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# Uptake and passage of β-lactoglobulin, palmitic acid and retinol across the Caco-2 monolayer

Pilar Puyol, M. Dolores Perez, Lourdes Sanchez, Jose M. Ena, Miguel Calvo *

*Tecnología y Bioquímica de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, 50013 Zaragoza, Spain*

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**Abstract**

Caco-2 cell line grown on collagen coated polycarbonate membranes in bicameral chambers has been used to study the effect of the binding of palmitic acid or retinol on the uptake and passage of iodinated β-lactoglobulin and albumin across cell monolayers. The percentage of β-lactoglobulin transported through the monolayer was higher than that of albumin, about 50% and 30% of the total protein after 24 h of incubation, respectively. In all cases, less than 1% of protein was retained intracellularly. No differences were found in the uptake and transport of β-lactoglobulin or albumin in the presence or absence of ligands. Furthermore, uptake and passage across Caco-2 monolayer of retinol or palmitic acid added either bound to β-lactoglobulin or to albumin have been compared. The percentage of retinol found in the lower chamber was about 35% of the total retinol after 24 h of incubation for both proteins. However, the amount of retinol associated to cells was higher when it was added bound to β-lactoglobulin than to albumin, about 26% and 10%, respectively. This fact suggests that the metabolic processing of retinol by Caco-2 cells is the rate-limiting step for retinol transport. The percentage of palmitic acid that crossed the monolayer was about 7%, remaining approx. 90% in the cells for β-lactoglobulin and albumin. These data support the hypothesis that palmitic acid internalized by Caco-2 cells is mainly destined to serve the structural and energy needs. These results show evidence of retinol and palmitic uptake by Caco-2 cells when β-lactoglobulin or albumin are the donors, and indicate that the type of binding protein does not affect the transport of both ligands through Caco-2 monolayer.

*Keywords:* β-Lactoglobulin; Serum albumin; Palmitic acid; Retinol; Caco-2 cell line; (Bovine)

## 1. Introduction

Retinol and fatty acids are lipophilic compounds which are bound to proteins when present in an aqueous environment, both extracellularly and intracellularly. They may also be esterified for transport by lipoproteins, or storage in cytoplasmic lipid droplets [1–4]. Unesterified retinol is transported in the plasma bound to retinol-binding protein (RBP) [1,3] and fatty acids bound to serum albumin [2,4]. This binding increases the solubility of fatty acids and retinol in water, allowing them to become a readily available substrates for various tissues.

The mechanism of cellular uptake of these lipophilic compounds is not yet fully elucidated and although retinol and fatty acid transport and metabolism share many similarities they are clearly distinct processes [1,4,5]. Retinol may partition into the plasma membrane or might enter cells as a result of fluid phase endocytosis of retinol-RBP complex, or it may be taken up by a receptor-mediated mechanism [1,3]. On the other hand, fatty acids may enter cells by passive diffusion, or may be absorbed through an energy-independent facilitated diffusion mechanism [2,4,6]. Recent studies have also proposed that a saturable process mediated by specific binding sites on the cell surface is involved in uptake of the albumin-ligand complex [4]. The relative importance of these different mechanisms depends on the tissue and organ involved and the concentration and properties of the ligand [1,2,4].

Once inside the cells, unesterified retinol specifically binds to small binding proteins which belong to the cellular retinol-binding protein (CRBP) family [1,3] and fatty acids to the fatty acid-binding protein (FABP) [2,4] family. The function of these proteins is to transport the ligands inside the cell and to facilitate their metabolic utilization, according to cell needs and function.

Bovine β-lactoglobulin, the major whey protein of ruminants [7], has been shown to bind fatty acids [8,9] and

* Corresponding author. Fax: +34 76 591994.


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retinol [10,11] in vitro. Each bovine β-lactoglobulin dimer has two binding sites for retinol and one binding site for fatty acids. Furthermore, ruminant β-lactoglobulin, when isolated by non-denaturing methods, has lipids physiologically bound, mainly fatty acids [12]. The binding of palmitic acid to bovine β-lactoglobulin increases the stability of this protein to tryptic degradation in vitro [13] and to thermal denaturation [14], indicating that fatty acid binding may contribute to stabilization of the protein structure. However, this increased stability is not observed when retinol is bound to β-lactoglobulin.

Bovine β-lactoglobulin shows a high degree of structural homology with proteins known as lipocalycins, among them the human retinol-binding protein (RBP) [7], whose function seems to be to participate in the transport of small hydrophobic substances [15,16]. This analogy, coupled with the ability of bovine β-lactoglobulin to bind retinol [11] or fatty acids [9,12], and also to increase the activity of ruminant pregastric lipase in vitro [17], has prompted the suggestion that β-lactoglobulin could participate in the metabolism of milk fatty acids [9,11] and retinol [18] in the newborn. Furthermore, bovine β-lactoglobulin is remarkably acid-stable [19] and quite resistant to hydrolysis by proteases of the gastrointestinal tract [20]. It is also absorbed by the small intestine, and evidence of transcellular passage across this epithelium has been reported [21–23].

To study such processes in more detail, a suitable in vitro model is required. It has been shown that the human colon adenocarcinoma Caco-2 cell line expresses retinol and fatty acid-binding proteins, and microsomal enzymes which are homologous to those involved in the metabolism of fatty acids [24–26] and retinol [27–29] in human enterocytes. Furthermore, Caco-2 cells are able to internalize retinol [27,28] and fatty acids, and to support the synthesis and secretion of retinyl esters [27,28], triacylglycerols and lipoproteins [24,26]. These cells constitute a good in vitro model system for the study of transport such as the uptake of fatty acids and retinol.

The aim of this work has been to study the effect of the binding of retinol or palmitic acid on the uptake and transport of β-lactoglobulin across Caco-2 cell monolayers. In addition, we have compared the passage of retinol and palmitic acid presented either bound to β-lactoglobulin or to serum albumin.

## 2. Materials and methods

### 2.1. Cell culture

Caco-2 cells were kindly donated by Dr. I. Freshney from the Beatson Institute, Glasgow. Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (Northumbria, Cramlington, UK), 1% (v/v) non-essential amino acids (Flow, Rickmansworth, UK), 1 μg/ml bovine insulin (Sigma, Dorset, UK) and antibiotics, 50 U/ml penicillin and 50 μg/ml streptomycin (Flow, Rickmansworth, UK). Cells were normally grown in 25 $cm^2$ tissue culture flasks and subcultured slightly before confluence to be seeded in Transwell bicameral chambers (Costar, High Wycombe, UK) of 0.3 μm pore size and 6.5 mm diameter. The polycarbonate membrane of the inserts was previously precoated with 50 μl/well of a 2 mg/ml solution of collagen type I (Boehringer, Mannheim, Germany) dissolved in 0.1 M acetic acid. The excess of collagen was removed, and the inserts were dried inverted under sterile conditions. Cells were plated on Transwell membranes at a density of $10^5$ cells/$cm^2$ and 200 μl and 800 μl of culture medium were added to the upper and lower chamber, respectively. In order to avoid clumps, the cell suspension was passed through a nylon membrane before being plated. The cells were maintained at 37° C in an atmosphere of 5% $CO_2$ and 90% relative humidity and the medium changed daily.

### 2.2. Preparation of iodinated proteins

Bovine $^{125}$I-β-lactoglobulin and bovine $^{125}$I-serum albumin (Sigma, Dorset, UK) were prepared by the chloramine T method [30] using carrier-free $^{125}$INa (306 mBq) (The Radiochemical Centre, Amersham, UK). Free $^{125}$I was removed by gel filtration on Sephadex G-25 columns (1 × 10 cm) (Pharmacia, Uppsala, Sweden). The specific activity of the radiolabelled proteins was about 0.25 μCi/mg for β-lactoglobulin and 1.5 μCi/mg for albumin.

### 2.3. Binding of palmitic acid or retinol to β-lactoglobulin and albumin

Ligands were bound to proteins previously delipidated by the method of Chen [31]. 80 μl of delipidated β-lactoglobulin (0.9 nM) or albumin (0.6 nM) in phosphate-buffered saline (pH 7.4) was incubated with [$^{14}$C]palmitic acid (0.8 μM, 47 Ci/mol) (The Radiochemical Centre, Amersham, UK) or [$^{3}$H]retinol (1.6 μM, 24 Ci/mol) (DuPont, Nemours, Germany) at 37° C overnight. Then, to saturate binding sites, protein solutions were incubated with 2–3 molar excess of unlabelled fatty acid or retinol for 2 h, as above. To eliminate free ligands, protein solutions were chromatographed in a Sephadex G-25 column (1 × 10 cm). These preparations contained between 2 and 6 Ci of labelled palmitic acid, or between 2 and 3 Ci of labelled retinol per mol of protein. Preparations of unlabelled fatty acid or retinol bound to iodinated proteins were obtained by the same procedure.

### 2.4. Transport studies

Caco-2 cell monolayers were grown on collagen coated polycarbonate membrane in bicameral chambers. Transport

studies were carried out when cells showed evidences of differentiation by optical microscopy and the monolayer was intact as checked by measuring phenol red exclusion [32]. After washing both upper and lower chambers with serum free medium, cells were incubated with medium supplemented with either radiolabelled palmitic acid or retinol bound to β-lactoglobulin or albumin, or with iodinated proteins containing unlabelled ligands. Single labelled preparations were used to avoid interference of $^{125}I$ on $^{14}C$ or $^{3}H$ radioactivity counting.

The lower chamber contained medium supplemented with serum albumin (1 mg/ml) or foetal calf serum (1%) (v/v) as palmitic acid or retinol acceptors, respectively. Medium from the lower compartment was removed at 3, 6 and 24 h of incubation and replaced by fresh medium. At the end of every experiment phenol red exclusion was determined and cells washed three times with Hank's solution and thereafter dissolved in 2% (w/v) sodium dodecylsulfate. Radioactivity associated to the medium from the upper and lower chambers at different times of incubation, and to the cells, was determined by liquid scintillation or gamma counter. Integrity of $^{125}I$-β-lactoglobulin or $^{125}I$-albumin found in the lower chamber was determined by precipitation of an aliquot with trichloroacetic acid at a final concentration of 10% (w/v).

### 2.5. Statistical evaluation

Shiffer's test was used to assess statistical significance of differences between passage of β-lactoglobulin and serum albumin with different ligands and as delipidated protein. The same test was also used to evaluate the statistical significance of ligand transport differences.

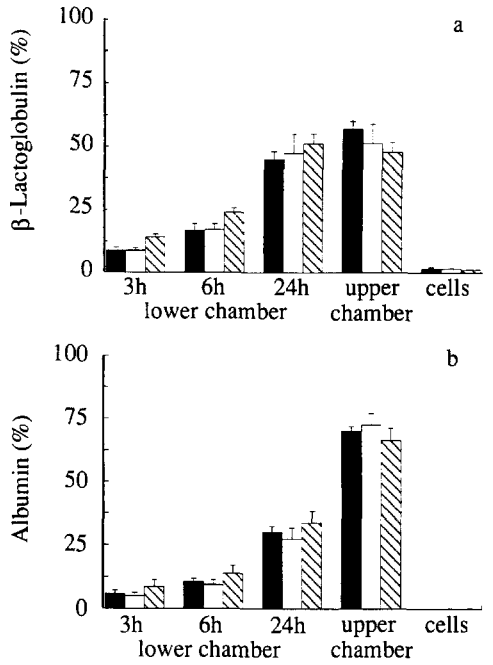


Fig. 1. Uptake and passage of bovine $^{125}I$-β-lactoglobulin (a) or $^{125}I$-albumin (b) across Caco-2 monolayers in bicameral chambers. 80 μl of delipidated $^{125}I$-β-lactoglobulin (0.9 mM, 9 Ci/ mol) or $^{125}I$-albumin (0.6 mM, 90 Ci/mol) were saturated with unlabelled palmitic acid or retinol by incubation of a large excess of ligand at 37° C overnight. The Caco-2 cells were grown on a collagen coated polycarbonate membrane in bicameral chambers and confluent monolayers of differentiated cells were obtained after 19 days of culture. Then, 200 μl of culture medium containing 40 μg of protein, either delipidated (hatched bar) or with bound retinol (□) or palmitic acid (■) were added to the upper chamber. Values represent the percentage of total radioactivity transferred to the lower chamber after 3, 6 and 24 h of incubation, as well as radioactivity in the upper chamber and that associated to cells after 24 h. Results represent the mean ± S.D. of six replicates from three independent experiments.

## 3. Results

### 3.1. β-Lactoglobulin and albumin transport studies

Uptake and passage of β-lactoglobulin and albumin have been studied using Caco-2 cell monolayers. In all experiments, the radioactivity transported to the lower chamber increased in a linear rate within 24 h of incubation.

β-Lactoglobulin and albumin labelled with $^{125}I$ were added to the upper chamber either delipidated or with bound palmitic acid or retinol. Fig. 1a and b shows the

Table 1
Trichloroacetic acid-insoluble fraction of $^{125}I$-β-lactoglobulin and $^{125}I$-serum albumin transported across Caco-2 monolayers in bicameral chambers

| Additions | Percentage of radioactivity (%) | | |
|---|---|---|---|
| | 3 h | 6 h | 24 h |
| Delipidated β-lactoglobulin | 54.2 ± 14.2 | 61.9 ± 9.1 | 75.2 ± 5.6 |
| β-Lactoglobulin with bound palmitic acid | 63.2 ± 1.8 | 68.9 ± 0.8 | 76.9 ± 1.5 |
| β-Lactoglobulin with bound retinol | 58.8 ± 2.4 | 61.6 ± 4.0 | 66.2 ± 9.0 |
| Delipidated serum albumin | 85.9 ± 1.8 | 86.6 ± 1.0 | 90.7 ± 0.7 |
| Serum albumin with bound palmitic acid | 81.6 ± 0.8 | 83.7 ± 0.9 | 89.4 ± 0.5 |
| Serum albumin with bound retinol | 75.9 ± 5.2 | 77.7 ± 4.7 | 78.5 ± 10.0 |

Proteins were previously iodinated and added to the upper chamber either delipidated or with bound retinol or palmitic acid. Values represent the percentage of radioactivity associated to the trichloroacetic acid-insoluble fraction of the lower chamber medium after 3, 6 and 24 h of incubation. Results are the mean ± S.D. of two replicates from three independent experiments.

percentage of radioactivity transported to the lower chamber at different times as well as radioactivity remaining in the upper chamber and the radioactivity associated to cells for β-lactoglobulin and albumin, respectively. As can be seen, more $^{125}$I-β-lactoglobulin than $^{125}$I-albumin crossed the monolayer, being about 50% and 30% for β-lactoglobulin and albumin, after 24 h of incubation, respectively. Presence or absence of ligand bound to proteins had no effect on β-lactoglobulin or albumin passage through monolayer. In all cases, less than 1% of total radioactivity was retained intracellularly after 24 h of incubation.

Protein degradation after passage through the monolayer was determined by measuring the radioactivity in the insoluble and soluble fraction after trichloroacetic acid precipitation of the lower chamber medium (Table 1). After 24 h of incubation trichloroacetic acid-insoluble radioactivity was about 70% and 90% for delipidated β-lactoglobulin and albumin, respectively, indicating that β-lactoglobulin transported to the lower chamber is more degraded than albumin. Furthermore, no significant differences have been detected in the degradation of delipidated proteins and proteins with bound palmitic acid or retinol for each protein. To eliminate the possibility of the protein being degraded by apical proteinases, before crossing the monolayer, integrity of the proteins in the upper chamber was also checked by trichloroacetic acid precipitation of culture medium at the end of incubation. It was found that insoluble-trichloroacetic acid radioactivity was more than 99%.

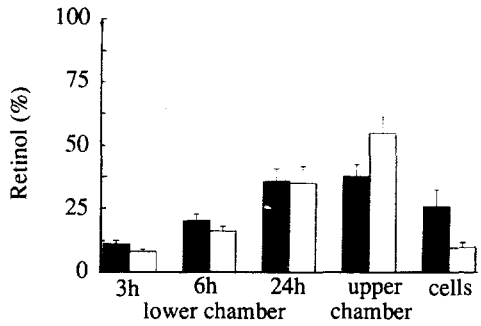


Fig. 2. Uptake and passage of [$^3$H]retinol across Caco-2 monolayers in bicameral chambers. 80 μl of delipidated β-lactoglobulin (0.9 mM) or albumin (0.6 mM) were incubated with [$^3$H]retinol at a final concentration of 0.4 μM, at 37° C overnight. Afterwards, the protein solution was incubated with a large excess of unlabelled ligand for 2 h to saturate the binding sites. Caco-2 cells were grown on a collagen coated polycarbonate membrane in bicameral chambers and confluent monolayers of differentiated cells were obtained after 19 days of culture. Then 200 μl of culture medium containing 40 μg of β-lactoglobulin (■) or albumin (□) with bound retinol were added to the upper chamber and 800 μl of the culture medium containing foetal calf serum (1%) (v/v) to the lower chamber as retinol acceptor. Values represent the percentage of total radioactivity transferred to the lower chamber after 3, 6 and 24 h of incubation, as well as the radioactivity remaining in the upper chamber and that associated to cells after 24 h. Results represent the mean ± S.D. of six replicates from three independent experiments.

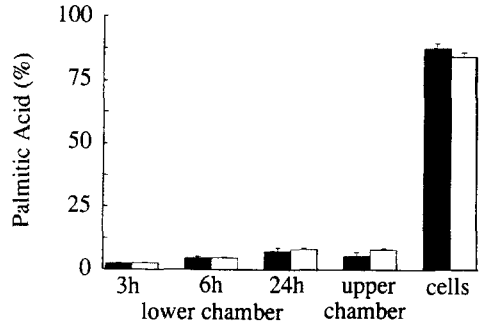


Fig. 3. Uptake and passage of [$^{14}$C]palmitic acid transport across Caco-2 monolayers in bicameral chambers. 80 μl of delipidated β-lactoglobulin (0.9 mM) or albumin (0.6 mM) were incubated with [$^{14}$C]palmitic acid at a final concentration of 0.8 μM, at 37° C overnight. Afterwards, the protein solution was incubated with a large excess of unlabelled ligand for 2 h to saturate the binding sites. Caco-2 cells were grown on a collagen coated polycarbonate membrane in bicameral chambers and confluent monolayers of differentiated cells were obtained after 19 days of culture. Then, 200 μl of culture medium containing 40 μg of β-lactoglobulin (■) or albumin (□) with bound palmitic acid were added to the upper chamber and 800 μl of the culture medium containing serum albumin (1 mg/ml) to the lower chamber as palmitic acid acceptor. Values represent the percentage of total radioactivity transferred to the lower chamber after 3, 6 and 24 h of incubation, as well as the radioactivity remaining in the upper chamber and that associated to cells after 24 h. Results represents the mean ± S.D. of six replicates from three independent experiments.

### 3.2. Palmitic acid and retinol transport studies

Uptake and passage of palmitic acid or retinol have been studied using the same model of Caco-2 cell monolayers. As with passage of β-lactoglobulin and albumin, the ligand bound to these proteins transported to the lower chamber increased in a linear rate with time, either for palmitic acid or retinol.

To study the effect of β-lactoglobulin and albumin on the uptake and passage of palmitic acid or retinol across cell monolayers, [$^3$H]retinol or [$^{14}$C]palmitic acid were added to the upper chamber bound to β-lactoglobulin or albumin. Fig 2 shows the radioactivity of retinol transported to the lower chamber at different times, as well as radioactivity in the upper chamber and that associated to cells, for β-lactoglobulin and albumin. No differences were found in the passage of retinol through the monolayers whether added complexed with β-lactoglobulin or albumin, being about 35% after 24 h of incubation in both cases. However, radioactivity associated to cells was higher when retinol was added bound to β-lactoglobulin than to albumin, about 26% and 10%, after 24 h of incubation, respectively. These differences were statistically significant ($p < 0.001$).

Fig. 3 shows the percentage of [$^{14}$C]palmitic acid, added to the upper chamber bound to β-lactoglobulin or albumin, which was transported to the lower chamber, as well as radioactivity retained intracellularly and in the upper chamber. In this study, no differences have been

found between the effect of β-lactoglobulin or albumin on palmitic acid uptake and passage through the monolayer. The percentage of total radioactivity transported to the lower chamber is about 2%, 4% and 7% after 3, 6 and 24 h of incubation for both proteins. It is remarkable that more than 90% of total radioactivity was retained by cells after 24 h of incubation.

## 4. Discussion

The Caco-2 cell line has been extensively used to study absorption of several substances such as proteins [33], ions [34], vitamins [27] and lipids [24]. This cell line exhibits many morphological and biochemical characteristics of absorptive intestinal cells, including polarization and expression of several brush border enzymes. Furthermore, it has been demonstrated that Caco-2 cell monolayers show barrier properties similar to those observed in the small intestine [35]. Therefore, we consider Caco-2 cell monolayers as an appropriate model to investigate cellular transport of fatty acids and retinol, as well as β-lactoglobulin and albumin.

The results obtained in this work indicate that the passage of β-lactoglobulin across Caco-2 cell monolayers is higher than that of albumin. However, β-lactoglobulin seems to be more degraded than albumin as suggested by trichloroacetic acid precipitation of the lower chamber medium. These results are in accordance with those obtained by Stern and Walker [36] using the rat everted gut sac technique. They found that more β-lactoglobulin than albumin was bound and taken up by cells. Furthermore, β-lactoglobulin was more extensively degraded inside the cells than albumin, resulting in fragments of lower molecular weight for β-lactoglobulin than for albumin. The reason for such differences is still unknown. However, it could be explained assuming the existence of different intracellular pathways for each protein with differential protein breakdown during binding and transport across the cells.

Passage of β-lactoglobulin and albumin across the intestinal mucosa has been reported to be a transcellular mechanism involving two functional pathways. The main pathway is degradative and implies lysosomal processing of the protein and the second one is a minor route by transcytosis that allows transport of the intact protein [22]. In this study, we have found that about 70% of β-lactoglobulin and 90% of albumin transported to the lower chamber is recovered in the trichloroacetic acid-insoluble fraction. These results are similar to those reported by Marcon-Genty et al. [20] using isolated rabbit ileum, who found that about 77% of transported β-lactoglobulin was recovered in the trichloroacetic acid-insoluble fraction. However, analysis of this fraction by chromatographic and immunological techniques showed that only 6-9% of the trichloroacetic acid-insoluble β-lactoglobulin corresponded to completely intact protein. Similarly, analysis of passage of albumin across the adult intestine in vivo showed that only about 2% of albumin in the blood remained completely undegraded [37]. Therefore, these findings indicate that the trichloroacetic acid-insoluble fraction obtained in our work contains not only intact protein but also large peptides from the hydrolysed protein.

It is well known that uptake of some molecules occurs by a specific mechanism of receptor-mediated endocytosis. An example is the cellular uptake of iron-saturated transferrin. This protein interacts with a specific receptor on the cell surface, which has a higher affinity for transferrin saturated with iron than for apotransferrin [38]. However, uptake and passage of β-lactoglobulin is similar whether or not ligands are bound. This suggests that any specific binding sites on Caco-2 cells, do not distinguish between different forms of the protein [36].

We have also used the Caco-2 cell monolayers to study the effect of β-lactoglobulin and albumin on retinol uptake and transport. We observed a significantly greater retinol uptake when bound to β-lactoglobulin, compared to that bound to albumin. However, under the same conditions, passage of retinol through the monolayers was similar with both proteins. Some workers have shown that β-lactoglobulin, like the retinol-binding protein from human serum, significantly increased retinol uptake [39,40], but others have found that neither β-lactoglobulin nor albumin, which also binds retinol in vitro [10], had any effect [41]. Although the existence of a specific binding site for β-lactoglobulin at the brush border membrane of rat intestine has been suggested [39], the existence of another mechanism, in which retinol is dissociated from its binding protein before associating with the cells has been proposed [40,42]. In this case, a specific interaction between proteins and cells would not be required. This last hypothesis seems to be more probable because rat milk does not have a protein homologous to β-lactoglobulin. Differences in experimental methods and intrinsic variation in the model systems may account for these contradictory findings. The existence of specific binding sites for β-lactoglobulin in Caco-2 cells would be surprising as human milk does not contain β-lactoglobulin [7]. However, the higher uptake of retinol bound to β-lactoglobulin compared to that bound to albumin might be related with different number of binding sites and affinity for retinol when bound to each of these proteins [8,10]. Since retinol is transported in a similar amount by cells when bound to either protein it is possible that the rate-limiting step for retinol transport is the metabolic processing by cells [3,28,42]. It has been reported that Caco-2 cells contain several microsomal enzymes that are involved in retinol esterification and it was therefore suggested that retinol crosses the monolayer as retinyl ester [27].

On the other hand, our data show that the uptake and transport of palmitic acid across cell monolayers is similar when the fatty acid is added to the upper chamber bound

either to β-lactoglobulin or to albumin. These results indicate that the type of binding protein does not affect palmitic acid internalization and transport by Caco-2 cells. Palmitic acid may therefore be dissociated from its binding protein before being taken up by cells, without binding to a specific cell surface site. The present results are strikingly similar to those reported by other authors, who observed that β-lactoglobulin and albumin have a similar effect on fatty acid uptake by parenchymal cells [43], lymphocytes [44] and Ehrlich ascites tumor cells [8]. Although β-lactoglobulin binds less fatty acids per mol of protein than serum albumin, they are bound with lower affinity and may therefore be transferred to cells more efficiently. Furthermore, we have found that most of the palmitic acid incorporated was still retained inside the cells after 24 h of incubation, as previously observed by Levin et al. [24] who studied the transport of oleic acid using the same cell line. These data support the hypothesis that fatty acids internalized by Caco-2 cells are destined to serve the structural and energy needs of the cells.

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