

Transport of β -lactoglobulin across rabbit ileum in vitro

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MARCON-GENTY, DOMINIQUE, DANIEL TOME, OMAR KHEROUA, ANNE-MARIE DUMONTIER, MARTINE HEYMAN, AND JEHAN-FRANÇOIS DESJEUX. *Transport of β -lactoglobulin across rabbit ileum in vitro*. Am. J. Physiol. 256 (Gastrointest. Liver Physiol. 19): G943-G948, 1989.—Intestinal transepithelial transport constitutes a major limiting step in the transfer of food protein antigens to the blood. This transport was studied in isolated rabbit ileum in Ussing chamber in vitro for the milk protein antigen β -lactoglobulin (β -Lg). The transepithelial passage of β -Lg was measured by enzyme-linked immunosorbent assay (ELISA) and radiolabeled protein transfer and compared with that of the nonmetabolizable marker polyethylene glycol (PEG)-4000. When 1 mg/ml of β -[^{14} C]Lg or [^{3} H]PEG was added to the mucosal side of the tissue, the total uptake, measured as the transfer of radiolabeled material across the ileum, was significantly higher for β -Lg than for PEG (5.46 ± 1.75 vs. $1.43 \pm 0.26 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$). Measured by ELISA, 6–9% of the total amount of β -Lg transported was absorbed in an intact antigenic form. This transport of intact β -Lg was inhibited by the metabolic inhibitors 50 mM 2-deoxyglucose and 1 mM azide added simultaneously, was reduced by the microtubule assembly inhibitor 0.05 mM colchicine, and was enhanced by 20 mM ammonia, which inhibits lysosomal proteolytic activity. These results indicate that β -Lg is efficiently absorbed by the intestinal mucosa of adult animals, partly in intact antigenic form and that β -Lg transport is probably transcellular, as observed for other proteins. The finding that β -Lg is absorbed in intact antigenic form agrees with other reports implying that β -Lg is the main factor responsible for milk protein immunoreactivity and intolerance.

protein absorption; milk proteins; intestine

AMONG FOOD PROTEINS, bovine milk proteins constitute a major source of dietary proteins for humans and are known to induce hypersensitive reactions in some predisposed subjects (2, 6, 20, 23). These proteins consist of two major fractions: a micellar casein fraction and a whey fraction. Caseins are rapidly degraded by proteolytic enzymes of the gastrointestinal tract (14, 16, 18). In the whey protein fraction, β -Lactoglobulin (β -Lg) seems to be the most resistant to intraluminal digestion and is generally considered the main factor responsible for milk protein immunoreactivity and intolerance (12–14, 16, 18, 20, 23, 25).

Minute absorption of intraluminal proteins by the intestine of adult animals is believed to play a key role in inducing sensitization to food proteins ingested orally (1, 4, 5, 8, 9, 22). The passage of intact proteins across the intestinal epithelium constitutes a major limiting

step in this absorption. In fact, previous studies with model proteins such as horseradish peroxidase (HRP) or bovine serum albumin (BSA) clearly established that macromolecules are absorbed across the intestinal epithelium of adult animals (7, 11, 27–29). They indicate that the intestinal transepithelial passage of proteins is mainly transcellular and proceeds by endocytotic uptake at the luminal membrane, intracellular transfer, and exocytosis through the basolateral membrane. The main route for this passage involves intracellular protein degradation by the lysosomal system and transport of degraded products to the extracellular space, but a small fraction of protein that escapes degradation is also transported intact (11).

Food proteins were also demonstrated to be absorbed by the gut, but few investigations have evaluated the magnitude and the mechanism of their transport (4). The present study was undertaken to investigate the pathway and the magnitude of β -Lg transport across the intestinal epithelium of the adult rabbit. For this purpose the rate of its transepithelial passage across isolated rabbit ileum in an Ussing chamber was measured by enzyme-linked immunosorbent assay (ELISA) and radiolabeled protein measurement. The absorption of β -Lg was compared with that of the nonmetabolizable macromolecular marker polyethylene glycol (PEG)-4000. The results indicated that the transport of β -Lg was mainly transcellular and that a fraction of this protein was transported intact.

MATERIALS AND METHODS

Chemicals. Bovine β -Lg (Sigma) was radiolabeled with ^{14}C by reductive alkylation of amino groups using [^{14}C]-formaldehyde (15). This labeling was verified by reverse-phase high-performance liquid chromatography (RP-HPLC). The specific activity was 1 $\mu\text{Ci}/\text{mg}$. PEG-4000 was purchased from Sigma, and [^3H]PEG (2 mCi/g) was from Amersham.

Ussing chamber experiments. Male New Zealand White rabbits weighing 2–3 kg were killed by intravenous pentobarbital sodium injection. Segments of distal ileum were removed and rinsed free of intestinal content. The external muscle layers were stripped off with fine forceps, and the tissue was opened along the mesenteric border. Pieces of stripped tissue were mounted as flat sheets between the two halves of a Lucite chamber (exposed area 3.14 cm^2) and bathed on each side at 37°C by 12 ml of isotonic Ringer solution containing the following (in

TABLE 1. *Electrical parameters and mucosal concentration of [^{3} H]PEG or β -[^{14} C]Lg after their addition to the mucosal side of rabbit ileum in an Ussing chamber*

	Control	PEG	β -Lg
Electrical parameters			
I_{sc} , μ A/cm ^{2}	35.6 $\pm$ 4.7	34.3 $\pm$ 5.2	36.4 $\pm$ 6.8
PD, mV	-2.3 $\pm$ 0.3	-2.2 $\pm$ 0.4	-2.3 $\pm$ 0.4
G , mS/cm ^{2}	15.1 $\pm$ 1.7	15.6 $\pm$ 1.6	15.9 $\pm$ 1.6
Mucosal labeled material equivalent, mg/ml			
$t = 0$ min		1.03 $\pm$ 0.07	0.99 $\pm$ 0.05
$t = 120$ min		0.97 $\pm$ 0.08	0.95 $\pm$ 0.06

Values are means $\pm$ SE for 18 tissues. β -[^{14} C]Lg (1 mg/ml) or [^{3} H]-PEG or Ringer only for control was added to the mucosal reservoir at time 0. I_{sc} , PD, and G values were measured between 60 and 120 min after such addition. During this period none of the electrical parameters ever varied significantly, and no significant differences were found between the mucosal radioactivities expressed in labeled material equivalent (mg/ml) at time 0 and 120 min for β -[^{14} C]Lg or [^{3} H]PEG.

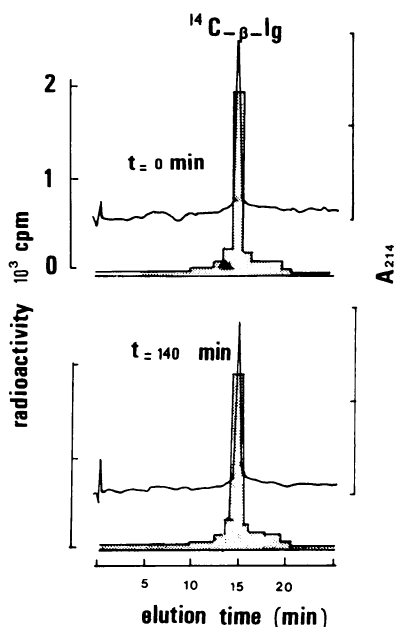


FIG. 1. Reverse-phase HPLC pattern at $t = 0$ and $t = 140$ min of the content of the mucosal reservoir of an Ussing chamber after addition of 1 mg/ml β -[^{14} C]Lg to the mucosal side of stripped rabbit ileum (exposed area 3.14 cm ^{2}). Sixty microliters of this mucosal reservoir's content were injected into the chromatograph. Absorbance at 214 nm (A_{214}) was directly recorded with an ultraviolet detector, and 2-ml fractions were collected for radioactivity determination (histogram).

mM): 140 Na $^+$, 5.2 K $^+$, 1.2 Ca $^{2+}$, 1.2 Mg $^{2+}$, 120 Cl $^-$, 25 HCO $_3^-$, 2.4 HPO $_4^{2-}$, 0.4 H $_2$ PO $_4^-$ (pH 7.4 with 95% O $_2$ -5% CO $_2$). As required, 2-deoxyglucose (50 mM, Sigma) and sodium azide (1 mM, Prolabo), colchicine (0.05 mM, Sigma), or ammonium chloride (20 mM) were added to each reservoir. The spontaneous transmucosal electrical potential difference (PD) was continuously short-circuited by a short-circuit current (I_{sc}) delivered by an automatic voltage clamp system (WPI, New Haven, CT) that corrected for fluid resistance. The electrical conductance (G) of the tissue was calculated according to Ohm's law.

Measurement of mucosal transport. After the stability of the electrical parameters of the tissue was checked for

30 min, 5 μ Ci of either β -[^{14} C]Lg or [^{3} H]PEG adjusted to 1 mg/ml with either cold β -Lg or PEG, or Ringer only for the control, was added to the mucosal reservoir. Aliquots were collected at different times from the mucosal and serosal reservoirs for radioactivity measurement and ELISA. After the above additions (140 min), the solution remaining in the serosal reservoir was collected, adjusted to 12% in trichloroacetic acid (TCA), and centrifuged. The resulting TCA-insoluble precipitate was washed with chloroform to eliminate TCA, freeze-dried, and used for ELISA, electrophoresis, and HPLC.

High-pressure liquid chromatography. Samples were injected in a Waters gradient system equipped with a Waters C $_{18}$ μ -Bondapak column (350 $\times$ 4.6 mm) and an ultraviolet detector at 214 nm. The column was eluted at 40°C with a linear gradient of 0–75% acetonitrile for 20 min in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. Fractions of 2 ml were collected and used either immediately for radioactivity determination or, after evaporation of the solvent, for ELISA and electrophoresis.

Enzyme-linked immunosorbent assay. Microtiter plates (Polylabo) were coated overnight at 4°C with 100 μ l of samples adjusted to pH 9.6 with 0.5 M carbonate buffer. Plates were washed and incubated for 1 h at 37°C with 1% gelatin. After washing, plates were again incubated for 1 h at 37°C with 100 μ l of rabbit anti- β -Lg serum (1/1,000). Samples were detected with goat anti-rabbit immunoglobulin G conjugated with HRP (Institut Pasteur, 1/3,000) using diaminooorthophenylene and H $_2$ O $_2$ as substrates. Calculation of the protein concentration in the samples was based on the standard curve obtained with purified β -Lg. The detection limit was 5 ng/ml.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10–20% polyacrylamide gels in the presence of 0.1% SDS. Gels were silver stained (17).

Calculations. ^{14} C was used to measure the apparent total transport of β -Lg (intact and degraded) and ELISA the intact β -Lg. PEG was used as an extracellular marker, and G was used as a measure of paracellular ionic permeability. Results are expressed as means $\pm$ SE. Statistical treatment was performed by analysis of variance.

RESULTS

Electrical parameters and transport of radiolabeled material. Addition of 1 mg/ml β -[^{14} C]Lg or [^{3} H]PEG on the mucosal side of the ileum mounted in an Ussing chamber had no effect on the electrical parameters of the tissue, and 140 min after this addition, the total radioactivity in the mucosal reservoir remained unchanged (Table 1). Moreover, in the chamber with mucosal β -[^{14} C]Lg, HPLC analysis of the mucosal content showed that at 120 min this radioactivity was still located on the intact protein fraction, thus indicating that none of the protein in the mucosal reservoir had been degraded (Fig. 1).

In the serosal reservoir, the radioactivity increased as a function of the time elapsing after the addition of 1 mg/ml β -[^{14} C]Lg or [^{3} H]PEG to the mucosal side of the rabbit ileum (Fig. 2). During the period from 60 to 140

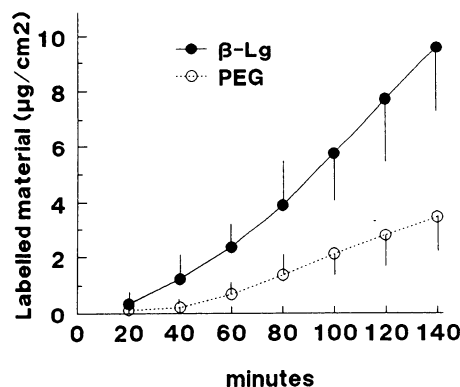


FIG. 2. Time-dependent accumulation of radioactivity on the serosal side of stripped rabbit ileum mounted in an Ussing chamber (exposed area 3.14 cm^2) after addition to the mucosal reservoir of $1 \text{ mg/ml } \beta\text{-}^{14}\text{C}[\text{Lg}]$ or $^{3}\text{H}[\text{PEG}]$. Results are expressed in $\beta\text{-}^{14}\text{C}[\text{Lg}]$ or $^{3}\text{H}[\text{PEG}]$ equivalents in micrograms per square centimeter of tissue. Values are means $\pm$ SE for 18 tissues.

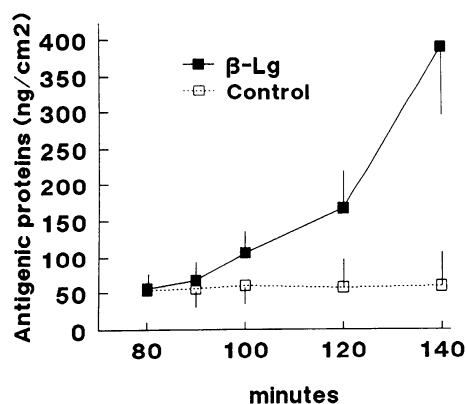


FIG. 3. Time-dependent accumulation of antigenic determinants of βLg on serosal side of stripped rabbit ileum mounted in an Ussing chamber (exposed area 3.14 cm^2) after addition to the mucosal reservoir of $1 \text{ mg/ml } \beta\text{-Lg}$ or Ringer only for control. Results are expressed in antigenic $\beta\text{-Lg}$ equivalents in nanograms per square centimeter of tissue. Values are means $\pm$ SE for tissues from 6 animals.

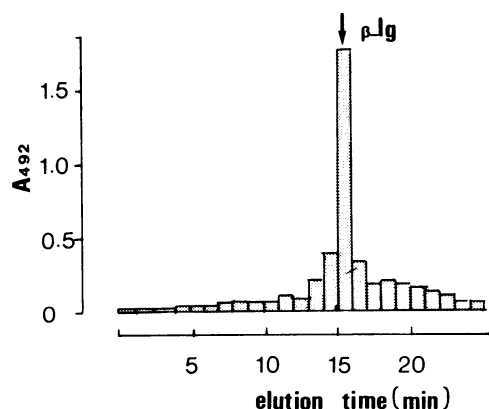


FIG. 4. Reverse-phase HPLC pattern with ELISA detection of the TCA-insoluble fraction of the serosal reservoir content of an Ussing chamber 140 min after addition of $1 \text{ mg/ml } \beta\text{-Lg}$ into the mucosal reservoir. Arrow represents position of elution of standard $\beta\text{-Lg}$. A_{492} , absorbance at 492 nm .

min after this addition, the rates of accumulation of serosal radiolabeled material were 5.46 ± 1.75 and $1.43 \pm 0.26 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$ for $\beta\text{-}^{14}\text{C}[\text{Lg}]$ and $^{3}\text{H}[\text{PEG}]$, respectively, i.e., significantly higher for $\beta\text{-}^{14}\text{C}[\text{Lg}]$ than for

$^{3}\text{H}[\text{PEG}]$ ($P < 0.01$).

Trans epithelial passage of antigenic intact $\beta\text{-Lg}$. After adding $\beta\text{-Lg}$ to the mucosal reservoir of the Ussing chamber, the antigenic response that emerged on the serosal side of the tissue was measured by ELISA (Fig. 3). This response became significantly different after 70 min from that of a control chamber without mucosal protein and then increased with time at a rate of $0.43 \pm 0.08 \mu\text{g} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$. In addition, 77% of the total serosal response measured after 140 min of incubation were recovered in the TCA-insoluble fraction.

To demonstrate that some intact $\beta\text{-Lg}$ crossed the epithelium, the TCA-insoluble fraction of the serosal reservoir was analyzed by HPLC coupled with ELISA detection after 140 min of incubation of $\beta\text{-Lg}$ on the mucosal side of the ileum (Fig. 4). On the chromatogram, an antigenic response was detected precisely at the position of the intact $\beta\text{-Lg}$, whereas the corresponding peak did not appear in the serosal reservoir of a control chamber without mucosal protein. This peak was collected and the solvent evaporated. The TCA-insoluble serosal fraction and this peak were analyzed by electrophoresis (Fig. 5). In both samples, a band was detected on the gel at exactly the position occupied by $\beta\text{-Lg}$, whereas the corresponding band did not appear in the control chamber without mucosal protein.

Effect of inhibitors on the transport of $\beta\text{-Lg}$ and PEG. The effect of various inhibitors on the passage of $\beta\text{-Lg}$ was tested to get some insight into the mechanism of its transport (Table 2).

The passage of both radiolabeled material and antigenic $\beta\text{-Lg}$ was reduced by 50% in the presence of 0.05 M colchicine, which is known to inhibit transcellular transport. In the same conditions the passage of $^{3}\text{H}[\text{PEG}]$ was reduced by 38%, but no effect was observed on the conductance of the tissue.

The metabolic inhibitors 50 mM 2-deoxyglucose and 1 mM azide added simultaneously reduced by 20% the passage of radiolabeled material and completely suppressed the transport of antigenic $\beta\text{-Lg}$. These inhibitors did not significantly modify the transport of $^{3}\text{H}[\text{PEG}]$ but significantly increased the conductance of the tissue.

In the presence of 20 mM ammonium chloride, which inhibits lysosomal proteolytic activity, the passage of radiolabeled $\beta\text{-Lg}$ remained unaffected, whereas the transport of antigenic $\beta\text{-Lg}$ was significantly increased. A significant increase in the passage of $^{3}\text{H}[\text{PEG}]$ and in the conductance of the tissue was also observed.

DISCUSSION

The present study confirms the idea that proteins are absorbed through the epithelium of the intestine and also demonstrates that the food protein $\beta\text{-Lg}$ is transported partly in an intact antigenic form, probably by a transcellular mechanism. This conclusion was supported by the following observations: 1) $\beta\text{-Lg}$ was found both intact and degraded in the serosal side of the ileum; 2) metabolic inhibitors reduced both total and intact transport of $\beta\text{-Lg}$, indicating that this transport requires energy; and 3) the transport of $\beta\text{-Lg}$ was modulated by modulating cellular metabolism.

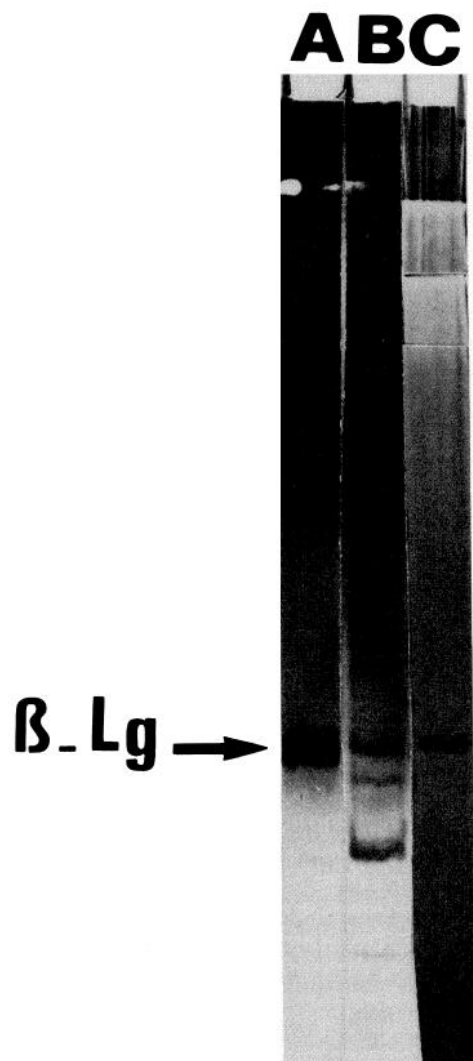


FIG. 5. SDS-PAGE analysis of β -Lg in serosal reservoir of an Ussing chamber 140 min after addition of 1 mg/ml β -Lg to mucosal reservoir. A: standard β -Lg. B: TCA-insoluble fraction of the serosal reservoir. C: β -Lg antigenic fraction prepared by RP-HPLC of the TCA-insoluble fraction of the serosal reservoir (see Fig. 4). Arrow represents position of elution of standard β -Lg.

Previous reports have established that proteins can be absorbed by the intestine (7, 11, 27, 29) and that the limiting step in the absorption of macromolecules is probably the initial endocytotic uptake at the luminal membrane of the enterocyte (7, 27). Furthermore, it is also assumed that tight junctions completely prevent the diffusion of macromolecules through the intercellular spaces across the epithelial cell layer (10). In the present study, the total uptake of macromolecules was measured by the equivalent transfer of radiolabeled material. This total uptake was significantly higher for β -Lg than for PEG, an extracellular marker that is not believed to enter the cells (19). The transport of intact β -Lg was probably not due to passage through a damaged edge because it was modulated by pharmacological drugs and because it exhibited different kinetics from those of PEG. The role of damaged edge was already found insignificant in the transport of HRP (11) or polypeptides (26) in previous experiments using Ussing chambers of different areas. The degradation of the major part of β -Lg during the transport and the inhibition of intact β -Lg absorption by metabolic inhibitors also suggested that its transport was transcellular. In addition, the modifications in the transport of PEG by the different inhibitors were highly correlated ($r = 0.98$) to their effect on the conductance of the tissue, whereas this correlation was not found for β -Lg ($r = 0.66$). Though it appeared difficult to establish whether the passage of PEG is entirely paracellular, these results further strengthen the hypothesis of a transcellular route for β -Lg and a paracellular route for PEG. Although the mechanism of β -Lg uptake remains to be clearly established, these observations strongly imply that in our experiments the absorption of β -Lg was transcellular and that this protein was efficiently taken up by the intestinal mucosa.

It was previously assumed that after internalization, the endocytotic vesicles are probably transferred to the lysosomal system, where their proteins are degraded (24, 27) but that a fraction of protein may escape this degradation and is transported intact (11, 29). In the present study, the antigenic intact β -Lg transported across the intestinal epithelium was measured by ELISA. Electrophoresis and HPLC showed that most of the antigenic

TABLE 2. Effect of drugs on the transepithelial passage of β - ^{14}C Lg and ^{3}H PEG across isolated rabbit ileum

	Electrical Parameters			Serosal Accumulation Rate, $\mu\text{g} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$		
	I_{sc} , $\mu\text{A}/\text{cm}^2$	PD, mV	G , mS/cm^2	β -Lg equivalent		^{3}H PEG equivalent
				Labeled material	Antigenic protein	
Ringer	42.50 $\pm$ 4.60	-2.30 $\pm$ 0.20	18.50 $\pm$ 1.90	6.21 $\pm$ 0.95	0.37 $\pm$ 0.14	1.11 $\pm$ 0.17
Colchicine (0.05 mM)	48.60 $\pm$ 2.50	-3.10 $\pm$ 0.40	15.70 $\pm$ 1.70	3.07 $\pm$ 0.99	0.11 $\pm$ 0.06	0.69 $\pm$ 0.10
P	NS	NS	NS	0.05	0.05	0.05
2-Deoxyglucose (50 mM) + NaN ₃ (1 mM)	57.90 $\pm$ 2.20	-2.02 $\pm$ 0.40	28.20 $\pm$ 2.60	4.90 $\pm$ 0.75	0.01 $\pm$ 0.01	1.55 $\pm$ 0.19
P	0.01	NS	0.05	0.05	0.01	NS
Ammonia (20 mM)	50.30 $\pm$ 6.3	-1.14 $\pm$ 0.20	38.10 $\pm$ 3.80	6.84 $\pm$ 1.60	1.15 $\pm$ 0.22	2.06 $\pm$ 0.32
P	NS	0.05	0.05	NS	0.05	0.05

Values are means $\pm$ SE for tissues from 6 rabbits. β - ^{14}C Lg (1 mg/ml) or ^{3}H PEG (1 mg/ml) were added to mucosal side of the ileum, which was either untreated (Ringer) or pretreated by an inhibitor. The radiolabeled material equivalent and the antigenic protein equivalent that appeared in the serosal reservoir were measured during the period from 70 to 140 min after such addition. I_{sc} , PD, and G values were also measured between 70 and 140 min after such addition.

activity that crossed the epithelium was linked to intact β -Lg. Comparison of total β -Lg uptake with intact transport indicated that 91–94% of the amount transported was degraded during its transepithelial passage and 6–9% was transported intact. An initial common mechanism of uptake is strongly suggested by the fact that colchicine reduced the transport of both degraded and intact β -Lg to the same extent. A subsequent lysosomal degradation of an important part of the absorbed protein is also confirmed by the reduction of this degradation by ammonia without alteration in the total uptake of β -Lg. The modification in the passage of PEG in the presence of colchicine and ammonia suggested that these compounds could also affect the paracellular route. These effects were however different for β -Lg and PEG. Ammonia increased the total uptake of PEG but not of β -Lg. An effect of colchicine at the tight junctions does not explain the reduction in the transport of degraded β -Lg.

The present results confirmed the existence of intact protein transport, as previously described for horseradish peroxidase (11). The amount of β -Lg transported intact here appeared larger than that previously observed for HRP (11). One possible reason is that the resistance of β -Lg and HRP to hydrolysis by lysosomal enzymes is different. Another is that protein molecules differentially escape the lysosomal system. As observed earlier, the intestinal mucosal breakdown process of different proteins generated fragments with different sizes (11, 24). Lastly, it is also possible that enzyme inhibitors are released during the intracellular hydrolysis of some proteins. A substantial difference had previously been observed between β -Lg and BSA inasmuch as more β -Lg was bound and taken up by rat jejunum in vitro (24).

One of the main points of interest of this study is the alimentary origin of β -Lg. This protein was previously demonstrated to be highly resistant to digestion by proteolytic enzymes of the gastrointestinal tract and was recovered intact at various levels of the alimentary canal, whereas other milk proteins, including caseins or α -lactalbumin, were rapidly degraded in the intestinal lumen (14, 16, 18). The present study demonstrated that some β -Lg was efficiently absorbed across rabbit ileum in intact antigenic form. This implies that it is able to stimulate the immunocompetent cells in the lamina propria from the luminal side of the intestine. This conclusion agrees with reports indicating anaphylactic sensitization to β -Lg and the secretion of β -Lg antibodies in the serum and intestinal mucosa of milk-drinking animals or humans (2, 3, 9, 20, 23, 25). However, it is known that there are important differences in the immunological response of different animals to milk proteins, and results obtained on rabbit ileum may not be necessarily directly relevant to other animals or humans. In addition, the protective role of the secreted mucosal surface immunoglobulin A of the intestine must also be evaluated as a barrier against macromolecular movements across the intestinal mucosa in vivo (4).

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