) was sprayed on a thin-layer chromatography plate (50  $\times$  100  $\times$  0.2 mm silica gel 60F-Merk) using a LINOMAT IV (Camag) and developed in cyclohexane–diethyl ether–glacial acetic acid 8.5:1.4:0.1 (v/v/v). Radioactivity was quantified using a Phosphorimager™ (Molecular Dynamics, Sunnyvale, CA).

### Analysis of apolipoprotein content by the three-step method (Fig. 1)

*Step one: lipoproteins isolation.* One hundred  $\mu\text{l}$  conditioned medium was supplemented with NaN<sub>3</sub> (0.01%), EDTA (0.01%), and thimerosal (0.001%); 10% (v/v) human serum was added as a carrier. The density was adjusted to 1.21 g/ml using KBr and overlaid by 10  $\mu\text{l}$  of



**Fig. 1.** Three-step method to analyze apolipoprotein content. First, the lipoproteins were isolated by flotation ultracentrifugation (a). Second, total lipoproteins were directly applied to native agarose-acrylamide gel electrophoresis in order to separate VLDL, IDL, LDL, and HDL (b). After migration, this gel was sliced and each fragment was eluted in a buffer containing SDS and analyzed by SDS-PAGE (c).

0.9% (w/v) NaCl. Total lipoproteins were isolated by flotation ultracentrifugation on a Beckman TL 120 ultracentrifuge with a TLS 55 swinging bucket rotor. After 18 h at 4°C and 259,000 g, 20 µl of the upper layer containing the lipoproteins was removed (Fig. 1a).

**Step two: lipoprotein separation.** The lipoproteins were separated by native agarose-acrylamide electrophoresis gel (Lipofilm, Sebia, Issy les Moulineaux-France). This gel was divided into two zones of different acrylamide concentration: (2% in the upper layer and 3% in the lower layer) and was designed to separate lipoproteins according to their size (18, 19). Five µl of the lipoprotein fraction was loaded without any desalting procedure. After 1.5 h at 150 V migration, the lane was sliced every 2 mm (Fig. 1b).

**Step three: apolipoproteins analysis.** Each slice of lipofilm gel was eluted for 72 h in 30 µl of Laemmli Buffer containing 3% SDS and 5% mercaptoethanol. The apolipoproteins were analyzed by SDS-PAGE under reducing conditions by the method of Laemmli (20), with resolving gels containing a discontinuous gradient of 5–15% and a stacking gel of 4% acrylamide (Fig. 1c). Apolipoproteins radioactivity was quantified using a Phosphorimager™.

#### Yield of the three-step method

**Lipoproteins isolation yield.** After flotation ultracentrifugation (see above), the top fraction (20 µl) was removed. Then, four fractions of 20 µl (fractions 2–5) were successively collected. The remaining fraction was brought up to 20 µl (fraction 6) with deionized water. In order to recover all proteins, the empty tube was rinsed with 100 µl

of a denaturing buffer containing SDS (fraction 7). The apolipoproteins were analyzed by SDS-PAGE under reducing conditions by the method of Laemmli (20) on a resolving gel containing a discontinuous gradient of polyacrylamide (5–15%) and a stacking gel of 4%. Apolipoproteins radioactivity was quantified using Phosphorimager™.

**Lipofilm elution yield.** After flotation ultracentrifugation, the total lipoproteins secreted by [<sup>35</sup>S]protein labeling mix-labeled HepG2 cells were separated on a lipofilm (5 µl loading). The whole lane was sliced and each piece was eluted in 1 ml of Laemmli Buffer for 6, 24, 48, 72, and 96 h. In the control experiment, 5 µl of the same sample of labeled cells was added to 1 ml of Laemmli buffer.

After elution for the different time periods, supernatants were analyzed by SDS-PAGE. After quantification by a Phosphorimager™, the elution recoveries of apoB-100, apoE, apoA-I, and of apoA-II + apoC were calculated with respect to the control.

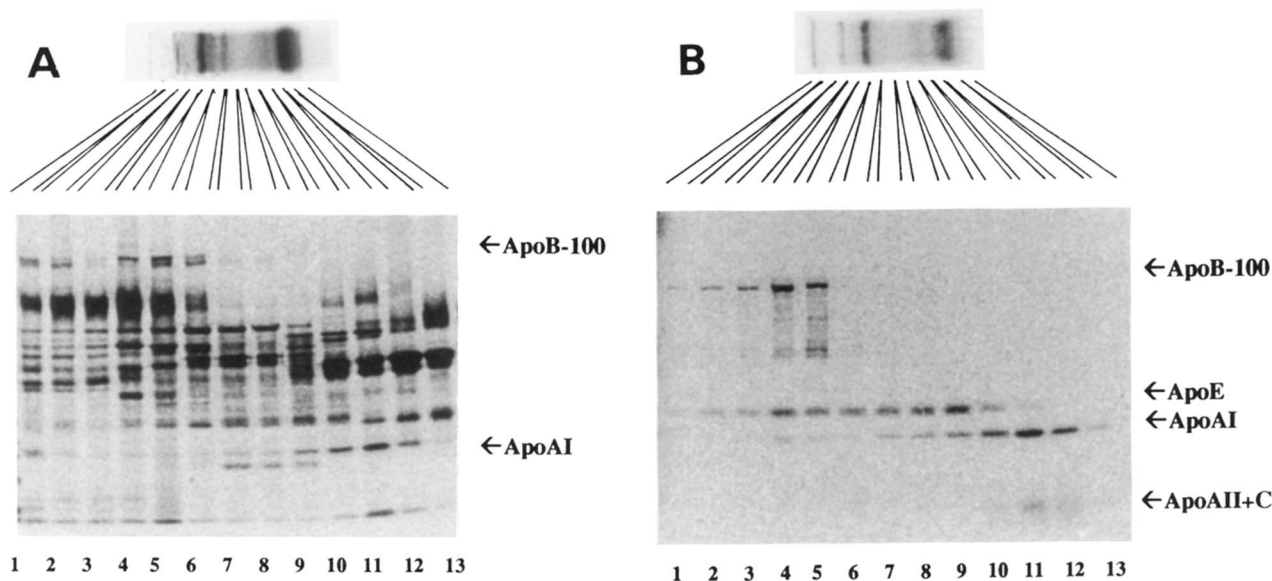
#### Statistical analysis

Statistical significance of differences was calculated by Student's *t* test for paired data with the level of significance selected as  $P < 0.05$ .

## RESULTS

#### Analysis of lipoproteins using a three-step method

To minimize potential reuptake and to illustrate the high sensitivity of this method, HepG2 cells labeled for 4 h with [<sup>35</sup>S]methionine before analysis of secreted



**Fig. 2.** Analysis of lipoprotein components. [<sup>35</sup>S]methionine-labeled proteins secreted by HepG2 cells were analyzed after a 4-h incubation by two methods: the two-step method (A) and the three-step method (B). Standard molecular weights were run on the same gel. The gels were analyzed by Phosphorimager™.

proteins. All the secreted proteins can be resolved by two-dimensional gel electrophoresis: a native agarose-acrylamide gel (i.e., a lipofilm) followed by denaturing SDS-PAGE after gel slicing and elution (Fig. 2a). In the lipofilm (native conditions), proteins migrate according to their charge; however, the migration of high molecular weight complexes such as lipoproteins could be slowed down by interaction with the 3% acrylamide gel matrix. As published (18, 19), we have confirmed using density subfraction of human serum that lipoproteins migrate on lipofilm according to their density; small dense HDL migrate faster than less dense HDL followed by LDL and IDL, whereas VLDL does not enter the 3% acrylamide gel and is stopped at the end of the 2% acrylamide stacking gel (data not shown). As shown in Fig. 2a, apoB-100 and apoA-I can be detected by 2D electrophoresis of total protein (the two-step method) but other apolipoproteins such as apoE or apoC co-migrate with other proteins and are difficult to localize. Moreover, it is not possible to determine the apolipoprotein composition of each lipoprotein class. For this reason, lipoproteins have been isolated by flotation ultracentrifugation prior to this 2D-electrophoresis. As shown in Fig. 2b, the composition of lipoproteins can be visualized: lanes 1-2, VLDL; and lanes 4-5, LDL containing apoB-100 and apoE; lanes 7-12, apoA-I is associated with HDL as well as traces of apoA-II. ApoE, present in VLDL, LDL, and HDL, can also be identified in the fraction including no other detectable apolipoproteins (lane 6). Proteins unassociated with lipids are discarded in the flotation ultracentrifugation step. Others investigators usually separate the various classes of lipoproteins by a density gradient ultracentrifugation. A drawback of this method is that recovered subfractions must be cleared of contaminating salts and concentrated. All these processes could introduce artefacts. By

contrast, we used a native electrophoresis on lipofilm in which KBr salts (up to 2.5 M) do not alter lipoprotein migration (data not shown). A discontinuous gradient was used to analyze apolipoproteins. It is formed by a 5% acrylamide gel in the upper half of the gel for optimal analysis of apoB-100 and by a 15% polyacrylamide gel in the lower half for analysis of other apolipoproteins.

#### Yield of the three-step method

**Lipoprotein isolation yield.** In order to evaluate lipoprotein isolation yield, all fractions, corresponding to six layers of 20  $\mu$ l from top to bottom, were collected. Moreover, the empty tube was rinsed with denaturing buffer containing SDS and this fraction constituted the seventh. Apolipoproteins B-100, A-I, E, and A-II + C and albumin were analyzed by SDS-PAGE and their radioactivity was quantified by Phosphorimager<sup>TM</sup>. The proportion of these proteins was calculated in each fraction. As shown in Table 1, the apolipoproteins were isolated from the other proteins in a replicative manner and the recovery yield was good. The loss of apolipoproteins quantified in fraction 7 was minimal and corresponded to the apolipoprotein adsorption. The fraction 6 did not contain any apolipoprotein except apoE which is also a lipid free protein. We did not detect any albumin in fraction 1, leading us to assume that this fraction is devoid of contaminating proteins.

**Lipofilm elution yield.** After flotation ultracentrifugation, total lipoproteins secreted by [<sup>35</sup>S]protein labeling mix-labeled HepG2 cells were separated on a lipofilm (5  $\mu$ l loading). The whole lane was sliced and each piece was eluted in 1 ml of Laemmli Buffer for 6, 24, 48, 72, and 96 h. In the control experiment, 5  $\mu$ l of the same sample of labeled cells was added to 1 ml of Laemmli buffer.

TABLE 1. Lipoprotein isolation yield

Fraction	Percent of Total for Each Protein				
	ApoB-100	ApoE	ApoA-I	ApoA-II + C	Albumin
1	85.3 $\pm$ 4.2	78.9 $\pm$ 2	78.1 $\pm$ 2	77 $\pm$ 4.3	0
2	11 $\pm$ 2.8	14.9 $\pm$ 1.6	16.2 $\pm$ 2.3	16.6 $\pm$ 2.7	0
3	1.3 $\pm$ 1.2	4.6 $\pm$ 0	5.2 $\pm$ 0.3	4.6 $\pm$ 0.5	0
4	0	0	0	0	0.7 $\pm$ 0.3
5	0	0	0	0	5.4 $\pm$ 0.3
6	0	0.8 $\pm$ 0.1	0	0	92.4 $\pm$ 0.4
7	2.5 $\pm$ 0.1	0.8 $\pm$ 0.1	0.6 $\pm$ 0.2	1.9 $\pm$ 0.5	1.6 $\pm$ 0.1

Total lipoproteins were isolated by flotation ultracentrifugation for 18 h at 4°C and 259,000 g. The upper layer containing lipoproteins (20  $\mu$ l) was removed (fraction 1). The next four fractions of 20  $\mu$ l, from top to bottom, were removed (fractions 2-5). The volume of the last fraction was brought up to 20  $\mu$ l with deionized water (fraction 6). In order to recover all proteins, the empty tube was rinsed with 100  $\mu$ l of Laemmli buffer (fraction 7). The apolipoproteins were analyzed by SDS-PAGE with resolving gels containing a discontinuous gradient of 5-15% and a stacking gel of 4% acrylamide. Apolipoprotein activity was quantified using Phosphorimager<sup>TM</sup>. The results are expressed as percentage of each protein in different fractions. Mean and standard deviation were calculated from four experiments.

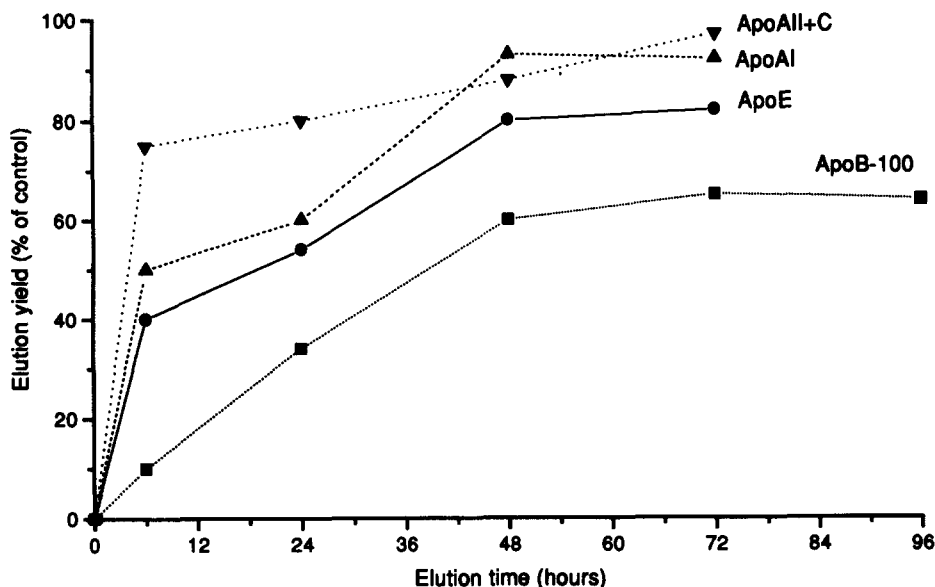


Fig. 3. Lipofilm elution yield. After flotation ultracentrifugation, total lipoproteins secreted by [<sup>35</sup>S]methionine-labeled HepG2 cells were separated on a lipofilm (5  $\mu$ l loading). The whole lane was sliced into several fragments and each of them was eluted in 1 ml of Laemmli buffer for 6, 24, 48, 72, and 96 h. After elution for the different time periods, supernatants were analyzed by SDS-PAGE. After quantification by Phosphorimager<sup>TM</sup>, the elution recoveries of apoB-100, apoE, apoA-I, and of apoA-II + apoC were calculated with respect to control. As controls, we used 5  $\mu$ l of samples collected directly after the flotation ultracentrifugation step. Each point represents the mean of three parallel experiments. Results varied by not more than 2%.

After elution for the different time periods, supernatants were analyzed by SDS-PAGE. After quantification by phosphorimager<sup>TM</sup>, the elution recoveries of the main apolipoproteins were calculated with respect to control. After 72 h of elution at 4°C, 65%, 82%, 92%, and 97% of apoB-100, apoE, apoA-I and of apoA-II + apoC, respectively, were eluted. A better recovery of apoB-100 could not be obtained for a long time of elution (Fig. 3).

#### Validation of the technique: intracellular lipid neosynthesis and lipoprotein secretion are modulated by oleic acid

Our new technique allows the study of the apolipoprotein composition of neosynthesized lipoproteins. It should allow the study of the relationship between the apolipoprotein content of lipoproteins secreted by HepG2 cells and the de novo synthesis of intracellular lipids. Numerous authors have reported that oleic acid stimulates apoB-100 secretion in HepG2 cells. Therefore, we have selected it to demonstrate the validity of our technique. [<sup>14</sup>C]acetate and [<sup>35</sup>S]methionine have been used to label intracellular lipids and secreted proteins simultaneously. A 4-h treatment of HepG2 by oleic acid increased the rate of triglyceride biosynthesis by 1.7-fold as evaluated by the incorporation of [<sup>14</sup>C]acetate into triglycerides (Table 2). Oleic acid did not induce any significant increase of the cellular neosynthesis of cholesteryl ester, cholesterol, and phospholipids.

To determine whether oleic acid-induced alteration of triglyceride neosynthesis had an effect on the composition of secreted lipoproteins, the supernatants of HepG2 cells incubated in the presence or absence of oleic acid (0.75 mM) were compared. The effect of oleic acid on the amount and distribution of apoB-100 secreted by HepG2 cells was analyzed by the three-step method. As shown in Fig. 4 and Table 3, addition of oleic acid resulted in a 2.4-fold increase in the secretion of apoB-100 by HepG2 cells without affecting the apoA-I secretion. This selective stimulation of apoB-100 secretion has been confirmed by ELISA:  $7.0 \pm 0.4$  and  $2.0 \pm 0.2$  ng/ $10^4$  cells per 4 h for apoB-100 and apoA-I in

TABLE 2. Quantification of neosynthesis of cellular lipids

Cellular Lipids	BSA	Oleic Acid + BSA
Triglycerides	2266 $\pm$ 297	3828 $\pm$ 691*
Phospholipids	19651 $\pm$ 881	16195 $\pm$ 3953
Cholesteryl ester	199 $\pm$ 21	153 $\pm$ 55
Unesterified cholesterol	515 $\pm$ 61	688 $\pm$ 123

Cellular lipids were extracted from confluent HepG2 cells after 4 h of metabolic radiolabeling with [<sup>14</sup>C]acetate in the presence of oleic acid + BSA or BSA alone. They were analyzed by quantitative thin-layer chromatography using LINOMAT IV sample application. After migration in cyclohexane-diethyl ether-glacial acetic acid 8.5:1.4:0.1 (v/v/v), radiolabeled lipids were quantified using Phosphorimager<sup>TM</sup>. Mean and standard deviation were calculated from three experiments. \*Significant difference at  $P < 0.05$ .

control conditions and  $17.0 \pm 1.2$  and  $2.0 \pm 0.3$  by oleic acid-treated cells respectively. Moreover, oleic acid treatment induced a redistribution of apoB among the lipoprotein fractions. The amounts of apoB-100 that accumulated in the density range of VLDL increased significantly by 12.7-fold ( $P = 0.0073$ ) while an increase of 2.5-fold ( $P = 0.0011$ ) was observed for apoB-100 in the LDL density range. Thus oleic acid-treated cells secreted a higher proportion of VLDL. In conclusion, oleic acid stimulation of intracellular triglycerides synthesis is correlated with the increase of lipid-enriched apoB-100-containing lipoproteins.

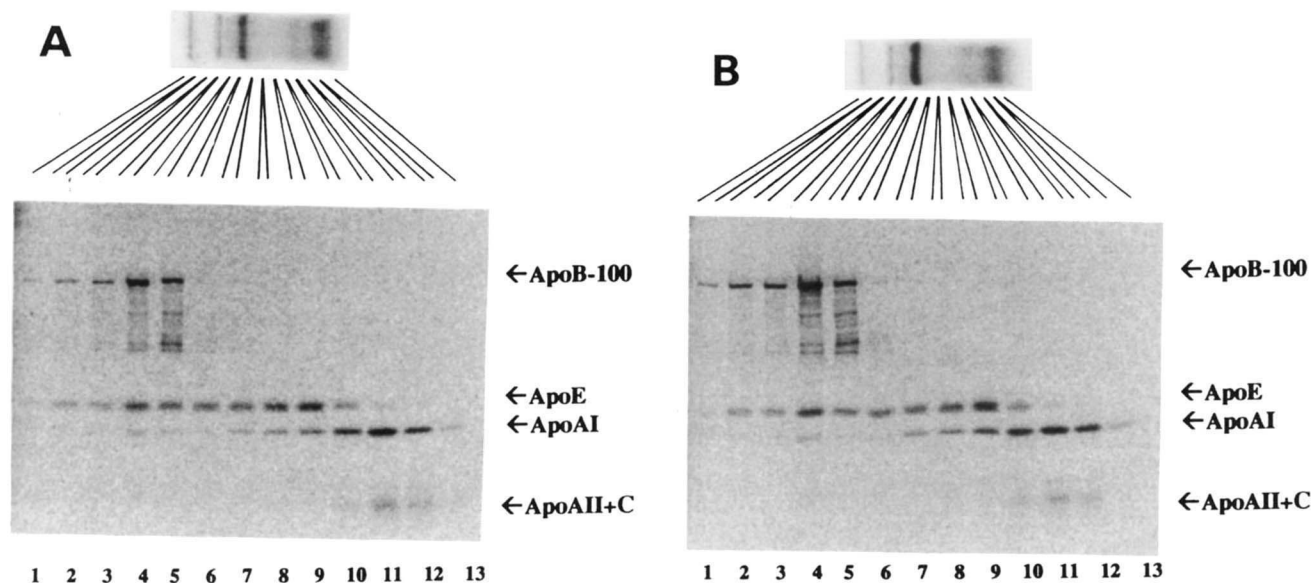
## DISCUSSION

In this paper, we describe a sensitive method to study the composition of lipoproteins secreted by cells in culture. Our method has several advantages: it requires neither delipidation of the lipoproteins nor dialysis of the sample, not even when high concentrations of salts are present (e.g., KBr used to isolate lipoproteins by ultracentrifugation). This method allows the analysis of both the amount and distribution of apolipoproteins within the different lipoproteins secreted by cells in culture. A low number of cells is sufficient for this study (e.g.,  $8 \times 10^4$  cells). This three-step method minimizes losses and changes in the composition of lipoproteins. The method has been evaluated using HepG2 cells but can also be used for the analysis of lipoproteins secreted by other cells in culture (e.g., primary cultures of rat or

human hepatocytes). Moreover, this technique allows for handling a large number of samples at once, thus permitting the analysis of the modulation of apolipoprotein secretion by various compounds in the same experiment. The amino acid sequence of all human apolipoproteins has been published and the number of methionine per molecule is known: 78 (21), 7 (22), and 3 (23) for apoB-100, E, and A-I, respectively. After the quantification of [ $^{35}\text{S}$ ]methionine in the apolipoproteins, it is possible to calculate the molar ratio of the different apolipoproteins present in one class of lipoprotein; for example, VLDL secreted by control HepG2 cells contains 1 mole of apoB-100 for 10 moles of apoE.

In HepG2-conditioned medium, apoA-I is secreted as constituent of HDL. Next to apoA-I, small amounts of apoA-II are found in the HDL fraction. ApoB-100 is present in VLDL and IDL, but predominantly in LDL, whereas apoE is observed in several fractions including VLDL and HDL. These results are in accordance with the characterization of lipoprotein particles from human serum described by Fruchart and Bard (24). In addition to the detection of apoE in VLDL and HDL, we found apoE in a lipoprotein without any other apolipoprotein with a migration between LDL and HDL fractions. This is in line with the existence of  $\gamma\text{LpE}$  which has recently been described by Huang et al. (25).

In order to validate our novel method, we have studied the effect of oleic acid, a well-known modulator of apoB-100 production by HepG2 cells (10–13). In addition to protein labeling, we measured the incorporation of [ $^{14}\text{C}$ ]acetate into intracellular lipids (triglycerides,



**Fig. 4.** Effects of oleic acid + BSA on apolipoproteins. HepG2 cells were incubated for 4 h in medium RPMI 2640 containing either 0.08 mM BSA (A) or 0.75 mM oleic acid complexed to 0.08 mM BSA (molar ratio = 9:1) (B). Two  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]acetic acid (56 mCi/mmol Amersham) and 48  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]methionine per well were added in methionine-free medium. Lipoproteins were analyzed by the three-step method (see Materials and Methods). Standard apolipoproteins of known molecular weights were run on the same gel. The gels were analyzed by Phosphorimager<sup>TM</sup>.

cholesteryl ester, free cholesterol, and phospholipids) for 4 h. We have shown that the intracellular content of neosynthesized triglycerides was increased 1.7-fold and the apoB-100 secretion was increased 2.4-fold upon treatment with 0.75 mM oleic acid during a 4-h incubation period. These results on HepG2 cells are in line with those of other investigators (10–13). This indicates that the rate of intracellular triglyceride neosynthesis regulates the apoB-100 secretion rate. Indeed, with our 4-h incubation period, there is no other neosynthesized lipid accumulation. When [<sup>14</sup>C]glycerol was used instead of acetate, the stimulation of triglycerides neosynthesis was even higher: 3-fold (data not shown) but this did not allow the detection of the neosynthesis of cholesterol and cholesteryl esters. Incubation of HepG2 cells with oleic acid modified the composition of secreted lipoproteins. The ratio of VLDL/LDL was increased by oleic acid treatment. Similar results were observed by Ellsworth, Erickson, and Cooper (26), who reported that treatment of HepG2 cells with oleic acid caused a major redistribution of both secreted apoB and triglycerides from LDL to VLDL. However, in contrast to our results and those of many other laboratories (10–13), Ellsworth et al. (26) did not observe a stimulation of apoB-100 secretion. We do not know why the data of Ellsworth et al. differ from our data as well as the data of several other laboratories describing a stimulatory effect of oleic acid on apoB-100 secretion.

As described by others, the secretion of apoB-100 in vivo might occur in two ways (27, 28). The VLDL pathway could be activated in the presence of oleic acid, perhaps because the secretion of triglyceride-rich VLDL is limited by the triglyceride neosynthesis rate. Without oleic acid, the triglyceride synthesis is low and apoB-100 is nearly exclusively secreted as lipid-poor LDL. Our observation about the stimulation of the VLDL secretory pathway by oleic acid in HepG2 cells might explain the observed in vivo increase in VLDL upon lipid intake (29).

In preliminary studies using primary rat hepatocytes, analysis of the secreted lipoproteins with the three-step

TABLE 3. Effect of oleic acid + BSA on the radiolabeled amount of apoB and distribution between VLDL and LDL

	BSA Control (% of ApoB-100)	Fold Increase with Oleic Acid
	%	-fold
Total apoB-100		2.4 ± 0.5
VLDL apoB-100	1.4	12.7 ± 2.6
LDL apoB-100	98.6	2.5 ± 0.5

HepG2 cells were incubated with [<sup>35</sup>S]methionine for 4 h with or without 0.75 mM oleic acid + BSA. The conditioned medium was analyzed by the three-step method (see Materials and Methods). ApoB-100 in VLDL and LDL was quantified by Phosphorimager<sup>TM</sup>. Results are expressed as percentage of total apoB-100 secretion of the control sample and as relative fold increase of control with oleic acid. Mean and standard deviation were calculated from three experiments.

method described in this paper showed that treatment with oleic acid caused a major redistribution of both apoB-100 and apoB-48 from LDL to VLDL but without any increase of the apoB-100 secretion. The latter finding is in accordance with those of published data (30, 31). In fact, it is now well established that in contrast to the observation in HepG2 cells, exogenous oleic acid stimulates triglyceride secretion, but it does not stimulate total apoB secretion in rat hepatocytes in culture.

Our method allows for the study of the modification of apoB distribution within lipoproteins. It offers a new way to study the effect of pharmacological agents on the distribution of the apolipoproteins within subclasses of the lipoproteins. ■

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