

# Regulation of retinol uptake and esterification in MCF-7 and HepG2 cells by exogenous fatty acids

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**Abstract** The influence of extracellular fatty acids on the uptake and esterification of [<sup>3</sup>H]retinol bound to human retinol-binding protein (RBP), to RBP-transthyretin (TTR), or in dispersed form by the human hepatoma, HepG2, and human mammary epithelial carcinoma, MCF-7, cell lines was studied. The esterification of [<sup>3</sup>H]retinol was significantly increased in cells incubated with myristic, palmitic, stearic oleic, or linoleic acid-albumin complexes and was observed for all forms of [<sup>3</sup>H]retinol. Enhancement of [<sup>3</sup>H]retinol uptake was also observed in cells incubated with these fatty acids, but this increase was relatively small for the dispersed form as compared to that observed for [<sup>3</sup>H]retinol bound to RBP or RBP-TTR. Comparing equal concentrations of the [<sup>3</sup>H]retinol donors, cell uptake and esterification was greatest from the dispersed form and least from that bound to RBP-TTR. After preincubation of cells with oleate, uptake and esterification of [<sup>3</sup>H]retinol was increased but not to the extent observed when oleate and [<sup>3</sup>H]retinol donor were co-incubated. Incubation of cells with oleate resulted in rapid and correlated increases in the rates of [<sup>3</sup>H]retinol uptake and esterification which persisted until the steady state for [<sup>3</sup>H]retinol uptake was achieved. Beyond this time, net esterification of [<sup>3</sup>H]retinol continued in the presence of oleate. This kinetic pattern was observed for all [<sup>3</sup>H]retinol donors. These effects on [<sup>3</sup>H]retinol uptake and esterification were dose-dependent as the oleate to albumin ratio was varied from 0.5 to 3.0 and were observed across a physiological concentration range of RBP-<sup>3</sup>H-retinol. ■ The data indicate that: 1) the fatty acid status of cells is a determinant of retinol uptake and esterification; and 2) the form of retinol presentation to cells is not qualitatively important for these processes. — **Randolph, R. K., and A. C. Ross.** Regulation of retinol uptake and esterification in MCF-7 and HepG2 cells by exogenous fatty acids. *J. Lipid Res.* 1991. 32: 809-820.

**Supplementary key words** retinol-binding protein • transthyretin

Vitamin A is a nutrient that is essential for the proper growth, development, and function of many animal tissues and organs. A critical role for vitamin A in supporting the structure and function of epithelial tissues was indicated in 1925, when Wolbach and Howe (1) characterized the histopathological changes that accompanied vitamin A deficiency. Subsequent studies have confirmed and extended these observations in demonstrating the effects of vitamin A on inducing and

maintaining the differentiated state of epithelial tissues. The specific effects of retinoids, however, are diverse between tissues and vary with the organism's development (2). Despite these differences, all vitamin A-dependent tissues share the necessity of obtaining this lipid nutrient from their respective extracellular environment.

Retinol is transported in two major forms in the plasma and lymph: as retinyl esters of the intestinal chylomicron (3) and as unesterified retinol bound to plasma retinol-binding protein (RBP) (4). In the postprandial state, after the intravascular conversion of chylomicrons to chylomicron remnants, these particles, including their retinyl ester component, are rapidly cleared from the circulation, principally by hepatocytes (5, 6). As a result of their very large size, the access of chylomicrons and their remnants is probably restricted to cells that reside in tissues that possess large fenestrated endothelium. In many species including humans, retinol is secreted from the liver into the circulation bound to RBP, a 21 kDa plasma protein that possesses a single binding site for one molecule of retinol (4, 7). The concentration of RBP-retinol in normal human plasma is constant at about 2 to 3  $\mu$ M, despite variation in liver stores of vitamin A (4, 7). In the plasma, RBP-retinol binds reversibly with transthyretin (TTR), a 55 kDa plasma protein that is also secreted from the liver, to form an RBP-retinol-TTR complex (4, 7). Owing to its small size, this form of retinol is thought to have access to most extracellular spaces in the body and, thus, likely represents a major route by which retinol is delivered to cells.

The concentrations of vitamin A and its fatty acid esters vary widely between tissues but are generally characteristic of the particular tissue (5, 8). The

Abbreviations: RBP, retinol-binding protein; TTR, transthyretin; LDL, low density lipoproteins; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LRAT, lecithin:retinol acyltransferase; ARAT, acyl coenzyme A:retinol acyltransferase.

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mechanism whereby this apparently selective delivery and storage of retinol occurs is not completely understood. The uptake of chylomicron remnants by hepatocytes (6), hydrolysis of newly assimilated retinyl ester (5, 6, 9), the reesterification of retinol (5, 9), the intra- (10) and intercellular movement of retinol in liver (6) have been described in some detail. In contrast, the mechanism by which retinol bound to RBP is delivered to and taken up by cells remains unknown. A rapid, reversible binding event between a cell surface receptor or binding site and RBP has been proposed by some investigators to mediate the uptake of retinol by cells (11–17). Saturable, specific binding of RBP to cells that correlates with retinol transfer to cells has not been shown, however. This line of research has revealed that the uptake of RBP, or RBP-TTR does not seem to be involved in the transfer of retinol to cells (11, 13, 14). A role for RBP in the specific delivery of retinol to cells is, in fact, called into question by observations that solvent-dispersed retinol can be taken up and elicit physiological processes such as the secretion of RBP from liver (18), cellular differentiation (2), and retinol esterification by cells in culture (19, 20). For example, Creek, Silverman-Jones, and De Luca (21) have shown that retinol was esterified to a similar extent regardless of whether it was presented to cells bound to RBP or nonspecifically in dispersed form. Furthermore, Noy and Xu (22) have recently demonstrated that retinol can dissociate from RBP, diffuse through the aqueous phase, and partition into cell membranes. Thus, presentation of retinol to cells in dispersed form may model its delivery *in vivo*.

Although evidence that suggests how cells might regulate the uptake of RBP-retinol is lacking, there is evidence that the esterification of retinol by cells can be regulated. Previous work from this laboratory (19) and others (20) has shown that the esterification of dispersed retinol was regulated by exogenous fatty acids. These experiments demonstrated a three- to sevenfold increase in the proportion of total cell retinol as retinyl esters in J774 macrophages incubated with dispersed retinol and fatty acid-albumin complexes. The effects of fatty acids on the uptake and esterification of retinol bound to its physiological carriers, RBP and RBP-TTR, were not addressed.

Free fatty acids, in addition to their well-known structural and metabolic functions, may fulfill other physiological roles in lipid and lipoprotein metabolism. Recent studies have reported that fatty acids can modulate the binding of low density lipoproteins to the LDL receptor (23) and the binding of lipoprotein lipase to endothelial cells (24). Free fatty acids are present in the plasma at concentrations ranging from 300 to 1,200  $\mu\text{M}$ , depending on the nutritional and metabolic status of the individual (25). Almost all are transported bound to albumin and, hence, would be expected to be normal constituents of an extracellular environment consisting of plasma or its

ultrafiltrate. Despite this, the effects of fatty acids on cellular retinol metabolism have not been systematically examined. In the present work we have characterized the effects of extracellular fatty acids on the uptake and esterification of retinol presented to cultured human cells bound to human RBP, to RBP-TTR, and in dispersed form. We have examined these parameters in a human hepatoma, the HepG2 cell line, which possesses many of the pathways characteristic of normal hepatocyte retinol metabolism (26, 27) and the human mammary epithelial carcinoma, MCF-7 cell line, which is related to an extrahepatic cell type with the capacity to transport retinol from the plasma into milk retinyl ester.

## MATERIALS AND METHODS

### Chemicals

Bovine serum albumin (BSA; fatty acid-free from fraction V), human transthyretin (TTR), all-*trans* retinyl acetate, dimethylsulfoxide (DMSO), myristic, palmitic, stearic, oleic, and linoleic acids, and butylated hydroxytoluene were obtained from Sigma. Alumina (Brockman activity 1, 80–200 mesh) and organic solvents were obtained from Fisher. Solvents utilized for extraction and storage of retinol or retinyl esters contained 100  $\mu\text{g}$  butylated hydroxytoluene/ml.

### Isotopes

[15(n)- $^3\text{H}$ ]Retinol (all-*trans*; 23.5 Ci/mmol) was purchased from New England Nuclear. Ethanolic stock solutions (0.1  $\mu\text{M}$ ) of [ $^3\text{H}$ ]retinol (sp act 2.2 mCi/ $\mu\text{mol}$ ) were made by mixing labeled retinol with unlabeled retinol that was obtained by saponification of retinyl acetate. Stock solutions were stored in the dark at  $-20^\circ\text{C}$  under  $\text{N}_2$  and were purified by aluminum oxide chromatography and checked for purity by ultraviolet spectroscopy and high performance liquid chromatography (28).

### Cell culture

Stock cultures of the human breast epithelial carcinoma (MCF-7) and human hepatoma (HepG2) cell lines were maintained on growth medium consisting of Eagle's minimum essential medium (MEM; Gibco) containing 10% fetal bovine serum and 50  $\mu\text{g}$  gentamicin/ml. Cells were grown in T-75 flasks (Corning) at  $37^\circ\text{C}$  in a 95% air/5%  $\text{CO}_2$  humidified atmosphere.

### RBP- $^3\text{H}$ retinol, BSA-fatty acid preparation

Human plasma RBP was purified as described previously (27) from pooled, outdated blood bank plasma and stored desiccated at  $-70^\circ\text{C}$ . RBP- $^3\text{H}$ retinol was prepared essentially as described by Futterman and Heller (29). All procedures involving the preparation of

[<sup>3</sup>H]retinol-protein complexes were performed in dim light. After reconstitution of the apoRBP with [<sup>3</sup>H]retinol, the reconstituted RBP-[<sup>3</sup>H]retinol was purified by gel permeation chromatography on Sephadex G-75 with 0.1 M NaCl, 30 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.0 buffer at 4°C. After chromatography, the RBP-[<sup>3</sup>H]retinol was concentrated over a YM-10 membrane in an Amicon ultrafiltration cell and subjected to ultraviolet spectroscopy to check for extent of incorporation of the [<sup>3</sup>H]retinol ligand. Both the overall spectrum and the ratio of absorbance at 330 to 280 nm for the reconstituted material corresponded to that of the parent unlabeled RBP (0.9–1.0). Recovery of RBP in an average preparation was 60%. The RBP-[<sup>3</sup>H]retinol preparations were further characterized and were shown to comigrate with the purified parent holoRBP under nondenaturing condition on polyacrylamide electrophoresis. The RBP-[<sup>3</sup>H]retinol preparations were also completely precipitable with polyclonal antibodies specific for human RBP (27). For some experiments, RBP-[<sup>3</sup>H]retinol was complexed with TTR. These complexes were prepared by mixing a 1.5 mole excess of RBP-[<sup>3</sup>H]retinol with TTR in a 0.1 M NaCl solution. The mixture was then chromatographed on Sephadex G-100 in the buffer described above for RBP-[<sup>3</sup>H]retinol. The RBP-[<sup>3</sup>H]retinol-TTR eluted slightly ahead of BSA in the included volume of the column. Peak fractions were pooled and concentrated in column buffer. The ultraviolet spectrum of the isolated complexes was similar to that reported by Heller and Horowitz (30) with A<sub>330</sub> to A<sub>280</sub> ratios of approximately 0.5.

The effect of BSA-fatty acid complexes on RBP-fatty acid complexes on RBP-retinol binding affinity was measured by determining the fluorescence emission intensity of RBP-retinol (31) in the presence or absence of BSA or BSA-oleate complexes at 485 nm with an excitation wavelength of 350 nm. The A<sub>280</sub> of the RBP-retinol solution before addition of BSA or BSA-oleate was less than 0.06.

Complexes of BSA and fatty acids were prepared by a method adapted from van Harken, Dixon, and Heimberg (32).

### Experimental protocol and analyses

Cells for experiments were plated into 35-mm dishes after trypsinization of stock cultures and were maintained under the same conditions as stock cultures prior to experiments. Cells were allowed to grow to confluence (5–6 days) prior to the initiation of experiments. They were refed with growth medium on day 3. On the day of experiment, cells were washed with phosphate-buffered saline and were then incubated with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-buffered MEM containing 150 μM BSA alone or 150 μM BSA complexed with fatty

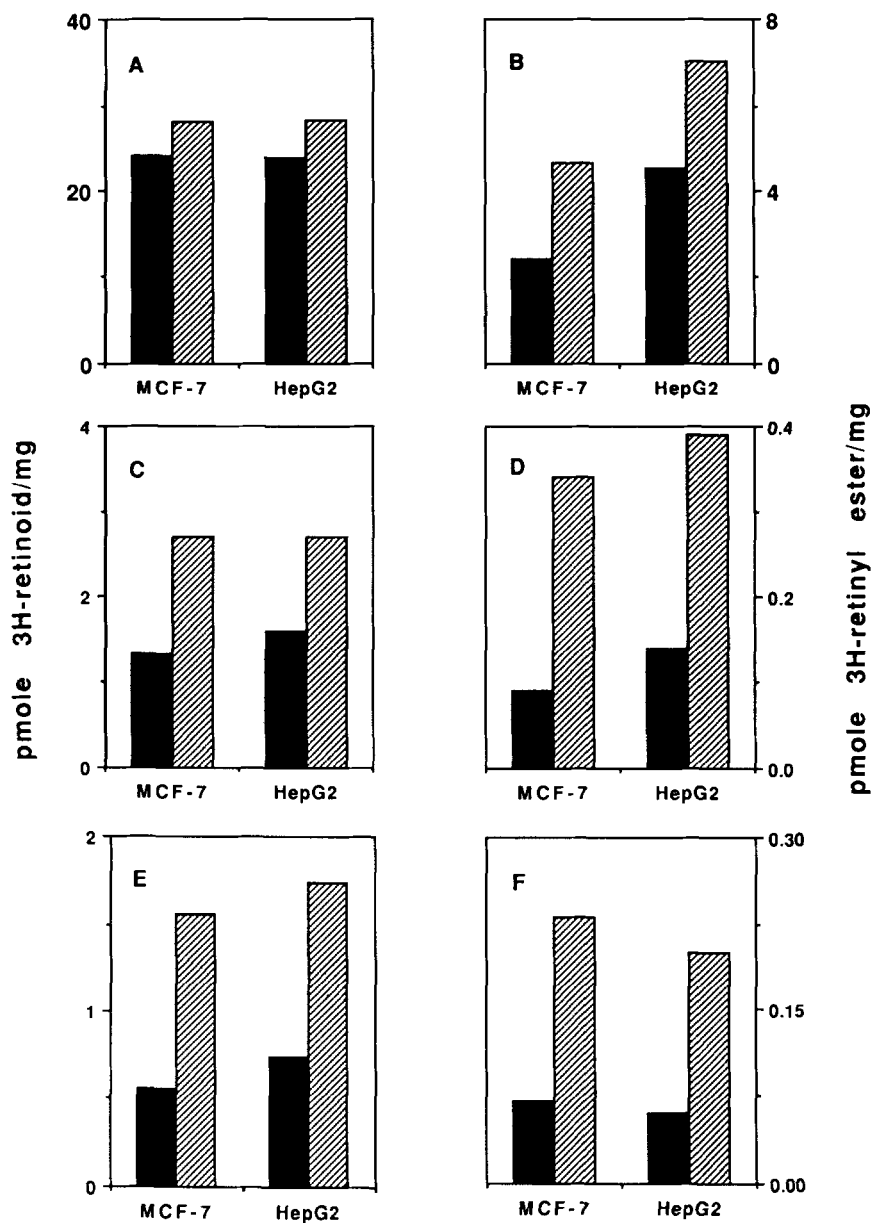
acid (with a fatty acid to BSA molar ratio of 1.4), unless otherwise specified, and either RBP-[<sup>3</sup>H]retinol, RBP-[<sup>3</sup>H]retinol-TTR or [<sup>3</sup>H]retinol in DMSO (final DMSO concentration was 0.1%). In some experiments, media containing RBP-[<sup>3</sup>H]retinol also contained DMSO to control for any solvent effects with the dispersed [<sup>3</sup>H]retinol. In these experiments no effect of DMSO was detected. After an appropriate incubation, experimental medium was removed, cell monolayers were chilled on crushed ice, washed with ice-cold PBS, and cell-associated [<sup>3</sup>H]retinoids were extracted *in situ* with isopropanol overnight at 4°C. The isopropanol extracts were collected, partitioned into hexanes, and washed with water (19). A portion of the hexane extract was taken for scintillation counting and uptake (total cell-associated <sup>3</sup>H) was calculated. Cell content of [<sup>3</sup>H]retinyl esters was determined by scintillation counting after aluminum oxide chromatography of the remaining hexane extract. In some experiments cells were released from dishes by trypsinization, washed by repeated centrifugation, and disrupted by sonication in PBS. The disrupted cells were then extracted by the method of Bligh and Dyer (33). After extraction, portions of the organic phase were taken and analyzed exactly as described above for total cell-associated <sup>3</sup>H and [<sup>3</sup>H]retinyl esters. The results were similar to those obtained with the isopropanol extraction. Cell protein was determined on the extracted monolayers by the method of Markwell et al. (34) after solubilization with sodium dodecylsulfate. Cell [<sup>3</sup>H]retinol uptake and esterification are presented as pmole cell-associated [<sup>3</sup>H]retinoid/mg cell protein and pmole [<sup>3</sup>H]retinyl ester/mg cell protein, respectively. Unless otherwise indicated, results represent the average of duplicate cultures.

The effects of fatty acids on the uptake and esterification of [<sup>3</sup>H]retinol have been expressed as an uptake ratio and an ester ratio, respectively. These ratios represent the ratio of the indicated parameter in the presence of fatty acid to that observed in its absence, e.g., the ester ratio equals cell [<sup>3</sup>H]retinyl ester content in incubations containing fatty acid divided by cell [<sup>3</sup>H]retinyl ester in the absence of fatty acid. In some cases the relative effects of fatty acids on [<sup>3</sup>H]retinol uptake and esterification have been compared by the calculation of an ester to uptake ratio that equals the ester ratio divided by the uptake ratio.

## RESULTS

### Dependence of retinol uptake and esterification on exogenous fatty acids and form of retinol presentation

In initial studies we compared the effects of oleate on the uptake and esterification of retinol presented to cells



**Fig. 1.** Effect of oleate on the uptake and esterification of [ $^3\text{H}$ ]retinol by HepG2 and MCF-7 cells. Uptake (panels A, C, E) and esterification of [ $^3\text{H}$ ]retinol (panels B, D, F) were measured in cells after a 6-h incubation with  $0.2 \mu\text{M}$  [ $^3\text{H}$ ]retinol in dispersed form (panels A, B), bound to RBP (panels C, D) or RBP-TTR (panels E, F), and BSA alone ( $150 \mu\text{M}$ ) (solid bars) or BSA-oleate (oleate to BSA ratio of 1.4) (hatched bars).

in dispersed form, bound to RBP or to RBP-TTR (Fig. 1). The measurements of retinol uptake and esterification in this experiment were obtained from cells that had reached the steady state with regard to cell  $^3\text{H}$  content (see below). The uptake of retinol was increased in both MCF-7 and HepG2 cells for all retinol donors incubated with oleate as compared to those incubated in its absence (Fig. 1, panels A, C, E). This difference was small (15%) in cells incubated with dispersed retinol (panel A) and much greater (80–100% and 150%) for cells incubated with RBP-retinol (panel C) or RBP-retinol-TTR (panel

E), respectively. Although these incubations contained equal concentrations of retinol ( $0.2 \mu\text{M}$ ), the uptake of dispersed retinol was tenfold greater than that observed in cells incubated with retinol bound to RBP or to RBP-TTR. The uptake of retinol from RBP-retinol-TTR was approximately 40% less than that from RBP-retinol.

The esterification of retinol was also greater in incubations containing oleate (Fig. 1, panels B, D, F). This was observed for all forms of retinol and for both cell types. The degree to which retinol esterification was increased by incubation with oleate varied between 90 and 300%.

In every case the effect of oleate on retinol esterification was greater than on uptake in the same incubation. Overall, the differences in absolute retinol esterification that were observed between retinol donors correlated to the corresponding differences in absolute uptake.

Several experiments similar to the one shown in Fig. 1 but with different retinol concentrations were performed to determine the reproducibility of oleate's effects on sustaining high levels of retinol uptake and esterification in cells. The summarized results of these experiments are shown in **Table 1** in ratio form (see Methods). Inclusion of oleate in incubations with dispersed retinol resulted in small but significant increases in retinol uptake (uptake ratio: 1.15–1.16). A greater relative increase was observed for the uptake of RBP-bound retinol (1.62–1.86). The fraction of cell retinol stored as ester was greater for incubations that presented retinol bound to RBP as compared to the dispersed form. This difference, however, could be accounted for by the difference in cell retinol content in the incubations containing RBP. In fact, when these differences were normalized for cell retinol content (ester to uptake ratio), the relative magnitude of oleate's effect on cell retinyl ester content was similar for dispersed and RBP-retinol over this range of retinol concentrations. No significant differences were detected between cell types. Since subsequent studies revealed no differences between cell types, only data from the MCF-7 cell line will be shown.

Oleic and palmitic acids are the most abundant free fatty acids in human plasma, accounting for approximately 35 and 25% of total plasma fatty acids, respectively (35). Previous work from this laboratory (19) demonstrated that these two fatty acids as well as a variety of other fatty acids stimulated the esterification of dispersed retinol by a mouse macrophage cell line. To determine whether these effects were common to fatty acids in general, the uptake and esterification of retinol bound to RBP was compared in cells incubated with BSA complexes of myristic, palmitic, stearic, oleic, and linoleic acids. In these experiments, both the uptake and esterification of retinol were enhanced comparably to the data shown in

Fig. 1 (two- to fourfold for every fatty acid tested; data not shown). Thus, a variety of fatty acids varying in chain length and saturation effectively increased the cellular uptake and esterification of retinol.

#### Stability of RBP-retinol in the presence of fatty acids

The effect of BSA-oleate on stimulating the uptake of retinol presented to cells bound to RBP was surprising. If fatty acids decreased the avidity of RBP for retinol or destabilized the RBP-retinol complex, displacing retinol from RBP onto BSA or into the aqueous phase, retinol could partition into cells to a greater extent than that which was bound to RBP. Two experiments were performed to test these possibilities. First RBP- $^3\text{H}$ retinol ( $0.2 \mu\text{M}$ ) was incubated for 6 h at  $37^\circ\text{C}$  in cell-free dishes with medium containing BSA-oleate or BSA alone under conditions identical to those of cell experiments. After the incubation period, aliquots of media were chromatographed on Sephadex G-75. Bovine serum albumin and RBP- $^3\text{H}$ retinol eluted at volumes corresponding to their respective unincubated controls. There was no  $^3\text{H}$  detected in the fractions containing BSA under conditions where displacement of 5% of the retinol from the RBP- $^3\text{H}$ retinol preparation would have been detected. Second, the affinity of retinol-RBP binding was measured by monitoring the fluorescence of RBP-retinol in solutions containing BSA alone or oleate complexed with BSA at BSA to RBP concentration ratios comparable to those in the cell culture medium. When the oleate to BSA mole ratio was varied from 0.25 to 3.0, there was no detectable change in the fluorescence intensity of the RBP-retinol complex. Thus, these two experiments provide evidence that fatty acid albumin complexes did not alter the avidity of RBP for retinol or the stability of the RBP-retinol complex.

#### Temporal requirements for fatty acid-enhanced retinol uptake and esterification

Since it was evident that fatty acids were promoting retinol uptake and esterification by altering some aspect of cell function, we determined whether this alteration re-

TABLE 1. Effect of oleate on the uptake and esterification of  $^3\text{H}$ retinol presented to cells in dispersed form and bound to RBP

Cell Line	n	Dispersed- $^3\text{H}$ retinol			RBP- $^3\text{H}$ retinol			
		Uptake Ratio	Ester Ratio	Ester to Uptake Ratio	n	Uptake Ratio	Ester Ratio	Ester to Uptake Ratio
MCF-7	8	$1.16 \pm 0.15$	$1.92 \pm 0.93$	$1.63 \pm 0.62$	7	$1.86 \pm 0.45$	$2.92 \pm 0.67$	$1.59 \pm 0.25$
HepG2	7	$1.15 \pm 0.18$	$1.71 \pm 0.44$	$1.49 \pm 0.32$	6	$1.62 \pm 0.61$	$2.70 \pm 1.25$	$1.66 \pm 0.37$

Table values are the mean ratios  $\pm 1$  SD for the indicated parameters in the presence or absence of oleate, e.g., the uptake ratio equals the ratio of uptake in the presence of oleate to that in its absence. The ester to uptake ratio equals a ratio of the ester ratio to the uptake ratio for the respective cell line. The number of separate experiments with each cell line and  $^3\text{H}$ retinol donor is designated by "n." Duplicate determinations were made in each experiment. All measurements were made after 6-h incubations with  $^3\text{H}$ retinol concentrations ranging from  $0.15 \mu\text{M}$  to  $0.7 \mu\text{M}$  and with a BSA concentration of  $150 \mu\text{M}$ . For incubations containing oleate, the oleate to BSA ratio was 1.4.

quired simultaneous exposure to fatty acid and RBP-retinol (Table 2). Prior to incubation with RBP-retinol, cells were preincubated overnight with medium containing BSA-oleate or BSA alone. After the preincubation, each group of cells was washed and coincubated with fresh medium containing 0.2  $\mu$ M RBP-retinol and BSA-oleate or BSA alone. Preincubation of cells for 12 h with BSA-oleate followed by coincubation with BSA alone resulted in small but significant increases in retinol uptake and esterification (23 and 83% greater than control, respectively). These increases were less than one-half those observed when cells were only coincubated with BSA-oleate for 7 h (97 and 183%, respectively). When cells were both preincubated and coincubated with oleate, the corresponding effects of oleate on enhancement of retinol uptake and esterification were approximately additive. Therefore, full enhancement of retinol uptake and esterification required the simultaneous exposure of cells to fatty acids and retinol donor.

#### Fatty acids and the kinetics of retinol uptake and esterification

The effects of oleate on the time course for the uptake and esterification of RBP-retinol were determined next (Fig. 2). The uptake of retinol was biphasic (panel A). After an initial rapid phase lasting for about 1 h, uptake continued at a slower rate, with net uptake ceasing by 2 h. This pattern was not affected qualitatively by oleate. Interestingly, the initial rate of uptake in incubations with BSA-oleate was twice that observed in incubations with BSA alone. Furthermore, this quantitative difference was apparent after only 10 min. By the time the steady state was achieved, cells incubated with BSA-oleate contained threefold more [ $^3$ H]retinoid than did cells incubated with BSA alone.

The kinetics of esterification of RBP-retinol (panel B) exhibited a pattern that was very similar to that for uptake during the first 2 h. Thereafter, retinyl ester continued to accumulate in cells incubated with BSA-oleate but, in contrast, reached a plateau in cells incubated with BSA

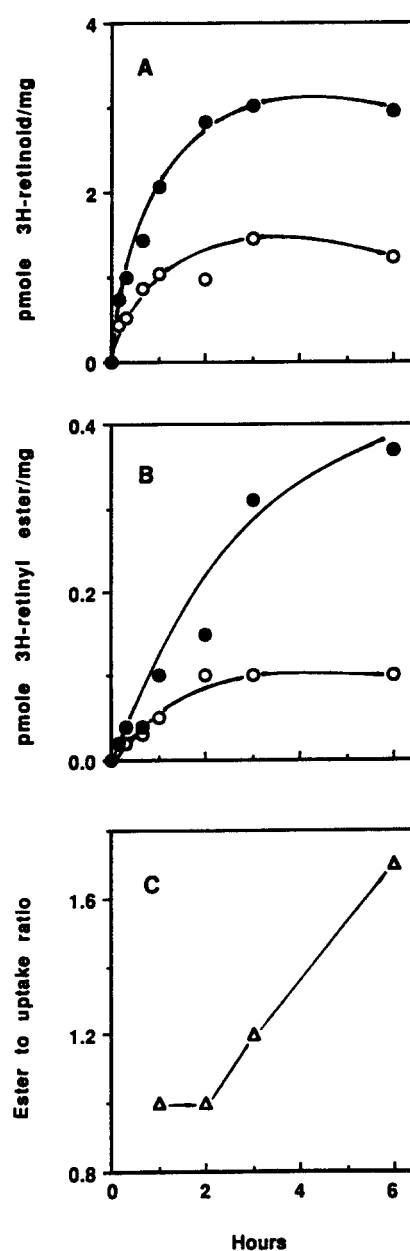


Fig. 2. Time course for the uptake and esterification of RBP- $^3$ H]retinol in the presence or absence of oleate by MCF-7 cells. Cells were incubated with 0.2  $\mu$ M RBP- $^3$ H]retinol and BSA alone (open circles) or BSA-oleate (closed circles). At the indicated times, incubations were stopped and uptake (panel A) and esterification of  $^3$ H]retinol (panel B) were measured. The ester to uptake ratio (panel C) was calculated as described in Methods.

alone. This plateau mirrored the retinol content of these cells. The ester to uptake ratio (panel C) was near 1 for the first 2 h of incubation, suggesting that the differences in cell retinyl ester during this time could be explained completely on the basis of differences in cell retinol uptake. Beyond 2 h this ratio increased steadily, pointing to an effect of oleate on promoting the storage of retinyl ester

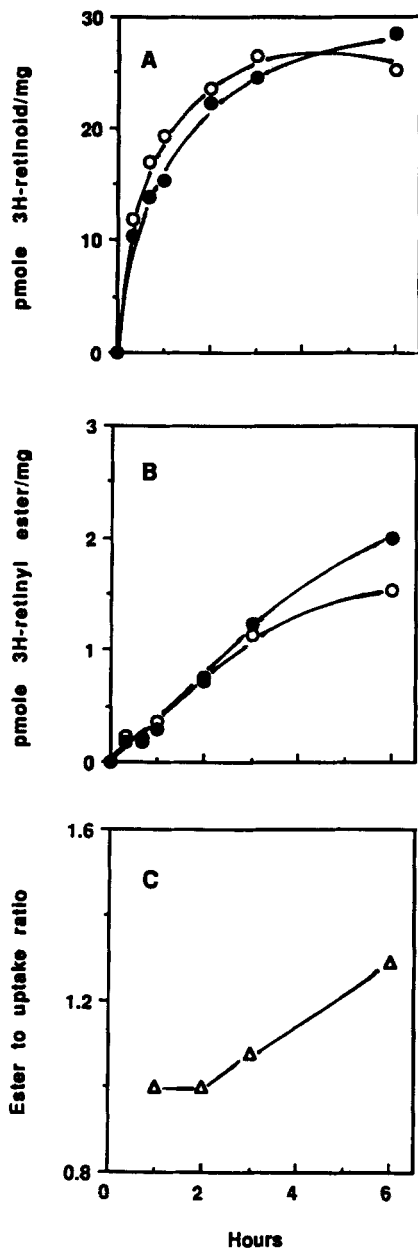
TABLE 2. Effect of preincubation and coincubation with oleate on the uptake and esterification of RBP- $^3$ H]retinol

Preincubation	Coincubation	Uptake	Ester
		<i>pmol retinoid/mg</i>	
BSA	BSA	0.96	0.06
BSA-oleate	BSA	1.18	0.11
BSA	BSA-oleate	1.89	0.17
BSA-oleate	BSA-oleate	2.59	0.30

MCF-7 cells were preincubated with medium containing 150  $\mu$ M BSA alone or BSA-oleate (oleate to BSA ratio was 1.4) for 12 h. After the preincubation, cells were washed and then coincubated with the same concentration of BSA alone or BSA-oleate and 0.2  $\mu$ M RBP- $^3$ H]retinol for an additional 7 h.

that was kinetically distinct from its effect on increasing cell content of retinol.

The kinetics of uptake and esterification of dispersed retinol are shown in Fig. 3. Dispersed retinol was added to incubations at a concentration identical to that for the experiment shown in Fig. 2 with RBP-retinol ( $0.2 \mu\text{M}$ ). The overall pattern for the time course of retinol uptake (panel A) was very similar to that observed in cells incubated with RBP-retinol but, in contrast, there was little



**Fig. 3.** Time course for the uptake and esterification of dispersed- $[^3\text{H}]$ retinol in the presence or absence of oleate by MCF-7 cells. Cells were incubated with  $0.2 \mu\text{M}$  dispersed- $[^3\text{H}]$ retinol and BSA alone (open circles) or BSA-oleate (closed circles). At the indicated times, incubations were stopped and uptake (panel A) and esterification of  $[^3\text{H}]$ retinol (panel B) were measured. The ester to uptake ratio is shown in panel C.

effect of oleate. The initial rate of retinol uptake from the dispersion was tenfold greater than uptake from RBP (compare Figs. 2 and 3, panel A).

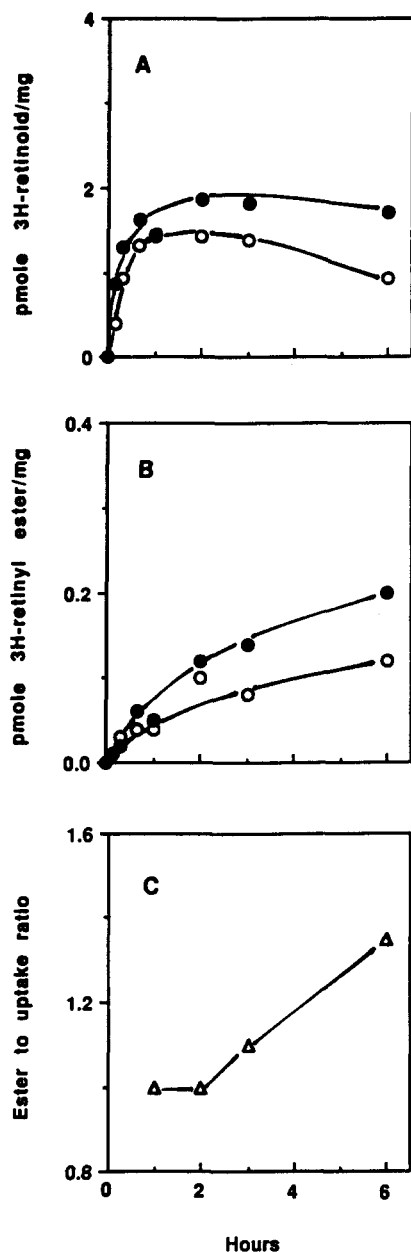
The time course for the accumulation of retinyl ester (panel B) shows that oleate had no effect during the first 2–3 h of the incubation. Beyond that time, cells incubated with BSA-oleate continued to accumulate retinyl esters whereas the retinyl ester content of cells incubated with BSA alone began to plateau. During the first 2 h of incubation, the ester to uptake ratio (panel C) remained near 1 because oleate produced little difference in either the cell content of retinol or retinyl ester. By 3 h, this ratio was increased and continued to rise in a manner very similar to that observed in the kinetic study with RBP-retinol (Fig. 2).

In an additional time course study (Fig. 4) the uptake and esterification of dispersed retinol were determined under conditions such that the rate of retinol uptake in the absence of fatty acid was comparable to that observed with RBP-retinol, also in the absence of fatty acid (see Fig. 2, panel A). This was achieved by decreasing the concentration of dispersed retinol to  $0.02 \mu\text{M}$  which was one-tenth that used in the experiments shown in Figs. 2 and 3. This experiment was conducted simultaneously with the experiment shown in Fig. 2 so that all variables except the form of retinol were equivalent. The kinetics of retinol uptake (panel A) were similar overall to those in the other time course experiments. Uptake of retinol during the first hour was increased 30–50% in incubations containing BSA-oleate. After attaining the steady state, this difference was maintained throughout the incubation.

The kinetics of retinol esterification (panel B) during the first 2 h closely reflected the pattern of uptake. Beyond 2 h, cell content of retinyl esters continued to increase in incubations containing BSA-oleate but did not change in incubations with BSA alone. A pattern very similar to that obtained in the two previous kinetic studies was observed for the ester to uptake ratio (panel C).

#### Concentration effects of RBP-retinol on uptake and esterification

The concentration of RBP-retinol in extracellular fluid is not known but is likely to be less than that in the circulation ( $2\text{--}3 \mu\text{M}$ ). Consequently, we examined the effects of oleate on retinol uptake and esterification across a range of RBP-retinol concentrations from  $0.1$  to  $3.2 \mu\text{M}$  (Fig. 5). The uptake of retinol increased in a curvilinear fashion as RBP-retinol concentration increased (panel A). Inclusion of oleate in the incubation medium resulted in increased uptake of retinol across the entire concentration range. The magnitude of this effect decreased in a dose-dependent manner from an uptake ratio of 2.2 at  $0.1 \mu\text{M}$  RBP-retinol to a ratio of 1.3 at  $3.2 \mu\text{M}$  RBP-retinol (panel B).



**Fig. 4.** Time course for the uptake and esterification of dispersed- $^3\text{H}$ retinol in the presence or absence of oleate by MCF-7 cells. Cells were incubated with  $0.02\ \mu\text{M}$  dispersed- $^3\text{H}$ retinol and BSA alone (open circles) or BSA-oleate (closed circles). Incubations were stopped at the indicated times and uptake (panel A) and esterification (panel B) of  $^3\text{H}$ retinol were measured. The ester to uptake ratio is shown in panel C.

Cell content of retinyl ester also increased in a curvilinear fashion across the RBP-retinol concentration range (panel C). Whereas the accumulation of cell retinyl esters reached a plateau at less than  $1\ \mu\text{M}$  RBP-retinol for incubations containing BSA alone, this was not observed in cells incubated with BSA-oleate until RBP-retinol concentrations exceeded  $2\ \mu\text{M}$ . The relative effects of oleate

on increasing cell retinyl esters became progressively greater as RBP-retinol concentration increased (panel D).

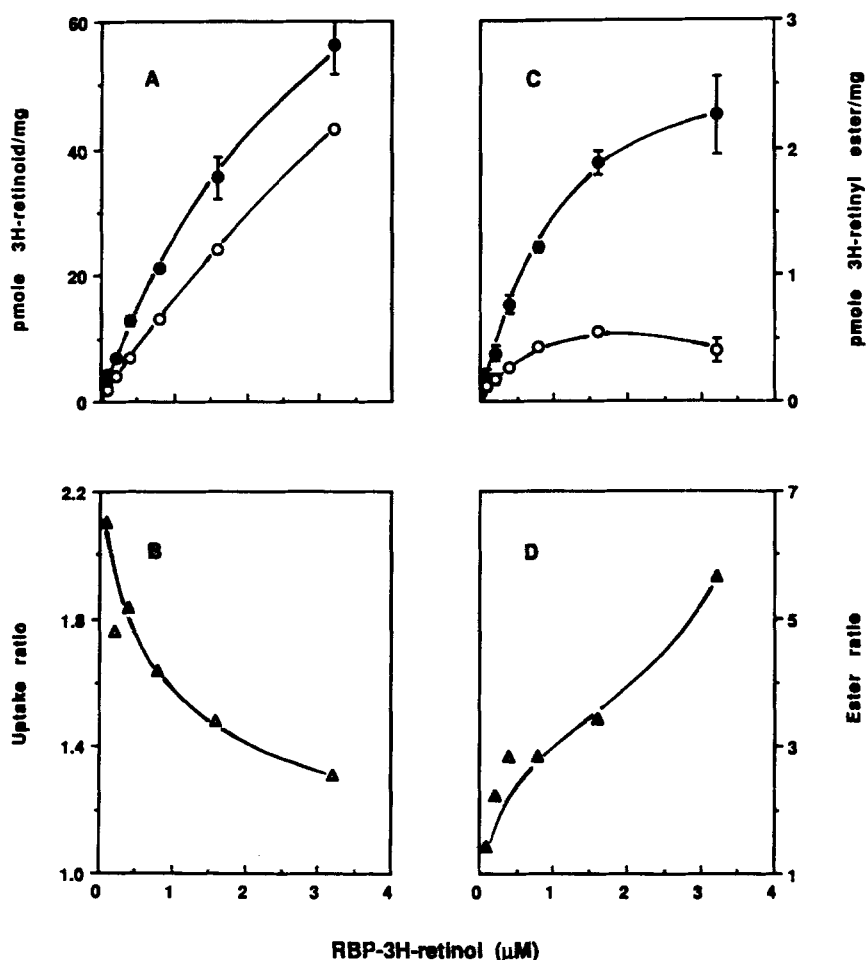
#### Concentration effects of fatty acids on retinol uptake and esterification

Plasma fatty acid-albumin ratios in humans are known to fluctuate between 0.5 and 2.0 by changes in the fatty acid component as compared to a relatively constant albumin concentration (25). We therefore measured retinol uptake and esterification across a range of physiological ratios of oleate to BSA (Fig. 6). Both the uptake and esterification of RBP-retinol increased in a dose-dependent manner as the oleate to BSA ratio was increased from 0.5 to 3.0.

#### DISCUSSION

In previous studies from our laboratory, the effects of extracellular fatty acids on the esterification of dispersed retinol by the transformed murine macrophage cell line, J774, have been reported (19). Extending these observations, the present work has characterized the effects of fatty acids on the uptake and esterification of retinol bound to its physiological carrier, RBP, in two human epithelial cell lines. These data suggest that the fatty acid status of cells and their environment are important determinants of the rate and extent of retinol uptake and esterification. Cells incubated with extracellular fatty acids consistently took up and esterified more retinol than did those incubated in their absence. Exogenous fatty acids were limiting for retinol esterification regardless of the manner in which retinol was presented to cells. The rate and extent of retinol uptake was also limited by fatty acids. This dependency was most pronounced when retinol was presented to cells bound to RBP or to RBP-TTR. Both uptake and esterification exhibited dose-dependent regulation by physiological concentrations of fatty acids ( $75\text{--}450\ \mu\text{M}$ ) with normal fatty acid albumin mole ratios (0.5–3.0). Such concentrations are comparable to that which would be found in culture medium containing 10% human (35) or bovine serum (36). Both the relative and the absolute magnitudes of fatty acid's effects were significant – uptake and esterification were enhanced from 30 to 300% and 30 to 600%, respectively, under these conditions. Oleate and palmitate, both of which have been demonstrated to be effective in these studies, together account for more than 50% of free fatty acids in human (35) and bovine sera (36). It follows that studies of retinol uptake and esterification in cell culture systems attempting to model a physiological setting should include extracellular fatty acids in their incubations. Excluding exogenous fatty acids in such studies would likely result in the underestimation of a cell's capacity to take up and esterify retinol.

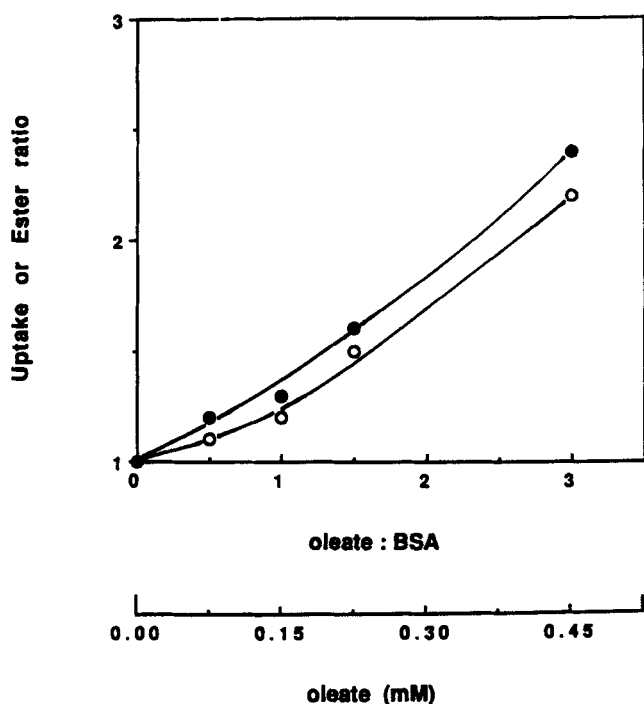




**Fig. 5.** Concentration dependence of RBP-[<sup>3</sup>H]retinol on its uptake and esterification in the presence or absence of oleate by MCF-7 cells. After a 7-h incubation with the indicated concentrations of RBP-[<sup>3</sup>H]retinol and either BSA alone (open circles) or BSA-oleate (closed circles), uptake (panel A) and esterification (panel C) of [<sup>3</sup>H]retinol were measured. The uptake ratio for data in panel A is shown in panel B and the ester ratio for data in panel C is shown in panel D. Error bars show  $\pm 1$  SD of triplicate determinations. Error bars that are not visible are within the dimensions of the symbol.

The concentrations of retinol and retinyl ester in different body tissues vary widely but are characteristic of the tissue (5, 8). Despite this, the mechanism whereby retinol is apparently selectively taken up and esterified in these tissues is unknown. With a view to exploring the role of the physiological transport protein, RBP, in this mechanism, we have compared the effects of fatty acids on the uptake and esterification of retinol presented to cells in a dispersed form and bound to RBP or to RBP-TTR. A number of investigators have suggested that a rapid reversible binding event between a cell surface receptor and RBP mediates the uptake of retinol by cells (11-17). Evidence for binding of RBP to cell membranes has been presented but lacking is the demonstration of saturable, specific binding which correlates with retinol uptake. The present data do not support a critical role for RBP or RBP-TTR in the uptake and esterification of retinol by

cells. In the steady state, the overall pattern of response of retinol esterification to fatty acids was very similar with the different retinol donors (Fig. 1, Table 1). In addition, the different forms of retinol were esterified to a similar extent. For example, cells incubated with fatty acids consistently esterified 10% of total cell retinol in 6-7 h, regardless of the retinol donor. Creek et al. (21) have reported similar findings for the esterification of dispersed versus RBP-bound retinol by cultured human keratinocytes. Kinetic similarities for uptake and esterification between the different forms of retinol were also observed. The overall patterns of retinol uptake and esterification with respect to time were similar (Figs. 2-4). When the uptake of dispersed and RBP-bound retinol were similar (Figs. 2 and 4), the pattern of oleate's effects on stimulating both uptake and esterification during the initial hour of incubation were qualitatively alike. Thus, these



**Fig. 6.** Effect of oleate to BSA ratio on the uptake and esterification of RBP- $^3\text{H}$ retinol by MCF-7 cells. After cells were incubated for 6 h with  $0.5 \mu\text{M}$  RBP- $^3\text{H}$ retinol bound to RBP and either BSA alone or BSA-oleate with the indicated ratio, uptake (open circles) and esterification (closed circles) of  $^3\text{H}$ retinol were measured.

results strongly argue that retinol uptake and subsequent esterification by cells occur by similar mechanisms for these two presentations of retinol.

The resemblance of the two forms of retinol also suggests that the disposition of dispersed retinol approximates that of RBP-bound retinol insofar as cell uptake is concerned. This is consistent with the idea that retinol is taken up by cells after its dissociation from RBP and diffusion in the aqueous phase as has been postulated by Noy and Xu (22). The differences in the rates of retinol uptake we have observed between the different forms of retinol are also compatible with this interpretation. At equal concentrations, the initial rate of uptake was greatest for dispersed retinol, intermediate for RBP-bound, and least for RBP-TTR-bound retinol. This ranking corresponds in reciprocal fashion to the reported avidities of the respective proteins for retinol (22).

Retinol that had newly entered cells quickly became available as a substrate for esterification (Figs. 2-4). Membrane-bound retinol has been reported to be a substrate for both lecithin:retinol acyltransferase (LRAT) and acyl coenzyme A:retinol acyltransferase (ARAT) (37). Retinol bound to the cellular retinol binding proteins, on the other hand, is thought to be available for esterification only by LRAT (37). Both of these activities have been localized to the microsomal fraction of cell

homogenates (38-40), although their precise distributions among cell membranes are not known. Likewise, the activities of both enzymes could be affected by the substrate effects of fatty acids that have been proposed in this work. Thus, it is not possible yet to distinguish the degree to which either enzyme might be responsible for the esterification of retinol in these cells. In either case, the original extracellular retinol donor does not seem to play a critical role in enhancing the capacity of retinol to undergo esterification by cells.

The precise nature of RBP's role in retinol transport to cells is still not fully understood. That association of retinol with RBP protects retinol from oxidation has been clearly demonstrated (4, 29). It is not clear, however, that RBP serves to facilitate retinol uptake by cells. Creek et al. (21) have proposed that one of RBP's functions may be to limit the transport of retinol to cells from the extracellular space. The present data, and those of others (21, 41), demonstrate that binding retinol to RBP or RBP-TTR retards rather than accelerates its uptake by cells. Thus, if protecting and solubilizing retinol describe the scope of RBP's role, still remaining is a satisfactory explanation for the apparent directed delivery of retinol to cells that acquire and store the vitamin.

Free fatty acids are known to partition into cell membranes and to change the fluidity of membranes directly by their presence in unesterified form or indirectly after incorporation into the structural lipids of the membrane (42). Such changes have been shown to be associated with a variety of alterations in cell function such as membrane permeability, enzyme activities, transporter and receptor functions (42). Fatty acid-mediated changes in membrane fluidity are generally specific to a particular fatty acid or class of fatty acids, however (42). In our studies, a variety of saturated and unsaturated fatty acids were equally effective in supporting high rates of retinol uptake and esterification. Consequently, the alteration of membrane fluidity does not seem to be a candidate mechanism for explaining these results.

The exposure of cultured cells to exogenous fatty acids results in their rapid incorporation into membrane phospholipid via acyl substitution and into cytoplasmic droplets of neutral lipid via synthesis of triacylglycerol (42). Fatty acyl coenzyme A is generated very rapidly upon entry of fatty acids to cells and can serve as an acyl donor for acyl substitution of phosphatidylcholine (43) and for triacylglycerol synthesis (44). As fatty acyl coenzyme A and phosphatidylcholine are thought to be fatty acyl donors for ARAT (45), and LRAT (38, 46), respectively, it follows that expansion of the pool size or regeneration of these substrates by the presence of extracellular fatty acids could conceivably increase the rate of retinol esterification.

The enhancement of retinol uptake could also be explained by a substrate effect if one hypothesizes a close

association between retinol uptake and esterification by cells. An increased rate of esterification would result in a corresponding decrease in the cellular content of retinol that could then increase the gradient of retinol into the cell, leading to an enhanced rate of uptake. An intermediate esterification step in the uptake of retinol as has been postulated by Ottonello, Petrucce, and Mariaini (47) to occur in retinol pigment epithelial cells. Our time course studies demonstrated that retinol esterification commenced very rapidly after uptake by the cell and exhibited kinetics similar to that for uptake during the first 2 h of incubation (Fig. 2-4). Furthermore, the relative extent of the effects of fatty acids on both of these parameters during this time were closely correlated. During the steady state, in contrast, the effects of fatty acids on retinol uptake and esterification were not correlated. This apparent dissociation was evident as a function of time (panel C, Figs. 2-4) and as a function of RBP concentration (panels B and D of Fig. 5). The turnover of a pool of retinyl ester that was increasing in size with time might result in a high concentration of substrate retinol at the site of deposition and, thus, render the net esterification reaction insensitive to additional increases in cell retinol content.

The uptake of free fatty acids and their subsequent conversion to fatty acyl coenzyme A, and incorporation into cell phosphatidylcholine and triacylglycerol are known to occur very quickly, i.e., within minutes (42)—a time frame that is consistent with the very rapid effects of fatty acids observed in these studies (see early time points in Fig. 2, panels A, B). Although the pool sizes and the turnover of these lipids can be acutely regulated by extracellular fatty acids, it is unlikely that enhanced retinol uptake and esterification were merely the result of a partitioning of retinol and retinyl ester into preexisting intracellular pool or sink of hydrophobic triacylglycerol. Such a model would predict that the extent to which fatty acids enhanced retinol uptake and esterification would be proportional to the time that cells were exposed to fatty acids and, hence, to the size of such an intracellular pool of lipid. This result was clearly not observed. The results of the preincubation study (Table 2) demonstrated that the full magnitude of increased retinol uptake and esterification required the simultaneous exposure of cells to fatty acid and RBP-retinol. A 12-h preincubation with fatty acids alone was less than one-half as effective in this regard as compared to a 6-h coincubation with fatty acid and RBP-retinol. Thus, these results point to an acute nature of fatty acid's effects on retinol uptake and esterification and suggest that the regulating factor(s) are very short-lived.

The present studies extend previous studies from this laboratory on the uptake and metabolism of chylomicron retinyl ester (26) and the secretion of RBP-retinol (27) by HepG2 cells to include the uptake and esterification

RBP-bound retinol. The rate of retinol uptake from RBP observed in these experiments (5-20 pmol retinol/mg cell protein in 6 h) was comparable to the rate of RBP secretion observed previously (approximately 20 pmol/mg cell protein in 8 h) (27). This, plus the striking effect of RBP on retaining retinol outside of cells, leads to the speculation that bidirectional transport of retinol between peripheral tissues and the liver could occur via RBP. This sort of recycling of retinol between tissues in the body has been proposed previously (48) and could function to protect extrahepatic tissues from excessive accumulation of retinol and to conserve retinol following glomerular filtration of RBP. ■

This work was supported by NIH grants HD-16484, HL-22633, HL-07443 and by funds from the Howard Heinz Endowment. We thank Diana T. Long for excellent technical assistance.

Manuscript received 17 September 1990 and in revised form 24 January 1991.

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