Chylomicron remnant-vitamin A metabolism by the human hepatoma cell line HepG2

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Abstract The binding and metabolism of [^{3}H]vitamin A-containing chylomicron (CM) remnants by the human hepatoma cell line HepG2 were studied. Mesenteric lymph chylomicrons were collected from [^{3}H]retinol-fed rats and incubated with lipoprotein lipase to obtain CM remnants. At 4°C, specific CM remnant binding was inhibited by an excess of unlabeled CM remnants. Specific binding predominated at low concentrations and approached saturation while total binding continued to increase over an extensive concentration range (0.45–32 μg triglyceride/ml). CM remnant uptake at 37°C was greater than that of CM and at least 70 times more efficient than the pinocytosis of sucrose. CM remnant binding increased with the extent of lipolysis. Addition of human apolipoprotein E enhanced both CM remnant and CM binding. After internalization, HepG2 cells hydrolyzed CM remnant-[^{3}H]retinyl esters, and radiolabeled metabolites accumulated. As a function of the concentration of [^{3}H]retinoid initially bound to cells, retinol and retinyl esters accumulated as the major cell-associated metabolites. In contrast, retinol was the major metabolite in the medium only at low retinoid concentrations; other more polar metabolites accumulated at higher concentrations (> 110 pmol retinoid/mg cell protein). The accumulation in the medium of labeled metabolites derived from CM remnant-retinoid was reduced when cells were preincubated in unlabeled retinol-supplemented media. The specific activity of retinol in the medium indicated that CM remnant-vitamin A had mixed with the cellular store prior to its secretion as retinol. These results indicate that HepG2 cells internalize CM remnants in part by specific binding sites, and that the metabolism of CM remnant-retinoids by the HepG2 cell involves retinyl ester hydrolysis and the secretion of retinol and other more polar metabolites. These processes were regulated in part by the concentration of retinoid delivered by the CM remnant and by the initial retinoid content of the cell. — **Lenich, C. M., and A. C. Ross.** Chylomicron remnant-vitamin A metabolism by the human hepatoma cell line HepG2. *J. Lipid Res.* 1987. **28:** 183–194.

Supplementary key words chylomicron remnant binding • retinoid metabolites • lipolysis

The tissue of key importance in regulating the metabolism and distribution of vitamin A is the liver. Newly absorbed vitamin A is delivered to liver mainly as retinyl ester in association with CM remnants (1). Studies have shown that remnant uptake by liver involves a receptor-mediated process (2) in which specific apolipoproteins play an important role (3–6). During the uptake process, CM remnant-retinyl esters are hydrolyzed, reesterified (1, 7) and are subsequently found in cytoplasmic lipid droplets (8). The liver also secretes retinol to plasma bound to retinol-binding protein (9).

It is generally thought that vitamin A stores in the liver serve to buffer the fluctuations in dietary intake and to maintain steady-state levels of plasma retinol for use by peripheral target tissues (10). Two cell types in liver, parenchymal and stellate cells, have been shown to be important in the hepatic metabolism of vitamin A. Parenchymal cells are mainly responsible for the initial uptake of CM-vitamin A by liver (11) and for the synthesis and secretion of retinol-binding protein (12). Whereas parenchymal cells have been shown to accumulate retinyl ester (8, 13), the majority of liver retinoids are now understood to be stored in the fat-storing stellate cell (14–16). In addition, recent studies in the vitamin A-sufficient rat have shown that CM remnant-vitamin A is transferred from hepatic parenchymal cells to stellate cells within a few hours of initial uptake (11, 17). How the reactions involved in the uptake, storage, and mobilization of vitamin A by liver are coordinated and regulated, however, is poorly understood.

To study the initial metabolism of dietary vitamin A by liver, we have chosen to examine the uptake and metabolism of CM remnant-vitamin A by the HepG2 cell line. Recently derived from human liver tumor biopsies (18), this cell line has retained several functional characteristics of liver parenchymal cells such as the secretion of plasma proteins including retinol-binding protein (19) and several apoproteins (20, 21). HepG2 cells have also been shown to possess specific receptors for asialoglycoproteins (22) and insulin (23), and to bind and catabolize

Abbreviations: CM, chylomicron; MEM, minimal essential medium; PBS, phosphate-buffered saline; apo, apolipoprotein; HPLC, high performance liquid chromatography.

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low density lipoprotein (24–26), high density lipoprotein (27), and apoprotein E-containing particles (28). Thus this cell appeared to be suitable for a study of the early events in the hepatic metabolism of retinoids delivered by the CM remnant.

MATERIALS AND METHODS

Isotopes, chemicals, and solvents

All tissue culture supplies were obtained from Flow Laboratories (McLean, VA). [10,11-$^3H_2$]Retinyl acetate (all-*trans*) was purchased from SRI International (Menlo Park, CA) and [1-^{3}H(N)]-vitamin A (all-*trans*) and [^{14}C]sucrose were purchased from New England Nucelar (Boston, MA). All retinoids were stored at −20°C under N_2 and protected from light. All-*trans*-retinol (crystalline, Type X), retinyl acetate, retinoic acid, butylated hydroxytoluene, sodium heparin (Grade II), bovine serum albumin (essentially fatty acid-free), and bacterial beta-glucuronidase (Type X) were obtained from Sigma Chemical Co. (St. Louis, MO). Delipidized serum protein was prepared as previously described (29). Hexanes (certified), liquid scintillation fluids (ScintiLene and ScintiVerse), and neutral aluminum oxide were purchased from Fisher Scientific Co. (King of Prussia, PA). Diethyl ether containing butylated hdyroxytoluene as preservative and high performance liquid chromatography (HPLC) grade acetonitrile were products of J. T. Baker (Phillipsburg, NJ). Solvents for extraction and chromatography of vitamin A compounds contained 5 μg/ml of butylated hydroxytoluene.

Preparation of labeled CM and CM remnants

Female Sprague-Dawley rats (180–250 g) were anesthetized and cannulas were inserted into the upper duodenum (Silastic, Dow Chemical Co., Midland, MI, 0.03 inch ID, 0.65 inch OD) and the main mesenteric lymph duct (0.025 inch ID, 0.047 inch OD). For the collection of radiolabeled lymph, 1 mCi of [^{3}H]retinol or [^{3}H]retinyl acetate was dissolved in 0.2 ml of safflower oil and infused through the duodenal cannula over a 1–2 hr period. In this study safflower oil was used for lymph collection in order to obtain chylomicrons that would not undergo the temperature-induced transitions that occur in chylomicrons collected from rats fed diets enriched in saturated fat (30). The labeled lymph was collected over ice in glass tubes containing EDTA (0.67 mg/ml) and sodium azide (0.2 mg/ml) as preservatives. The lymph was defibrinated, filtered through gauze, and CM were isolated by ultracentrifugation as previously described (31).

CM remnants were prepared by incubating CM (at 37°C) in 5% (v/v) rat postheparin plasma to which was added 1 mol of bovine serum albumin/mol of CM triglyceride. Postheparin plasma was prepared from aortic blood of fasted rats that had been injected intravenously with heparin (500 U/kg body wt) 10 min earlier. Generally, 50 mg of CM triglyceride, 1.7 g of bovine serum albumin, 1.5 ml of postheparin plasma, 0.02% sodium azide, and 0.2 M Trizma-base buffer, pH 8, in 30 ml were incubated until the initial turbidity of the mixture had cleared (2–4 hr). In some experiments CM remnants were prepared by incubation in media collected from cultured endothelial-like cells that secrete lipoprotein lipase (32). This medium was assayed for lipase activity by the method of Nilsson-Ehle and Schotz (33) and was added at 1–2 U of enzyme activity/mg of CM triglyceride (a unit is defined here as that amount of enzyme needed to generate 1 μmol of fatty acid from triolein per hr at 37°C). Human high density lipoprotein (HDL) was added to these incubations (1 mg HDL protein/2 mg triglyceride) as a source of apoprotein C-II. CM incubated in the absence of enzyme or in the presence of heat-inactivated postheparin plasma (60 min, 60°C) served as controls for the lipase treatment. Control CM and CM remnants were reisolated from the incubation mixture by the procedure of Gustafson, Alaupovic, and Furman (34) for the collection of lipoproteins of $S_f > 20$. Remnants were stored at 4°C under N_2 in the dark for up to 10 days.

Addition of apoproteins

Reisolated, radiolabeled CM and CM remnants prepared by incubation with lipoprotein lipase were incubated with human apoprotein (apo) E (20–800 μg/mg of CM remnant-triglyceride) in Eagle's minimal essential medium (MEM) containing bovine serum albumin (2.5 mg/ml). The mixtures were gently shaken for 2 hr at 37°C and added to cells immediately or after storage at 4°C for up to 24 hr. The human apoprotein was a generous gift from Dr. William Bradley of Baylor College of Medicine, Houston, TX. The protein had been lyophilized from 10 mM NH_4HCO_3 and contained mainly two isoforms, apoE-2 and apoE-3 (35). Before adding the apoprotein to lipoproteins, it was reduced with mercaptoethanol, relyophilized, and then solubilized in MEM.

Cell culture

Initial cultures of the HepG2 cell line were obtained from Dr. Barbara Knowles of the Wistar Institute, Philadelphia, PA and stock cultures were maintained in a humidified CO_2 incubator at 37°C in 75-cm^2 flasks containing 15 ml of MEM supplemented with basal-modified Eagle's vitamins, 10% (v/v) fetal bovine serum, and 0.005% (w/v) gentamicin. For lipoprotein incubations, cells were seeded in plastic dishes and grown to confluency. Confluent monolayers were washed three times with serum-free MEM containing 27 mM HEPES and incubated 12–36 hr prior to an experiment in either this medium or in medium also containing 2.5 mg/ml of de-

lipidized serum protein. Before the addition of labeled lipoproteins, the preincubation media was removed and the monolayers were washed three times with serum-free MEM.

Binding, uptake, and metabolism assays

For binding studies, cells in 35-mm dishes (typically 1–1.2 mg of cell protein per dish) were precooled for 30 min at 4°C. ^{3}H-Labeled CM and CM remnants in the presence or absence of a 20-fold excess of unlabeled lipoprotein were incubated with cells at 4°C in 1 ml of MEM containing 0.25% (w/v) bovine serum albumin. At the end of the incubation period, analysis of cells was carried out at 4°C. The medium was removed and the cells were washed three times with phosphate-buffered saline (PBS) containing bovine serum albumin (2 mg/ml) and finally three times with PBS alone. Cells were scraped from plates into 1.5 ml of PBS and homogenized by sonication. Aliquots of cell homogenates were assayed for protein and for radioactivity.

Uptake of ^{3}H-labeled CM and CM remnants was determined using similar incubation conditions at 37°C after which cell-associated radioactivity was measured. When surface-bound CM remnant-[^{3}H]retinoid was measured, monolayers were incubated with 0.5 ml of 0.25% trypsin for 15 min at 37°C as described by Bierman, Stein, and Stein (36). Heparin was also used (37) to measure surface-bound remnant-[^{3}H]retinoid; monolayers were incubated with 0.5 ml of sodium heparin (10 mg/ml) for 15 min at 37°C. The cells were centrifuged at 3000 *g* for 10 min at 4°C. Radioactivity released by either trypsin or heparin is the amount of CM remnant-[^{3}H]retinoid bound to the cell surface and that remaining cell-associated is the internalized remnant-[^{3}H]retinoid.

To measure the metabolism of remnant-[^{3}H]retinoid initially bound to the cell surface, cells in either 35-mm or 100-mm dishes were precooled for 30 min at 4°C. [^{3}H]Vitamin A-labeled CM remnants were incubated with cells for 1 hr at 4°C, after which the medium was removed and cells were washed as previously described. Fresh, unlabeled MEM was added (1 ml to 35-mm dishes and 5 ml to 100-mm dishes) and the incubations were continued at 37°C. At designated times the medium was collected and the cells were washed once with cold PBS. The media and cell sonicates were immediately added to ethanol for lipid extraction. The uptake of [^{14}C]sucrose by HepG2 cells was measured as descrcibed previously (28). Media containing different concentrations of [^{14}C]sucrose (12.5–100 μM), which had been screened by preincubation with cells to remove any [^{14}C]glucose, were incubated with HepG2 cells at 37°C for up to 2 hr. Cells were then washed and analyzed for protein and radioactivity.

Glucuronidase incubations

Portions of media from cells that had been incubated for 8 hr with CM remnant-[^{3}H]vitamin A were subjected to enzymatic hydrolysis. Two concentrations of beta-glucuronidase (8 and 16 U/ml) were used; units of enzyme activity were determined by measuring the amount of phenolphthalein (absorbance at 540 nm) liberated from phenolphthalein glucuronide under similar incubation conditions. Incubations with remnants were carried out at 37°C for 30–60 min. The incubation mixtures were extracted sequentially with hexane under basic and acidic conditions as described below and the hexane extracts were assayed for radioactivity.

Lipid extractions and chromatography

To determine the distribution of radioactivity between free and esterified retinol, neutral lipids were quantitatively extracted from 1 ml of sample (cells, media, or lipoproteins) into 4 ml of hexanes (38). After centrifugation, an aliquot of the upper phase (hexane) was taken to measure total extracted radioactivity. A second aliquot was taken for chromatography on columns of aluminum oxide as described previously (31). [^{3}H]Retinyl esters were eluted with hexane containing diethyl ether (3%, v/v).

To determine the distribution of radioactivity between neutral and acidic lipids, samples were sequentially extracted by the procedure of Borgström (39). Neutral and basic lipids were partitioned into 5 ml of hexane from 50% ethanol containing 0.01 N NaOH (pH 10.5). After centrifugation, thet hexane phase was collected. The extraction was repeated twice and hexane fractions were combined. HCl was added to the lower aqueous phase to give a final concentration of 1 N (pH 1.5) and extractions were repeated twice with hexane as described above. The hexane fractions were concentrated under nitrogen and an aliquot was taken to measure total extracted radioactivity. A second aliquot of the basic lipid extract was taken for aluminum oxide chromatography as described above. After eluting [^{3}H]retinyl esters, [^{3}H]retinol was eluted using 20 ml of hexane containing diethyl ether (50%, v/v). In some experiments, eluates from aluminum oxide columns and hexane fractions from acid-extractions were subjected to HPLC. Samples for analysis were concentrated under nitrogen, dissolved in acetonitrile, and analyzed on a Supelcosil LC-8 column as previously described (40). The mobile phase, pumped at 2.4 ml/min, consisted of acetonitrile–water 75:25 (v/v) containing 0.01 M ammonium acetate. Fractions were collected every 15 sec and portions were assayed for radioactivity.

Radioactivity measurements

^{3}H- and ^{14}C-radioactivity were determined in a Beckman LS 7500 liquid scintillation system. Efficiencies were

determined using calibrated [^{3}H]- or [^{14}C]toluene as internal or external standards.

Chemical analysis

Chylomicron and CM remnant triglyceride was extracted (38) and measured by the procedure of Sardesi and Manning (41). Cell protein was determined by the method of Markwell et al. (42).

RESULTS

Characteristics of the binding and uptake of CM remnants by HepG2 cells

When HepG2 cells were incubated with [^{3}H]retinoid-labeled CM remnants (8 μg of triglyceride/ml) at 4°C, binding to the cell surface was nearly complete within the first 15 min of incubation (**Fig. 1,** insert).

HepG2 cells were then incubated with increasing concentrations of CM and CM remnants (added at equivalent vitamin A concentrations to normalize for particle number) for 4 hr at 4°C (Fig. 1). Remnants were also incubated in the presence of excess unlabeled remnants to measure nonspecific binding. CM remnants bound to HepG2 cells more readily than did CM. The concentration-dependent binding of remnants was biphasic in nature; the rate at which binding increased was greater at low concentrations but continued to increase over an extensive concentration range (0.45–32 μg of triglyceride/ml). Saturation was apparent only when very high lipoprotein concentrations were used. Nonspecific binding increased as a linear function at low remnant concentrations where it remained a low proportion (approximately 20%) of the total binding measured. Because of the large volume of unlabeled CM remnants required, nonspecific binding could not be measured at higher concentrations. Specific binding was calculated by subtracting nonspecific binding from total binding (in the absence of unlabeled lipoprotein). At lower CM remnant concentrations, specific binding approached saturation (Fig. 1).

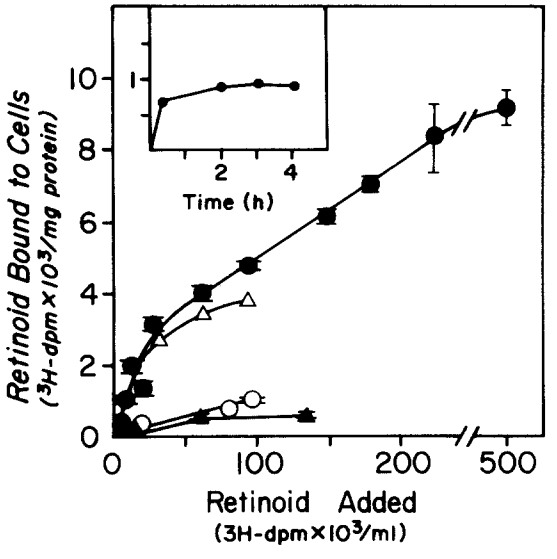

Fig. 1. Binding of CM and CM remnants at 4°C by the HepG2 cell. Cells that had been incubated for 36 hr in medium containing delipidated plasma (2.5 mg of protein/ml) were incubated for 4 hr at 4°C with increasing concentrations of CM or CM remnants. CM remnants (having 85% of triglyceride hydrolyzed and containing 0.45 to 72 μg of remnant triglyceride/ml medium) were incubated with cells and specific remnant binding (△) was calculated by subtracting the amount of [^{3}H] remnants bound in the presence of a 20-fold excess of unlabeled remnants (○) from the amount bound in the absence of unlabeled remnants (●). Cells were also incubated with CM, 12 to 136 μg of triglyceride/ml (▲). Lipoproteins differing in extent of hydrolysis were added on the basis of [^{3}H]retinoid content to normalize to equal particle number. Each point is the mean ± SD of three plates of cells. Insert: Total CM remnant binding at 4°C was determined after incubation times of 15 min to 4 hr. In this experiment, remnants having 82% of triglyceride hydrolyzed were added at a concentration of 8 μg of triglyceride/ml. Each point is the mean of two plates of cells.

To examine the internalization of CM remnants by the HepG2 cell, time course studies were conducted at 37°C in which trypsin was used to distinguish surface-bound (trypsin-releaseable) from internalized (trypsin-resistant) lipoprotein (36). The amount of radioactivity released by trypsin reached a steady-state level during the first 10 min of incubation, while internalized radioactivity continued to increase throughout the 4 hr incubation (**Fig. 2**). During the longer incubations (Fig. 2, insert), the accumulation of radioactivity by cells reached a steady-state level by approximately 10 hr. The proportion of radioactivity as ester in the trypsin-released material (86.2 ± 0.1%, mean ± SD, n = 9) remained constant with time and similar to that of the CM remnant added (88.2 ± 1.0%) indicating that hydrolysis of retinyl esters did not occur on the cell surface.

The uptake of [^{14}C]sucrose by HepG2 cells was measured to estimate to what extent fluid-phase pinocytosis might contribute to CM remnant uptake. The uptake of sucrose increased as a linear function of time (up to 1 hr) and concentration (12.5–100 μM). The clearance of sucrose from the medium was calculated to occur at a rate of 0.31 μl/mg of cell protein/hr. By contrast, the rate at which HepG2 cells take up CM remnant-[^{3}H]vitamin A from the medium corresponded to a clearance of approximately 23–55 μl of media/mg of cell protein/hr (38 ± 14 μl/mg of cell protein, mean ± SD for four independent experiments).

CM remnant uptake by cells has been shown to be at least partly regulated by alterations in the chemical composition of the CM including the hydrolysis of its lipid components (43, 44) and changes in its apoprotein composition (4–6). The uptake process has also been shown to be temperature-dependent (2, 45). Experiments were done to determine whether CM remnant uptake by the

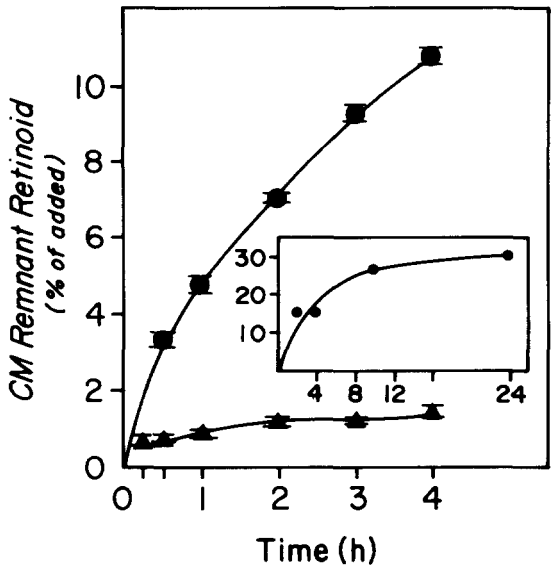

Fig. 2. Surface-binding and internalization of CM remnants as a function of time. After a 24-hr incubation in medium containing delipidated plasma (2.5 mg of protein/ml), cells were incubated at 37°C with CM remnants (having 86% of triglyceride hydrolyzed and containing 72 μg of triglyceride/ml). Cells were trypsinized as described under Methods and trypsin-released (▲, surface-bound) or trypsin-resistant (●, internalized) radioactivity was measured. Data are expressed as the percentage of CM remnant radioactivity added and each point is the mean ± SD of two or three plates of cells. Insert: CM remnants (having 72% of triglyceride hydrolyzed and containing 31 μg of triglyceride/ml) were incubated with cells at 37°C after which total cell-bound radioactivity was measured. Each point is the mean ± SD of three plates of cells.

HepG2 cell is similarly regulated. The effects of temperature on CM remnant binding and internalization were examined by incubating cells with [^{3}H]retinoid-labeled CM remnants at either 4°C or 37°C. As shown in **Table 1,** there was no significant difference in the amount of radioactivity released by trypsin at either temperature. The amount of radioactivity that was resistant to trypsin treatment, however, was much greater at 37°C indicating internalization of lipoprotein at this temperature. When these experiments were conducted using heparin in place of trypsin to release surface-bound material, heparin released nearly the same amount of cell-bound radioactivity as trypsin at either temperature.

To determine the contribution of lipid hydrolysis to CM remnant binding, remnants of different degrees of triglyceride hydrolysis were prepared by incubating one preparation of CM with postheparin plasma for various times. When remnants thus prepared were isolated and incubated with cells at 4°C, the concentration-dependent binding of the remnants (added at equivalent vitamin A concentrations since vitamin A per particle is expected to remain constant) increased as the degree of triglyceride hydrolysis increased, at least up to 72% removal of triglyceride (**Fig. 3**).

TABLE 1. Chylomicron remnant surface-binding and internalization as a function of temperature

	Chylomicron Remnant-[^{3}H]Retinoid	
	4°C[a]	37°C[a]
	dpm/mg cell protein	
Released by trypsin	408 ± 73	468 ± 40[b]
Resistant to trypsin	794 ± 32	3150 ± 13[c]
Released by control	256 ± 4	210 ± 23[b]
Resistant to control	967 ± 95	3584 ± 184[c]

After a 16-hr preincubation in serum-free media, monolayers were incubated with CM remnants (having 72% of triglycerides hydrolyzed and containing 50 μg of triglyceride/ml) for 1 hr at either 4°C or 37°C. Cells were washed and incubated with trypsin to release surface-bound lipoproteins. Trypsinized cells were collected by centrifugation and control cells (incubated with MEM alone) were collected by scraping. Supernates were collected and assayed for "released" radioactivity and the cells were washed and assayed for "resistant" radioactivity as described under Methods. Values are means ± SD of thre plates of cells and are representative of two experiments. In this experiment, cells were also incubated with heparin in place of trypsin to release surface-bound lipoproteins. Heparin released the same amount of CM remnant-[^{3}H]retinoid as trypsin at either temperature.

[a] Incubation temperature.
[b] Not significantly different from 4°C value.
[c] $P < 0.05$ compared to 4°C value.

To investigate the contribution of factors in plasma other than lipase activity that stimulate CM remnant uptake by HepG2 cells, remnants were prepared by incubating CM with lipoprotein lipase (obtained from cell

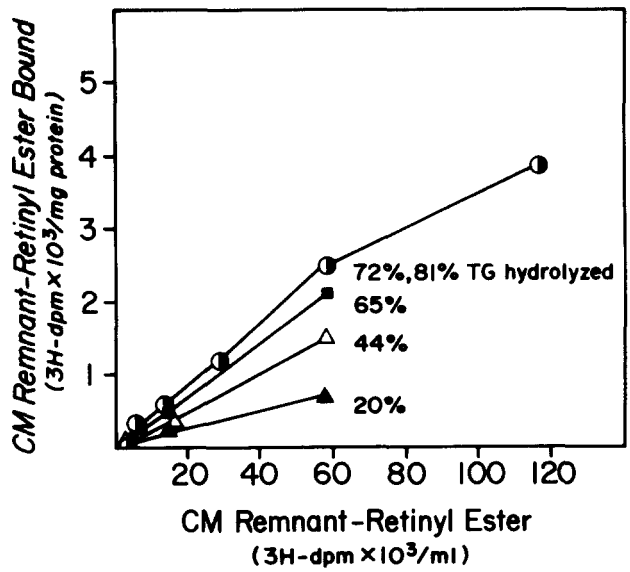

Fig. 3. Binding of CM remnants of differing degrees of triglyceride hydrolysis. One preparation of CM was incubated with postheparin-plasma for different times (0 to 7 hr) to produce remnants varying in triglyceride removal. Lipoproteins were reisolated and incubated with cells as in Fig. 1. Each point is the mean ± SD of three plates of cells. Over the concentration range used, CM remnant binding in this study was nearly linear. In general, the lipoprotein concentration required for saturable binding was variable and apparently depended on the preparation of CM used as well as the degree of triglyceride hydrolysis.

culture) in the presence or absence of plasma. Despite significant triglyceride hydrolysis (93%), remnants prepared in the absence of plasma bound poorly to cells, similar to the binding of control CM that were prepared either in the absence of enzyme or in the presence of heat-inactivated postheparin plasma. A similar observation was made by Nilsson, Ehnholm, and Floren (46) using remnants prepared by incubation with pure bovine milk lipoprotein lipase and rat hepatocyte monolayers. These data indicate that modification by lipase activity alone does not enhance CM remnant binding to liver cells, but that other factors present in plasma are also required. The hypothesis that a transfer of apoproteins between CM and other plasma lipoproteins is necessary for CM recognition by liver is supported by these data.

To investigate the role of specific plasma apoproteins in directing CM remnant uptake by HepG2 cells, experiments were done to determine whether the binding of CM and remnants would be altered by the addition of apoproteins C and E. These apoproteins had been isolated from rat plasma in buffer containing 0.1 M deoxycholate. The apoproteins were added to CM remnants by a dialysis procedure designed to prevent detergent solubilization of the lipoprotein (27) and the dialyzed apoprotein-lipoprotein mixtures were then incubated directly with cells. The addition of apoE (24 μg/50 μg of CM remnant triglyceride) increased CM remnant binding 3.5-fold over the dialyzed control CM remnants, while addition of apoC (61 μg/50 μg of triglyceride) decreased CM remnant binding 3-fold. The ability of apoE to enhance CM remnant binding to HepG2 cells was further investigated using apoE obtained from human plasma. Increasing concentrations of human apoE were added to one concentration of either intact CM or CM remnants having either 45% or 85% of triglyceride removed. As shown in **Fig. 4,** apoE enhanced the binding of both CM and CM remnants. The relationship between the concentration of apoE added and the binding of the lipoprotein was curvilinear and approached saturation at approximately 10 μg of apoE/ml for the three lipoprotein preparations. The binding of remnants hydrolyzed 45% and 85% were similar, indicating that enhanced remnant uptake in the presence of apoE is relatively insensitive to differences in triglyceride hydrolysis. Higher concentrations of apoE were also able to stimulate the binding of intact CM. Nonetheless, CM binding remained lower than that of the CM remnants at all concentrations of apoE.

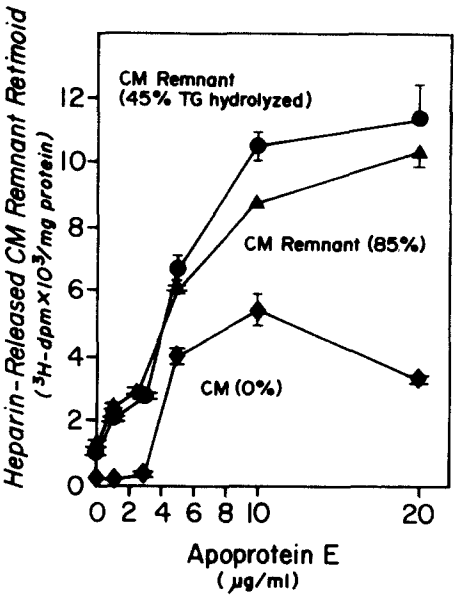

Fig. 4. Binding of CM of different degrees of triglyceride hydrolysis as a function of added human apoE. CM (315 μg of triglyceride/ml) (♦) and CM remnants having 45% (●) and 85% (▲) of triglyceride hydrolyzed were preincubated with increasing concentrations of human apoE for 2 hr at 37°C. The lipoproteins were directly added to cells and incubated for 1 hr at 37°C, after which surface-bound radioactivity was measured as described under Methods. Points are mean ± SD of three plates of cells.

Metabolism of CM remnant-[^{3}H]retinoid as a function of time and temperature

To study metabolism, remnants were bound initially to HepG2 cells during an incubation at 4°C, unbound remnants were washed away, and cells were then warmed to 37°C. A time course of metabolism at 37°C (**Fig. 5**) shows that there was a transfer of radioactivity from cells to medium (panel A). In contrast, when cells were incubated at 4°C for 8 hr, only 10% of the radioactivity initially cell-bound was transferred to the medium (Fig. 5 panel A, insert). During the 37°C incubation, extensive retinyl ester hydrolysis was observed (Fig. 5, panel B). Initially, 88% of the cell-associated radioactivity was present as esterified retinol, similar to the distribution of radioactivity in the CM remnant preparation added to cells (87% as ester). During the 8-hr incubation, the cell-associated retinyl ester radioactivity decreased until only 10% remained as ester. Additionally, only a small amount of radioactivity (≤ 5% of the total) appeared in the medium as retinyl ester. When incubations were conducted at 4°C, the cell-associated retinyl ester radioactivity remained relatively constant for at least 8 hr (Fig. 5 panel B, insert). These data demonstrate a time- and temperature-dependent hydrolysis of CM remnant-[^{3}H]retinyl esters by HepG2 cells.

Characteristics of radiolabeled metabolites of chylomicron remnant-[^{3}H]vitamin A

Cell and medium samples were analyzed further to determine some of the characteristics of radiolabeled metabolite(s) formed during the hydrolysis of CM remnant-[^{3}H]retinyl esters. Samples were extracted with hexane at a basic pH (10.5) to recover neutral plus basic lipids and at an acidic pH (1.5) to recover acidic lipids. The hexane

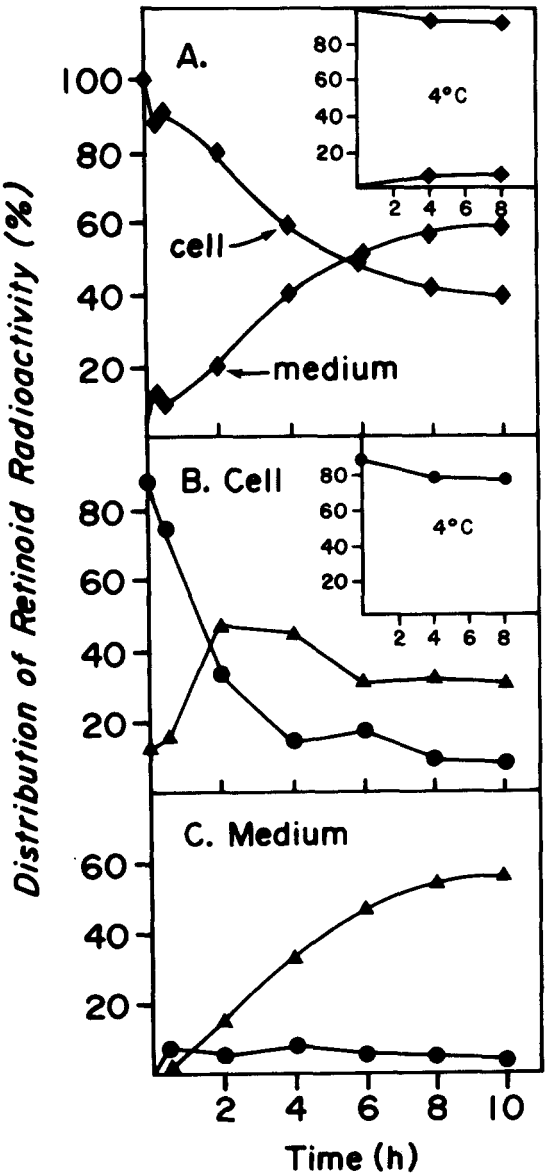

Fig. 5. Distribution of radioactivity after metabolism of CM remnant-[^{3}H]retinoid by HepG2 cells. An apoE-CM remnant mixture (1.4 nmol of total retinoid/ml, 50 μg of apoE/ml) was incubated with cells for 1 hr at 4°C. Cells were washed and either harvested to measure cell-bound radioactivity, or unlabeled media was added and the incubations were continued at 4°C or 37°C. At indicated times, cells and media were harvested and assayed for total ratioactivity (♦) and for radioactivity present as retinyl ester (●) or as material more polar than retinyl ester (▲) (calculated by subtracting retinyl ester radioactivity from total radioactivity).

extracts were subjected to alumina column chromatography and HPLC to separate and identify esterified retinol, retinol, and retinoic acid (see Methods). **Table 2** shows the distribution of radioactivity in hexane extracts of cell and media samples after cells that had been incubated with CM remnants for 1 hr at 4°C were then incubated for 4 hr and 8 hr at 37°C. At all times examined, the radioactivity that remained cell-associated was mainly hexane-extractable (> 89%). By contrast, only approximately 50% of the radioactivity transferred to the medium was recovered by hexane extraction.

When analyzed by alumina column chromatography, nearly all of the base-extracted radioactivity (87–94%) eluted in the retinyl ester and retinol fractions. The identity of the metabolites recovered in these column fractions was confirmed by HPLC which showed that, after saponification, greater than 95% of the radioactivity comigrated with a retinol standard. The acid-extracted radioactivity eluted as two peaks; one peak comigrated with a retinoic acid standard and the other as a more polar peak. Because the acid-extracted radioactivity was only a minor portion of the total hexane extract (< 8% from cell samples and < 20% from media samples), the identity of this material was not investigated further and is simply designated here as "acidic" metabolites. These data thus indicate that the major cell-associated metabolites of CM remnant-[^{3}H]-retinoid were free and esterified retinol, whereas in the medium a large portion of the radioactivity was present as a metabolite(s) more polar than retinol.

Samples of media were further analyzed to determine whether the hexane-insoluble radioactivity might be due to [^{3}H]retinol present as a glucuronic acid conjugate. However, incubation with several concentrations of beta-glucuronidase indicated that only a small and variable portion of the total media radioactivity (8–16%) was present as glucuronide conjugates of either retinol or retinoic acid.

TABLE 2. Distribution of radioactivity in cells and media after incubation with CM remnants containing [^{3}H]retinoid

Tissue	Incubation Time	% of Total ^{3}H	Distribution of Hexane-Extractable Radioactivity: Retinyl Ester	Retinol	Acidic Metabolites
	hr			%	
CM remnant	0	100	90	10	1
Cell	0	99	80	18	2
Cell	4	91	44	51	5
Cell	8	89	54	39	7
Medium	4	67	45	36	19
Medium	8	56	29	55	16

Cells were maintained in media containing fetal bovine serum (10% v/v) until the addition of CM remnants when cells were washed and the lipoproteins were added in serum-free media. CM remnants (2.40 nmol of [^{3}H]retinyl esters plus retinol/ml, 50 μg of triglyceride/ml) were incubated with cells for 1 hr at 4°C. Cells were washed and either harvested (0 hr) or unlabeled media was added and the incubation was continued at 37°C for 4 or 8 hr. Cells and media were sequentially extracted into hexanes under acidic and basic conditions as described under Methods. Portions of the CM remnant were similarly extracted and assayed for radioactivity.

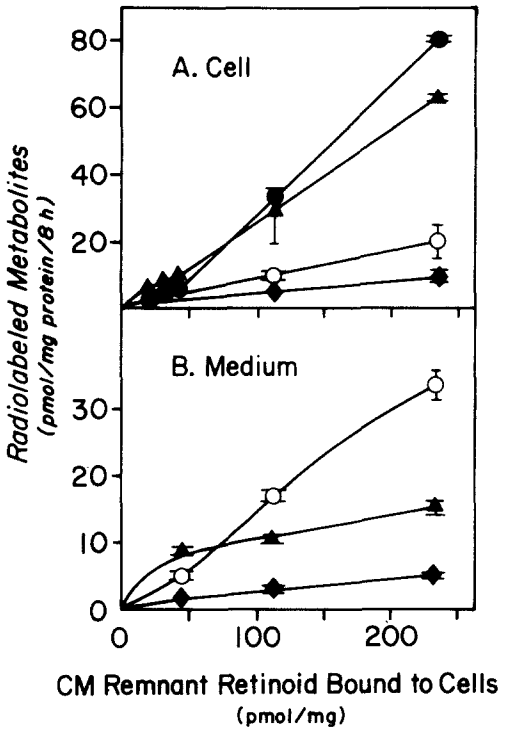

Fig. 6. Accumulation of radiolabeled metabolites in (A) cells and (B) media as a function of the concentration of CM remnant-[^{3}H]retinoid initially cellbound. CM remnant preparations containing different concentrations of [^{3}H]retinoid were incubated with cells at 4°C for 1 hr to allow surface binding and then at 37°C for 8 hr to allow metabolism of the lipoprotein. The radioactivity measured in cells and media after the 37°C incubation was converted to mass using the specific activity of [^{3}H]retinoid associated with the respective CM remnant preparation. Retinyl ester (●), retinol (▲), acidic retinoid(s) (♦) and the radioactivity not recovered by hexane extraction (○) are plotted as the mean ± SD of three plates of cells.

Relationship of retinoid uptake to metabolite formation

It was of interest to determine the relationship between the formation of radiolabeled metabolites and the concentration of CM remnant-vitamin A delivered to cells. The results from several independent time-course experiments were combined to form **Fig. 6** in which the mass of various radiolabeled metabolites formed during 8-hr incubations at 37°C is plotted as a function of the concentration of CM remnant-[^{3}H]retinoid initially bound at 4°C to the cell surface. In cells (panel A), retinol accumulated as a linear function of remnant concentration. Retinyl ester accumulation by cells, however, was a relatively low portion of the total cell radioactivity (20%) at low vitamin A concentrations (≤ 42 pmol total retinoid/mg of cell protein) but became a higher percentage (46%) at higher vitamin A concentrations (230 pmol retinoid/mg of cell protein). In the medium, retinol was the major radiolabeled metabolite at low vitamin A concentrations (≤ 42 pmol/mg of cell protein), but its accumulation increased only slowly as a function of concentration. In contrast, radiolabeled material that was not hexane-extractable accumulated at a much greater rate and represented the predominant metabolite(s) in the medium at higher retinoid concentrations (> 100 pmol of retinoid/mg of cell protein). Acid-extracted radioactivity accumulated in both cells and media as a linear function of concentration, but it remained a minor portion of the total radioactivity recovered (≤ 6.4% in cells and ≤ 17% in media) at all remnant concentrations examined.

Effect of the initial vitamin A concentration of the cell on remnant-retinoid metabolism

HepG2 cells maintained in the media supplemented with serum alone do not store detectable levels of retinoids. However, when incubated in media to which retinol has been added as an ethanol dispersion (20 nmol/ml; 1% ethanol, v/v), these cells readily take up, esterify, and store retinol. It is of interest to determine whether such a cellular store of vitamin A would affect the metabolism of newly delivered CM remnant-vitamin A. Cells were divided into four groups and preincubated for 12 hr in media containing serum with or without the addition of retinol (**Table 3**). Cells were then washed and incubated in the presence or absence of CM remnant-[^{3}H]retinoid for 1 hr at 4°C, after which incubations were continued in fresh, unlabeled media for 8 hr at 37°C. Cells (after incubation with labeled remnants for 1 hr at 4°C) and media (after an additional 8 hr at 37°C) were assayed for the presence of retinoid mass and radioactivity. Cells enriched in vitamin A by preincubation in retinol-supplemented media initially bound the same concentration of CM remnant-[^{3}H]retinoid (5741 ± 672 dpm of ^{3}H/mg of cell protein) as cells preincubated in media containing serum alone (5643 ± 532 dpm of ^{3}H/mg of cell protein). Although the secretion of remnant-derived [^{3}H]retinol by cells containing stored vitamin A was reduced 73%, nonetheless the mass of retinol secreted during 8 hr was nearly identical (approximately 6 pmol/mg of cell protein), whether cells had been preincubated with retinol as an ethanol dispersion or had taken up CM remnant-vitamin A. Thus, these data show that although the accumulation of vitamin A by HepG2 cells reduces the mobilization of newly delivered CM remnant-[^{3}H]vitamin A, the total mass of retinol secreted remains relatively constant over a wide range of cellular vitamin A concentrations (102–445 pmol/mg of cell protein). In previous experiments (Fig. 6) a similar mass of retinol (8–15 pmol/mg of cell protein per 8 hr) was secreted by cells that had initially bound a slightly lower range of CM remnant-[^{3}H]retinoid concentrations (42–233 pmol/mg of cell protein).

Finally, the specific activity of retinol in the medium of cells incubated with CM remnant-[^{3}H]retinoid was calculated. As expected, retinol recovered from the medium of cells previously grown in media supplemented with serum alone had a specific activity (94 ± 10 dpm/pmol) very

TABLE 3. Effect of a cellular store of vitamin A on the metabolism of CM remnant-[^{3}H]retinoid by HepG2 cells

Cell Incubation Conditions		Cell-Associated Retinoid (after 1 hr at 4°C)				Retinol in Medium (after 8 hr at 37°C)		
		Mass		Specific Activity				
Retinol[a]	Remnants[b]	Retinyl Ester	Retinol	Retinyl Ester	Retinol	Mass	^{3}H	Specific Activity
		pmol/mg cell protein		*dpm/pmol*		*pmol/mg cell protein*	*dpm/mg cell protein*	*dpm/pmol*
–	–	ND[c]	ND			ND		
+	–	90	274			6.5 ± 0.4		
–	+	76	26	78 ± 4[d]	87 ± 4	6.5 ± 0.5	606 ± 29	94 ± 10
+	+	169	276	35 ± 4	9 ± 0.4	5.9 ± 0.6	166 ± 4	29 ± 3

[a] Twelve hours at 37°C in media containing fetal bovine serum (10% v/v) with (+) or without (–) the addition of retinol (20 nmol/ml).

[b] One hour at 4°C in the presence (+) or absence (–) of CM remnants containing [^{3}H]retinoid with a specific activity of 72 dpm/pmol of retinyl ester and 92 dpm/pmol of retinol.

[c] Not detectable by HPLC.

[d] Mean ± SD for three plates of cells.

similar to that of the chylomicron remnant-[^{3}H]vitamin A added (92 dpm/pmol). In contrast, the specific activity of the retinol recovered from the medium of retinol-supplemented cells was much lower (29 ± 3 dpm/pmol). We calculated that if the cell surface-bound CM remnant-vitamin A had mixed completely with the cellular store of vitamin A before retinol was secreted, the specific activity would be 19 dpm/pmol, which is quite similar to that observed experimentally for retinol-supplemented cells (Table 3). These data indicate that the newly delivered CM remnant-[^{3}H]retinyl esters and retinol had mixed significantly with the vitamin A in the cell during the 8-hr time period.

DISCUSSION

The results of our study indicate that the uptake of CM remnant-vitamin A by the human hepatoma cell line HepG2 displays several characteristics similar to those reported previously for CM remnant-cholesteryl ester uptake by rat liver cells in vivo (2) and in vitro (47). Additionally, new information on the metabolism of CM remnant-retinoids by this cell line has also been obtained.

Chylomicron remnant uptake by liver involves high-affinity cell-surface receptors that do not take up nascent CM (2). The following evidence indicates that the HepG2 cell internalizes CM remnants by a similar receptor-mediated process. *1*) CM remnants bind to the HepG2 cell surface prior to internalization, as indicated by the observation that a portion of the cell-associated lipoprotein radioactivity was releasable by heparin or trypsin. *2*) Binding of CM remnants to the HepG2 cell was specific and saturable in that remnants were bound more efficiently than CM and, at the concentrations examined, a major portion of the binding of remnant-[^{3}H]retinoid could be inhibited by an excess of unlabeled remnants. *3*) Additionally, the rate of remnant internalization by the HepG2 cell exceeded the rate of internalization of sucrose by more than 70-fold. Taken together, these observations support the conclusion that the HepG2 cells possess receptors that bind and internalize CM remnants.

The extent of remnant association with the HepG2 cell was affected by changes in both the lipid and protein composition of the particle. When remnants were prepared using postheparin plasma, binding increased as a function of triglyceride hydrolysis, similar to observations made previously for the human skin fibroblast (44) and for rat hepatoma cells (43). Inclusion of plasma during lipolysis or addition of apoE to the remnant preparations also enhanced remnant binding to the HepG2 cell. Stimulation was observed with either human apoE or, in preliminary studies, apoE purified from rat plasma. The addition of rat apoC, on the other hand, reduced CM remnant binding indicating that the enhanced binding in the presence of apoE is not a general effect of all apoproteins. It has previously been shown that the addition of apoE enhances CM uptake by the perfused liver while apoC is inhibitory (4, 6). In the present study, apoE enhanced to the same extent the binding of remnants whose triglycerides had been hydrolyzed by 45% and 85%. The binding of intact CM was also enhanced, although it remained lower than that of the remnants at all concentrations of apoE examined, suggesting that some degree of modification by lipase is required for maximal enhancement of binding by the presence of apoE. One explanation for this observation would be that the CM contains a substantial amount of C apoproteins that

oppose the stimulatory effect of the added apoE (6, 48). Windler and Havel (48) have recently demonstrated that the addition of apoE to small CM did not displace C apoproteins and had no effect on the uptake of these lipoproteins by liver. In their study, however, only one concentration of apoE was examined. The present study indicates that the stimulatory effect of apoE on CM and CM remnant binding by liver is concentration-dependent. In the case of CM, enhancement was seen only above 3 μg of apoE/ml, and binding was also submaximal at 20 μg of apoE/ml. It should be noted that the binding observed in these apoE studies might have occurred in the presence of "free apoE" not bound to the lipoprotein since the reconstituted particle was not reisolated after the addition of the apoprotein. It is possible that free apoE might inhibit lipoprotein binding either by competing for the cell receptor, or at the higher concentrations by associating with the lipoprotein in a form that might reduce its receptor interaction. The higher concentrations of apoE that were less than maximally effective in stimulating CM remnant binding in this study ($>$ 10 μg/50 μg of CM remnant triglyceride) have been reported by others to alter the appearance and flotation characteristics of synthetic triglyceride emulsions (4).

HepG2 cells metabolized CM remnant vitamin A in a time- and temperature-dependent manner in which retinyl esters were hydrolyzed rapidly (a decrease of 80% within 2–4 hr) and metabolites of vitamin A were subsequently released to the medium. Recently, it has been shown that newly assimilated CM remnant-retinoid is transferred within a few hours of initial uptake from the hepatocyte either to the stellate cell in vitamin A-sufficient rats or from liver into plasma in vitamin A-deficient animals (11). Thus the progressive decrease of remnant retinyl ester and concomitant release of retinol and metabolites appears consistent with the functions of the liver parenchymal cell.

The majority of radioactivity that remained cell-associated after incubation with CM remnant-[^{3}H]retinoid was either free or esterified retinol. In contrast, only 56% of the radioactivity was extractable into hexane; the major portion of this was retinol. The nature of the hexane-insoluble radioactivity that accumulated in the medium is not yet known. Only a small portion appeared to be a glucuronide conjugate of either retinol or retinoic acid. Goodman, Huang, and Shiratori (1) observed that a portion of administered CM-vitamin A was excreted from liver into bile as a mixture of polar compounds of which only a small percentage were glucuronide conjugates. Others have observed the appearance of water-soluble metabolites of vitamin A in the urine or bile of rats given physiological doses of radiolabeled vitamin A (49, 50). The presence of polar metabolites of retinol in the medium of the HepG2 cell suggests that the hepatic parenchymal cell contributes directly to these transformations.

In the present study, the metabolism of CM remnant-retinoids by the HepG2 cell was examined in terms of *1*) the concentration of remnant-vitamin A initially delivered, and *2*) the retinoid status of the cell. The accumulation of both free and esterified retinol in cells increased as the concentration of remnant-vitamin A initially delivered increased, indicating the ability of the HepG2 cell to store retinoids. However, unlike liver cells in vivo, where most of vitamin A is stored as retinyl ester, HepG2 cells accumulated both free and esterified retinol in nearly equal amounts. The reason for this difference is not clear; however, recent studies of isolated rat liver parenchymal cells have indicated a greater proportion of unesterified retinol in these cells than in either stellate cells or unfractionated liver (51). The amount of retinol released to the medium remained relatively constant over an extensive concentration range of CM remnant-retinoid added to cells. The secretion of retinol by this cell thus appears to be limited. Hicks, Gunning, and Olson (50) have observed a similar relationship in the intact rat between liver vitamin A stores and the secretion of hepatic metabolites of vitamin A. In their study, plasma retinol concentrations remained relatively constant over a wide range of liver vitamin A stores (50–100 pmol/mg of protein) while the secretion of biliary metabolites increased sharply when liver stores rose above 100 pmol vitamin A/mg of protein. In the HepG2 cell, formation of more polar metabolites also increased markedly as more remnants were bound and subsequently metabolized.

The mobilization of newly delivered vitamin A from intact liver is more extensive in vitamin A-deficient rats (11, 52) than in those animals with adequate vitamin A stores. Since HepG2 cells maintained in serum-supplemented media (10% fetal calf serum) do not contain a detectable level of vitamin A, it was of interest to determine whether or not a cellular store of vitamin A would alter the metabolism of CM remnant-vitamin A by this cell. When cells were preincubated in retinol-supplemented media, the binding and uptake of CM remnant-vitamin A was not affected; however, the secretion of newly delivered CM remnant-vitamin A was reduced by 73%. In addition, the specific activity of the retinol secreted by these HepG2 cell was similar to that predicted if newly delivered vitamin A mixed with the cellular store of vitamin A before secretion. These data indicate that the retention and release of newly delivered CM remnant-retinol by this cell can be at least partly regulated by its vitamin A status. While the uptake and release of CM remnant-vitamin A by parenchymal cells in vivo has been recently described (11), the metabolism of remnant-vitamin A by an isolated parenchymal cell has not been previously characterized. The mechanism involved in the storage and release of CM remnant-vitamin A by hepatic parenchymal and fat-storing cells is not known and requires further study. Because the HepG2 cell both stores and secretes CM

remnant-retinoid, it provides a useful tool to further investigate the subcellular location(s) and the regulation of these processes.

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