

## Chloroplast thylakoids reduce glucose uptake and decrease intestinal macromolecular permeability

Caroline Montelius<sup>1</sup>†, Karolina Gustafsson<sup>1</sup>†, Björn Weström<sup>2</sup>, Per-Åke Albertsson<sup>3</sup>, Sinan Cem Emek<sup>3</sup>, Marilyn Rayner<sup>4</sup> and Charlotte Erlanson-Albertsson<sup>1</sup>\*

<sup>1</sup>Department of Experimental Medical Science, Appetite Control Unit, BMC B11, Lund University, Lund, Sweden

<sup>2</sup>Department of Biology, Lund University, Lund, Sweden

<sup>3</sup>Department of Biochemistry and Structural Biology, Lund University, Lund, Sweden

<sup>4</sup>Department of Food Technology, Engineering, and Nutrition, Lund University, Lund, Sweden

(Received 2 September 2010 – Revised 20 January 2011 – Accepted 10 February 2011 – First published online 11 May 2011)

### Abstract

Thylakoid membranes, derived from chloroplasts, have previously been shown to retard fat digestion and lower blood glucose levels after oral intake. The purpose of the present study was to investigate the effect of thylakoid membranes on the passage of methyl-glucose, dextran and ovalbumin over rat intestine *in vitro* using Ussing chambers. The results show that thylakoids retard the passage of each of the test molecules in a dose-dependent way. The thylakoids appear to be attached on the mucosal surface and a mechanism is suggested that the thylakoids delay the passage of the test molecules by sterical hindrance. The present results indicate that thylakoid membranes may be useful both to control intestinal absorption of glucose and to enhance the barrier function of the intestine.

**Key words:** Membranes: Hyperglycaemia: Barrier function: Fatty acids

Hyperglycaemia is a major symptom in non-insulin-dependent diabetes, the most prevalent disease affecting individuals with a Westernised lifestyle<sup>(1,2)</sup>. Management of hyperglycaemia relies mainly on the reduction of dietary carbohydrate intake, the use of low-glycaemic instead of high-glycaemic carbohydrates and the use of hypoglycaemic agents that stimulate the uptake of glucose from the blood into peripheral cells. After digestion of dietary carbohydrates, glucose is taken up through the apical brush-border membrane of the epithelial intestinal cells, mainly by the sodium-glucose-linked transporter-1, to the enterocyte<sup>(3)</sup>. Control of the intestinal absorption of glucose would help to regulate levels of glucose and is the subject of various strategies to improve glucose homeostasis.

In previous work we have demonstrated that thylakoids, i.e. chloroplast membranes from green leaves, retard fat digestion both *in vitro* and *in vivo*<sup>(4)</sup>. This retardation in turn stimulates the release of satiety hormones, such as cholecystokinin<sup>(4,5)</sup> and leptin<sup>(6)</sup>, while reducing the hunger hormones ghrelin and insulin<sup>(6)</sup>. The long-term effect of thylakoid administration was thus a reduced food intake and body weight, as demonstrated both in mice<sup>(5)</sup> and in rat models<sup>(4)</sup>. To our surprise, a reduction in blood glucose levels after oral administration of thylakoids was also observed<sup>(5)</sup>.

The uptake of glucose into the intestinal cell is influenced by several factors. Most importantly, glucose itself regulates its uptake by stimulating the expression of the GLUT<sup>(7)</sup>. Thus carbohydrate-rich food stimulates an increased uptake of glucose in the intestine, while carbohydrate-depleted food, such as high-fat diets, reduce the uptake of dietary glucose. In addition, several gut hormones influence the glucose uptake in the intestine. The hunger hormone ghrelin has been shown to stimulate the uptake of glucose, while cholecystokinin<sup>(8)</sup>, leptin<sup>(9)</sup> and resistin<sup>(10)</sup> inhibit the uptake of glucose. Since thylakoids stimulate the release of cholecystokinin and leptin<sup>(6)</sup>, it may well be that the observed reduction in blood glucose levels were an effect of these hormones. Another explanation could be that thylakoid membranes have a direct effect on glucose uptake, by interacting with the intestinal absorptive surface.

In order to address the question why blood glucose levels after the intake of thylakoid-enriched food was reduced, we set up an experiment where the methyl-glucose uptake was studied *in vitro*, both in the absence and the presence of thylakoids, using intestinal segments from rats mounted in Ussing diffusion chambers<sup>(11)</sup>. This method is used both in physiological and pharmacological contexts, where an

**Abbreviations:** AUC, area under the curve; chl/ml, chlorophyll/ml; FITC-dextran, fluorescein isothiocyanate-labelled dextran.

\*Corresponding author: Dr Charlotte Erlanson-Albertsson, fax +46 46 222 40 22, email charlotte.erlanson-albertsson@med.lu.se

†Contributed equally to the scientific research and report.

intestinal tissue sheet is mounted between two buffer-containing chambers, and the passage of molecules through the tissue from one chamber to the other is measured<sup>(12)</sup>. In addition to methyl-glucose, the passage of the macromolecules fluorescein isothiocyanate-labelled dextran (FITC-dextran) and ovalbumin was examined.

We observed a reduced uptake of methyl-glucose through the intestinal wall in the presence of thylakoid membranes *in vitro*. The reduced uptake appears to be a general phenomenon since also the passage of FITC-dextran and ovalbumin was decreased in the presence of thylakoids.

## Materials and experimental methods

### Materials

Fresh baby spinach leaves (*Spinacia oleracea*) were donated by SABA Fresh (Helsingborg, Sweden). Sprague–Dawley rats were obtained from Taconic M & B A7S (Ry, Denmark) and the Rat and Mouse Autoclavable Diet from B & K Universal AB (Sollentuna, Sweden). Oleic acid (18:1*n*-9) was obtained from Merck Chemicals (Darmstadt, Germany). Sodium dodecylsulfate (NaTDC), FITC-dextran, ovalbumin-antiserum (A6075), purified ovalbumin (A5503), trypsin type VI from bovine pancreas (lyophilised powder) and phenylmethylsulfonylfluoride were obtained from Sigma-Aldrich (St Louis, MO, USA). Methyl- $\text{D}$  glucose ( $^3\text{H}$ ]-*O*-methyl) was obtained from NEN Life Science Products (Boston, MA, USA), and methyl- $\text{D}$  glucose ( $^{14}\text{C}$ ]-*O*-methyl) from PerkinElmer (Boston, MA, USA). Isoflouran was obtained from Shering-Plough a/s (Ballerup, Denmark) and Ready Safe scintillation cocktail was obtained from Beckman (Fullerton, CA, USA).

### Preparation of thylakoids

Thylakoid membranes were prepared from fresh baby spinach leaves<sup>(13)</sup> by mixing the leaves with homogenising buffer (50 mM-phosphate buffer (pH 7.4)–5 mM-MgCl<sub>2</sub>–300 mM-sucrose) for 5 min until a homogeneous slurry was obtained. The slurry was filtered through four layers of 20  $\mu\text{m}$  monodur polyester mesh, and centrifuged (2000 g; 15 min; 4°C). The centrifugation step was repeated four times; the pellet was re-suspended in the homogenising buffer the first time, blast media (5 mM-MgCl<sub>2</sub>) for osmotic lysis the second time and washing media (10 mM-tricine–5 mM-MgCl<sub>2</sub>–300 mM-sucrose; pH 7.4) the final two times<sup>(13)</sup>. As a final step, the pellet was dispersed in a glass/Teflon potter Elvehjem homogeniser until a homogeneous slurry of thylakoid membranes was obtained.

The concentration of thylakoids was expressed as mg chlorophyll/ml (chl/ml) and determined by the method of Porra *et al.*<sup>(14)</sup> (using an Ultrospec 2000 spectrophotometer, Pharmacia Biotech, Uppsala, Sweden). As calculated from Flores *et al.*<sup>(15)</sup>, 1 mg chlorophyll corresponds to about 12 mg thylakoids.

Trypsin-treated thylakoid membranes were obtained by incubating thylakoids with 1 mg/ml trypsin for 2 h (37°C).

The activity of trypsin was then inhibited by 1 mM-phenylmethylsulfonylfluoride followed by a washing step<sup>(16)</sup>. By this treatment, trypsin targets specific sites and remove external proteins on the thylakoid membrane surface.

### Animals

The present study was performed on female rats of the Sprague–Dawley stock (Mole: SPRD Han), conventionally bred under a controlled environment (20  $\pm$  1°C, 50  $\pm$  10% relative humidity; 12 h light–12 h dark cycle). The rats were housed five per cage on chopped wood bedding in polycarbonate cages and with free access to tap water and a pelleted breeding chow. The study was approved by the Lund University Ethical Committee for Animal Experiments and conducted according to the European Community regulations concerning the protection of experimental animals.

### Ussing experiments and analyses

A total of eight female rats (220–280 g) were anaesthetised in the morning with isoflouran. Then the proximal and distal parts of the small intestine were collected, rinsed and immediately immersed in modified room temperature Krebs buffer (0.1 M-NaCl–3 mM-CaCl<sub>2</sub>–5.5 mM-KCl–14 mM-KH<sub>2</sub>PO<sub>4</sub>–29 mM-NaHCO<sub>3</sub>–5.7 mM-sodium-pyruvate–7 mM-sodium fumarate–5.7 mM-sodium glutamate–13.4 mM-glucose; pH 7.4) oxygenated with carbogen (O<sub>2</sub>–CO<sub>2</sub>, 95:5, v/v). The intestine was cut in 3 cm sections, opened along the mesentery and pinched to the nails of the pre-heated (37°C) Ussing half-cell<sup>(17)</sup> (Precision Instrument Design, Los Altos, CA, USA), modified in accordance with Grass & Sweetana<sup>(17)</sup>. The half-cell was connected to another half-cell, and thus the arrangement had two sides: one facing the mucosal side of the intestine and the other facing the serosal side, with an exposed intestinal area of 1.78 cm<sup>2</sup>. The Ussing chambers were each filled with 5 ml Krebs buffer, kept at 37°C and connected to a carbogen supply. All intestinal segments were mounted in the chambers within 30 min after the intestine was collected. The intestinal segments were considered viable for a minimum of 2 h after mounted in the chambers<sup>(18)</sup>.

At the start of the experiment, the buffer was exchanged to fresh buffer in the serosal half-cell, and test solution in the mucosal half-cell. The test solution (5 ml) consisted of modified Krebs buffer supplemented with oleic acid (18:1*n*-9; 5.07 mM), NaTDC (5.2 g/l) and the marker molecules methyl- $\text{D}$  glucose, [ $^3\text{H}$ ]-*O*-methyl (190 Da, 1.85  $\times$  10<sup>-2</sup> GBq, 3.1  $\mu\text{l/ml}$ ) or methyl- $\text{D}$  glucose, [ $^{14}\text{C}$ ]-*O*-methyl (190 Da, 1.85  $\times$  10<sup>-3</sup> GBq, 0.8  $\mu\text{l/ml}$ ), FITC-dextran (4000 Da, 1 g/l) and ovalbumin (45 000 Da, 25 g/l). Thylakoid membranes – both non-treated and treated with trypsin – were also added to the test solution to obtain final concentrations of 1.2, 2.9 and 5.8 mg chl/ml. Serosal samples of 1 ml were collected every 20 min during 120 min, with replacement of the sampled volume by fresh buffer.

The amount of radiolabelled methyl-glucose in 0.5 ml serosal samples was measured in a beta counter (Scint TriCarb Liquid Scintillation Analyzer 2100TR; PerkinElmer), after

mixing the serosal sample with 5 ml liquid scintillation cocktail. FITC-dextran was quantified by fluorescence spectrophotometry (CytoFlour™ 2300; Millipore Corp., Bedford, MA, USA) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, where FITC-dextran dissolved in modified Krebs buffer was used as standard. Quantification of ovalbumin was performed by electro-immunoassay<sup>(19)</sup> using a specific antiserum to ovalbumin (A6075) with purified ovalbumin (A-5503) as standard.

Following every experiment, a sample from the serosal half-cell was collected and analysed for chlorophyll content to investigate a possible thylakoid migration through the intestinal wall.

### *Binding of thylakoids to the intestinal wall*

Samples of intestine exposed to thylakoid membranes (2.9 mg chl/ml), in the same test solution as in the Ussing experiments, were collected after 120 min of incubation in the Ussing chambers and then washed with water to remove non-adsorbed thylakoids. The washed intestinal samples were mounted on a gel in a petri dish and covered with 3 ml 80% acetone and incubated on ice for 20 min on a shaker to extract the chlorophyll. The mucosa of the intestine was removed and collected in test-tubes, together with its surrounding acetone, and incubated for another 10 min on ice before being centrifuged (19 000 g; 5 min; 4°C). The chlorophyll in the supernatant fraction was determined.

### *Photographs*

Intestinal samples exposed to thylakoid membranes (2.9 mg chl/ml) during the 120 min of incubation in the Ussing chambers were photographed (Nikon Coolpix S3000; Kanagawa, Japan).

### *Electron microscopy*

Intestinal segments incubated for 120 min in the Ussing chambers in the absence of oleic acid and NaTDC but with thylakoid membranes (2.9 mg chl/ml) were fixed with 2.5% glutaraldehyde in 0.15 M-cacodylate buffer and embedded in Epon, and finally stained in 3% (v/v) uranyl acetate and lead citrate.

### *Interaction between marker molecules and thylakoids*

The test solution of modified Krebs buffer supplemented with thylakoids (5.8 mg chl/ml), oleic acid (18:1n-9; 5.07 mM), NaTDC (5.2 g/l) and the marker molecules methyl-D glucose (<sup>14</sup>C]3-O-methyl; 190 Da; 1.85 × 10<sup>-3</sup> GBq; 0.8 μl/ml), FITC-dextran (4000 Da; 1 g/l) and ovalbumin (45 000 Da; 25 g/l) was incubated in a shaker (70 rpm; 120 min; 37°C) (Innova™ 4310 Incubator Shaker; New Brunswick Scientific, Edison, NJ, USA). An identical test solution without thylakoid membranes was used as the control. The samples were centrifuged (16 100 g; 10 min; 4°C) and the supernatant fractions were gently transferred to test-tubes. The amount

of radiolabelled methyl-glucose in 50 μl of the supernatant fraction was measured in a beta counter (1414 Liquid Scintillation Counter; Wallac Guardian, PerkinElmer), after mixing the samples with 5 ml liquid scintillation cocktail. FITC-dextran in the supernatant fraction was quantified as described above. Quantification of ovalbumin in the supernatant fraction was analysed by measuring absorption at 280 nm using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

### *Calculations*

The uptake of methyl-glucose was determined as the percentage that had passed through the intestinal segment at indicated time points, by means of the formula:

$$\text{Uptake (\%)} = c_i/c_0 \times 100,$$

where  $c_i$  represents the concentration of the marker molecule present in the serosal half-cell (M) and  $c_0$  represents the initial concentration of the same marker molecule in the mucosal half-cell (M)<sup>(18)</sup>.

The apparent permeability coefficient ( $P_{app}$ ) was calculated for the macromolecules FITC-dextran and ovalbumin by means of the formula:

$$P_{app} \text{ (cm/s)} = (dc/dt) \times V \times 1/(c_0 \times A),$$

where  $dc/dt$  represents the change of serosal concentration in the 60 to 120 min period (M/s),  $V$  the volume in the half-cells (cm<sup>3</sup>),  $c_0$  the initial marker concentration in the mucosal half-cell (M), and  $A$  the area of exposed intestine in the Ussing chamber (cm<sup>2</sup>)<sup>(18)</sup>.

### *Statistics*

All data were normally distributed. The Student's  $t$  test (unpaired comparison) was used for the statistical analyses for the passage, permeability and affinity to thylakoids, for all marker molecules (at a constant time). All data are expressed as mean values with their standard errors and differences were considered significant when  $P < 0.05$  was reached.

The kinetics of glucose passage (uptake of methyl-glucose) over time (0 to 120 min) at the different levels of added thylakoids was evaluated by area under the curve (AUC) analysis. AUC was calculated for each experiment by numerical integration using the trapezoidal rule method. The effect of the thylakoid concentration (dose) on the level of passage over time AUC was evaluated for significance using a Student's  $t$  test to determine at what doses gave significantly different responses in glucose passage. In this way we can include the kinetic and dose-dependent effects.

## **Results**

### *Uptake of methyl-glucose*

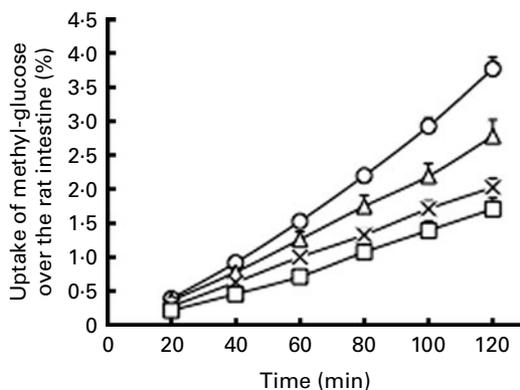
The passage of methyl-glucose through the rat intestinal segments increased during 120 min of incubation in Krebs buffer (without the addition of thylakoid membranes) in the

Ussing diffusion chambers. In the presence of thylakoids (1.2, 2.9 and 5.8 mg chl/ml) the uptake of methyl-glucose was significantly reduced in a dose-dependent way (Fig. 1). When considering the kinetic response by calculating the AUC, all concentrations of thylakoids had a significantly lower uptake of methyl-glucose ( $P < 0.005$ ) than the control. The differences among the concentrations were significantly different between 1.2 and 2.9 mg chl/ml ( $P < 0.05$ ), between 1.2 and 5.8 mg chl/ml ( $P < 0.001$ ) but not significantly different between 2.9 and 5.8 mg chl/ml, indicating perhaps a certain degree of saturation. Moreover, exposure to trypsin-treated thylakoids resulted in an even stronger reduction of the methyl-glucose uptake compared with the non-treated thylakoids, although only statistically significant at the concentration level of 5.8 mg chl/ml ( $P < 0.01$ ) (Fig. 2).

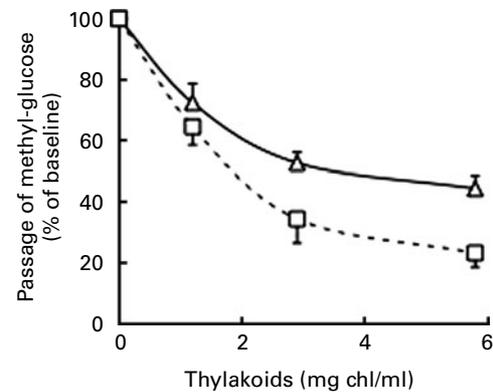
By calculating the apparent permeability coefficient ( $P_{app}$ ) it was shown that the transmucosal transport of methyl-glucose was significantly decreased in the presence of thylakoids (Fig. 3(a)). The presence of thylakoids at 1.2 mg chl/ml gave an initial strong reduction in permeability (63.0% of baseline;  $P < 0.001$ ). The permeability was further significantly reduced both in the presence of thylakoids at 2.9 mg chl/ml (43.5% of baseline;  $P < 0.001$ ) and at 5.8 mg chl/ml (41.5% of baseline;  $P < 0.001$ ) (Fig. 3(a)). Trypsin-treated thylakoids reduced the permeability even more (Fig. 3(a)), with a significantly reduced permeability of methyl-glucose in the presence of thylakoids at 5.8 mg chl/ml (24.8% of baseline;  $P < 0.01$ ), as compared with non-treated thylakoids of the same concentration.

### Macromolecular permeability

The macromolecular transmucosal passage over the rat intestinal segments increased in the absence of thylakoid membranes in the Ussing chambers during 120 min (data not shown). In the presence of thylakoid membranes, the



**Fig. 1.** Time-dependent uptake of [ $^3\text{H}$ ]methyl-glucose over the rat intestine in Ussing diffusion chambers (percentage of the amount added to the mucosal half-cell). The rat intestinal segments were incubated in the absence of thylakoid membranes, i.e. control (—○—;  $n$  8), and in the presence of three different concentrations of thylakoid membranes (—△—, 1.2 mg chlorophyll/ml (chl/ml); —×—, 2.9 mg chl/ml; —□—, 5.8 mg chl/ml;  $n$  2). Values are means, with their standard errors represented by vertical bars. At 120 min, the presence of thylakoid membranes, at all concentrations, resulted in a significant reduction in uptake, as compared with control (1.2 mg chl/ml v. control,  $P < 0.01$ ; 2.9 mg chl/ml v. control,  $P < 0.001$ ; 5.8 mg chl/ml v. control,  $P < 0.001$ ).



**Fig. 2.** Concentration-dependent mucosa to serosa passage of [ $^3\text{H}$ ]methyl-glucose/[ $^{14}\text{C}$ ]methyl-glucose over the rat intestine at 120 min in Ussing diffusion chambers (percentage of amount added to the mucosal half-cell) in the absence of thylakoid membranes (0 mg chlorophyll/ml (chl/ml);  $n$  8) and in the presence of three different concentrations of thylakoid membranes (1.2, 2.9 and 5.8 mg chl/ml;  $n$  2). The rat intestinal segments were incubated both with thylakoid membranes not treated with trypsin (—△—) and thylakoid membranes treated with trypsin (—□—). Values are means, with their standard errors represented by vertical bars. Incubation with thylakoid membranes at 2.9 and 5.8 mg chl/ml (both non-treated and treated with trypsin) resulted in a significant dose-dependent reduction of mucosa to serosa passage of methyl-glucose, as compared with control ( $P < 0.01$ ). Comparison of non-treated thylakoid membranes and trypsin-treated thylakoid membranes resulted in a significant reduction of passage of methyl-glucose only in the presence of thylakoid membranes at 5.8 mg chl/ml ( $P < 0.01$ ).

permeability for FITC-dextran was significantly reduced in a dose-dependent way (Fig. 3(b)). Incubation with thylakoids at 1.2 mg chl/ml resulted in a reduced permeability (64.6% of baseline;  $P < 0.01$ ) for FITC-dextran. In the presence of thylakoids at 2.9 mg chl/ml the permeability was reduced even further (37.8% of baseline;  $P < 0.001$ ) followed by a plateau (Fig. 3(b)). In the presence of thylakoids at 5.8 mg chl/ml no further reduction in permeability could be seen (35.0% of baseline;  $P < 0.001$ ) (Fig. 3(b)). Trypsin-treated thylakoids reduced the permeability of FITC-dextran to about the same extent as non-treated thylakoids, with an initial significant reduction, compared with non-treated thylakoids ( $P < 0.05$ ).

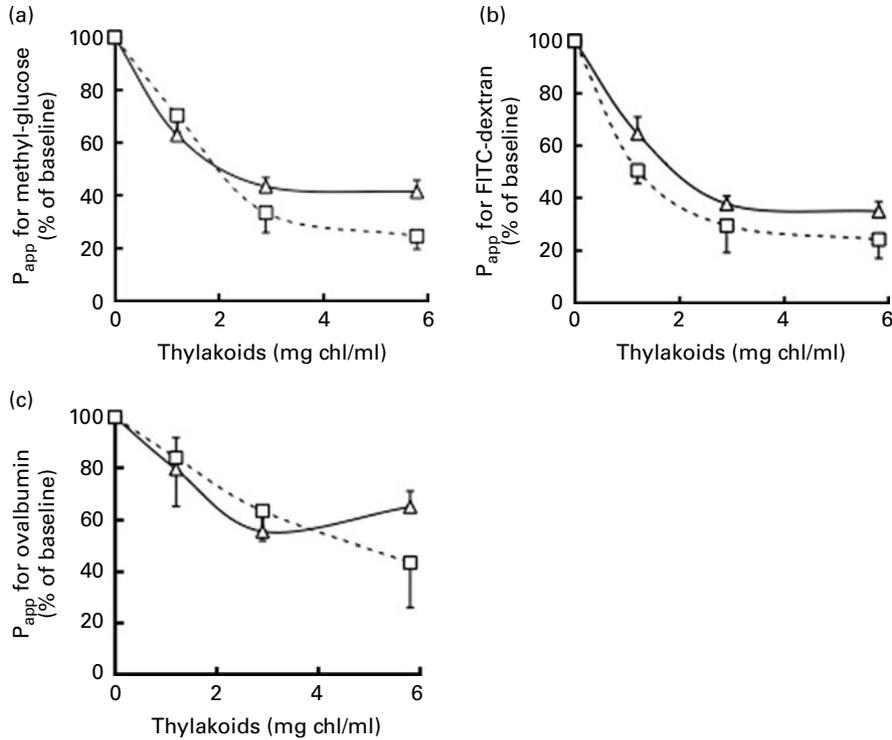
Also, the permeability for the protein marker, ovalbumin, was reduced in a dose-dependent way in the presence of thylakoids (Fig. 3(c)). The permeability was significantly reduced in the presence of thylakoids at both 2.9 and 5.8 mg chl/ml (55.4% of baseline,  $P < 0.01$ ; and 65.0% of baseline,  $P < 0.01$ ) (Fig. 3(c)). In the presence of trypsin-treated thylakoids the permeability was decreased in the same dose-dependent way as was observed for FITC-dextran (Fig. 3(c)).

### Trans-intestinal migration

Analyses of the chlorophyll content in the serosal half-cell after 120 min of incubation in the Ussing chambers showed that no thylakoid membranes had migrated over the intestinal wall.

### Binding of thylakoids to the mucosa

After termination of the incubations, all half-cells were dismantled and the exposed intestine was investigated. Intestinal samples incubated with thylakoids visually displayed a green



**Fig. 3.** Apparent permeability coefficient ( $P_{app}$ ) calculated from the transmural passage of methyl-glucose, fluorescein isothiocyanate-labelled dextran (FITC-dextran) and ovalbumin between 60 and 120 min, obtained in presence of different concentrations of thylakoid membranes (0, 1.2, 2.9 and 5.8 mg chlorophyll/ml (chl/ml)). The rat intestinal segments were incubated with thylakoid membranes not treated with trypsin ( $-\Delta-$ ), or treated with trypsin ( $-\square-$ ). The value 100% represents  $P_{app}$  without any thylakoid membranes present. Values are means, with their standard errors represented by vertical bars. (a)  $P_{app}$  (% of baseline) for methyl-glucose (190 Da). Incubation with both non-treated thylakoids and thylakoids treated with trypsin resulted in a significant dose-dependent reduction in permeability ( $P < 0.01$ ) as compared with control (0 mg chl/ml), with the exception of trypsin-treated thylakoids at 1.2 mg chl/ml, which were not significantly reduced compared with control. Comparison of non-treated thylakoid membranes and trypsin-treated thylakoid membranes resulted in a significant reduction in permeability for methyl-glucose only in the presence of thylakoids at 5.8 mg chl/ml ( $P < 0.01$ ). (b)  $P_{app}$  (% of baseline) for the carbohydrate marker FITC-dextran (4000 Da). Incubation with both non-treated thylakoids and trypsin-treated thylakoids resulted in a significant dose-dependent reduction in permeability ( $P < 0.05$ ) as compared with control (0 mg chl/ml). Comparison of non-treated thylakoid membranes and trypsin-treated thylakoid membranes resulted in a significant reduction in permeability for FITC-dextran only in the presence of thylakoids at 1.2 mg chl/ml ( $P < 0.05$ ). (c)  $P_{app}$  (% of baseline) for the protein marker ovalbumin (45 000 Da). Incubation with non-treated thylakoids resulted in a significant reduction in permeability for all concentrations except the lowest ( $P < 0.01$ ) compared with control (0 mg chl/ml). Incubation with trypsin-treated thylakoids did not result in any significant reduction in permeability. Comparison of the effects of non-treated thylakoid membranes and trypsin-treated thylakoids on the permeability for ovalbumin resulted in no significant difference.

layer completely covering the mucosal side, a layer that could not be washed away (Fig. 4). The serosal side of the intestine, i.e. the side not exposed to thylakoids, did not appear affected in any way (Fig. 4). Electron micrographs of the intestinal segments exposed to thylakoid membranes without oleic acid and NaTDC showed stacked thylakoid membrane structures attached to the mucosa covering the microvilli (Fig. 5).

Extraction of the chlorophyll from the bound thylakoids showed that 0.22% of the added thylakoid membranes (2.9 mg chl/ml) were bound to the mucosal side after 120 min of incubation in the Ussing chambers.

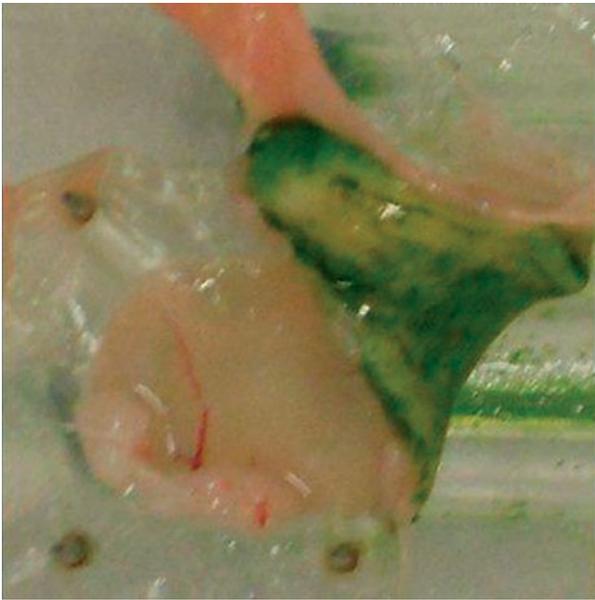
#### Interactions between marker molecules and thylakoids

All three marker molecules were found to bind at various degrees to the thylakoid membranes (Fig. 6(a)–(c)). FITC-dextran had the highest affinity for the thylakoid membranes; 22% ( $P < 0.001$ ) was found to bind to the thylakoid membranes followed by methyl-glucose and ovalbumin (17% ( $P < 0.001$ ) and 12% ( $P < 0.001$ ), respectively).

#### Discussion

In the present study we showed that thylakoid membranes substantially decrease the passage of methyl-glucose as well as decrease the permeability for the macromolecules FITC-dextran and ovalbumin over the rat intestine *in vitro* by using Ussing chambers. The thylakoids were also found to be strongly bound to the mucosal side of the intestine.

We propose that the mechanism behind this decrease of uptake by thylakoids is their binding to the mucosa surface (Figs. 4 and 5), thereby hindering the transport of marker molecules to the mucosa. This is based on the properties of the thylakoid membrane: in the chloroplast the thylakoid membrane forms a three-dimensional network of double membranes enclosing between them the lumen and separating it from the surrounding stroma of the chloroplast<sup>(20–22)</sup>. Thylakoid membranes contain a large number of intrinsic, i.e. membrane spanning, proteins. Their structure has been determined with high resolution<sup>(23)</sup>. The proteins, together with their tightly bound pigments, carotenoids and



**Fig. 4.** Photograph of both sides of rat intestine after 120 min incubation with thylakoids (2.9 mg chlorophyll/ml) in an Ussing chamber. The green surface is the mucosal side exposed to thylakoid membranes during the incubation. The pink surface is the serosal side, not exposed to thylakoids.

chlorophyll<sup>(24,25)</sup>, account for about 70% of the thylakoid mass. The membrane lipids, galactolipids, phospholipids and sulfolipids, account for the majority of the remaining 30% of the thylakoid mass<sup>(26)</sup>. The thylakoid membranes are negatively charged at neutral pH, with an isoelectric point of 4.7<sup>(27)</sup>. Thylakoids expose both hydrophilic and hydrophobic surface groups and have also been found to adsorb onto the surface of oil droplets and form stable oil-in-water emulsions<sup>(4,28)</sup>. Due to the large size of the thylakoid surface the number of exposed groups per thylakoid particle is large so that, even if the individual group interactions are weak, the sum of them will be large. Hence one would expect the surface properties of the thylakoid membrane to allow strong interaction with the intestinal surface.

Upon isolation the membrane network partly unfolds but still remains in a double membrane form. However, when bile salts (NaTDC) and oleic acid are added to the test solution, as done here, they are incorporated into the thylakoid membrane causing swelling into a single membrane form and unfolding, thereby increasing the exposed surface and net negative charge<sup>(16,29)</sup>. The swelling and unfolding may allow the thylakoids to cover a larger surface of the intestine. Native thylakoids have an area of about 2 m<sup>2</sup>/mg chlorophyll<sup>(4,15)</sup>, and the exposed area may increase significantly upon the unfolding caused by bile salts and fatty acids. It thus appears that this thylakoid network is responsible for the decreased passage through the intestinal wall.

Important to note is that the fraction of thylakoid membranes attached to the mucous layer is about 0.22% of the amount of thylakoid membranes added (2.9 mg chl/ml), which means that the overall nutritional status of the intestine is not negatively affected by thylakoid membranes, even after

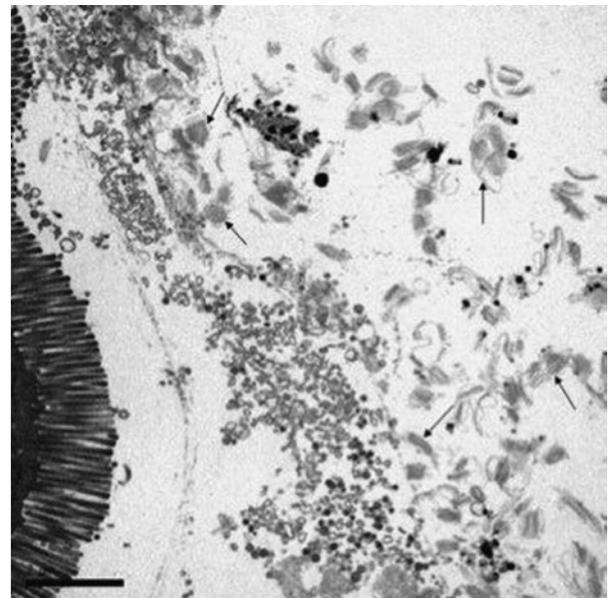
supplementation of a relatively high concentration of thylakoid membranes.

In the *in vitro* model used, thylakoid membranes were exposed directly to the small intestine. However, if supplemented *in vivo*, thylakoid membranes are exposed to various digestion enzymes and acids secreted from the gastrointestinal tract. In a recent study it was shown that thylakoids, when treated with gastric and pancreatic enzymes at 37°C, kept their membrane vesicular form for at least 2 h<sup>(16)</sup>. Trypsin treatment together with NaTDC caused swelling of the thylakoids, leading to a larger exposed surface<sup>(16)</sup>, and this may be the explanation for the increased capacity of trypsin-treated thylakoids for decreasing the permeability (Fig. 3).

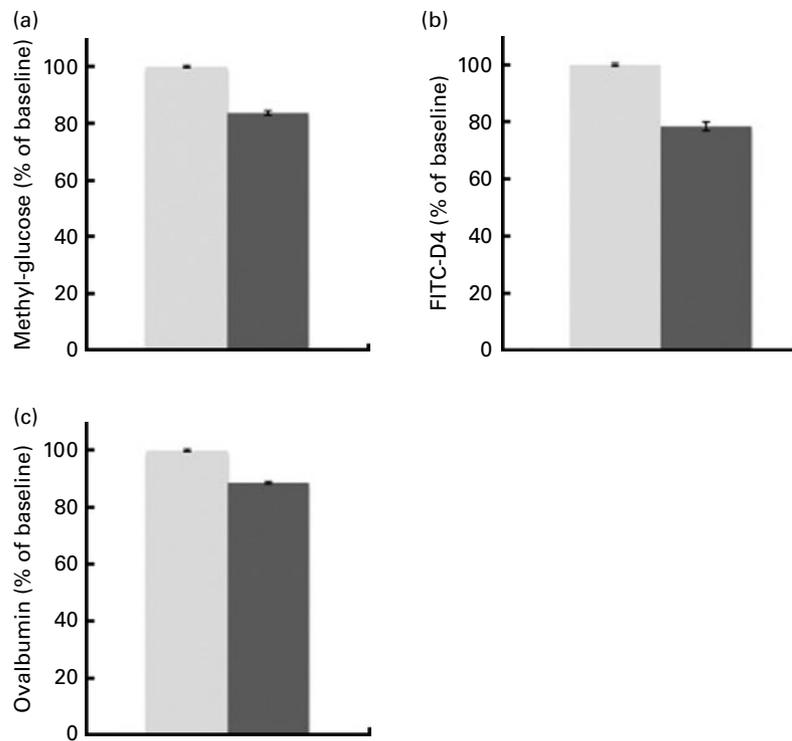
No thylakoid membranes had migrated through the intestinal wall during the incubation in Ussing chambers, an expected result since isolated thylakoid membranes have a size in the range of 1–5 μm.

Thylakoid membranes have an affinity ( $P < 0.001$ ) for all three marker molecules (Fig. 6). Methyl-glucose and FITC-dextran expose small hydrophobic groups which may bind to the thylakoids. Ovalbumin, being a protein, has charged hydrophilic and hydrophobic groups and might also bind to thylakoids. These results may also be explained by the trapping of some of the marker molecules in the thylakoid membrane network in the pellet after centrifugation. However, the main point is that the main fraction of the marker molecules was still present in the test solution. It is therefore concluded that the main reason for the decreased passage through the intestinal wall, as examined in the Ussing chambers, is attachment of the thylakoid membranes to the mucosa.

Uptake of glucose occurs through the brush-border membrane of the intestinal epithelial cells, both by active transport and diffusion, where the active uptake is driven by the Na<sup>+</sup>-K<sup>+</sup>-ATPase pump, mainly via the sodium-glucose-linked



**Fig. 5.** Electron micrograph of a mucosal segment of rat intestine, incubated with thylakoids (2.9 mg chlorophyll/ml) and Krebs buffer in an Ussing chamber for 120 min. ↑, Thylakoids.



**Fig. 6.** Affinity between the marker molecules (methyl-glucose, fluorescein isothiocyanate-labelled dextran (FITC-dextran) and ovalbumin) and thylakoid membranes (5.8 mg chlorophyll/ml; ■). The marker molecules and thylakoids ( $n$  9) were incubated for 120 min on a shaker (37°C) and the amount of marker molecules in the supernatant fraction was analysed. Values for each marker molecule are given as percentage of control (□), where the control is 100%. Values are means, with their standard errors represented by vertical bars. (a) Methyl-glucose (190 Da) (% of control). Incubation with thylakoids resulted in a significant binding ( $P < 0.001$ ) between methyl-glucose and thylakoids. (b) FITC-dextran (4000 Da) (% of control). Incubation with thylakoids resulted in a significant binding ( $P < 0.001$ ) between FITC-dextran and thylakoids. (c) Ovalbumin (45 000 Da) (% of control). Incubation with thylakoids resulted in a significant binding ( $P < 0.001$ ) between ovalbumin and thylakoids.

transporter-1. The present study showed that the uptake of methyl-glucose was significantly reduced in the presence of thylakoid membranes at 5.8 mg chl/ml *in vitro* (Fig. 1). Earlier studies<sup>(18,30)</sup>, using the same model system, have shown a similar glucose uptake, approximately 4% at time point 120 min, as presented for the control samples in the present study. This excludes that the transit of the examined marker molecules occur through leakage of the intestinal wall, i.e. the physiological integrity of the intestinal segments mounted in the Ussing chambers is maintained. This has also been demonstrated by several electron micrographs of the mounted intestine (results not shown). A controlled glucose absorption could help to improve levels of blood glucose, a desirable effect for the treatment of hyperglycaemia. Thylakoid membranes will therefore be further investigated both *in vitro* and *in vivo* to establish such an effect.

Thylakoid membranes also decreased the permeability of the macromolecular molecules FITC-dextran and ovalbumin *in vitro*. In Fig. 3(C), a sudden increase in the permeability of ovalbumin was seen in the presence of the highest concentration of thylakoid membranes. The increase is though not significantly ( $P > 0.05$ ) different from the permeability in the presence of the second highest concentration of thylakoid membranes. Since the intestinal mucosa has a fundamental role in sustaining a barrier against translocations of pathogenic

bacteria, intact proteins and other noxious substances, a decreased permeability of the intestinal wall is desirable, especially in conditions with impaired barrier function<sup>(31,32)</sup>. A reinforced intestinal barrier may result in the avoidance of severe implications such as allergies, inflammatory bowel disease, irritable bowel syndrome and Crohn's disease. The present results indicate that thylakoid membranes notably improve the barrier function of the intestine *in vitro* by decreasing the macromolecular permeability, and thereby may constitute a protection against harmful translocations, a phenomenon that will be further investigated.

If the attachment of thylakoids to the intestinal mucosa also takes place *in vivo* it would also affect the uptake of other food constituents. However, since the thylakoids are food themselves they will eventually be digested and their barrier function will only work temporarily in slowing down the uptake of food constituents.

Carotenoids, also present in thylakoid membranes, and retinol have previously been proposed to be important factors to enhance the intestinal barrier following stressful conditions, as demonstrated both *in vivo* in vitamin A-deficient children<sup>(33)</sup>, and *in vitro* by the use of Caco 2-cell lines<sup>(34)</sup>. The underlying mechanism to these observations is though not known. In the present *in vitro* study, the carotenoids are strongly bound to the thylakoids and it is not likely that they play a role in

retarding the uptake of the marker molecules. However, they may have a barrier function *in vivo* after digestion of the thylakoids and release of the carotenoids.

In summary, we have demonstrated that chloroplast thylakoid membranes decrease the uptake of methyl-glucose through the intestinal wall *in vitro*. In addition, macromolecular permeability was decreased in our model in the presence of thylakoid membranes. This indicates that thylakoid membranes might be useful both to control intestinal absorption of glucose, and to enhance the barrier function of the intestine, thus preventing leakage, especially during stressful intestinal conditions such as various bowel and intestinal diseases.

### Acknowledgements

The authors are thankful for the financial support from VINNOVA (Swedish government agency that administers state funding for research and development), FORMAS (Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning), The Carl Trygger Foundation, The Royal Physiographic Society of Lund, The Swedish Medical Research Council, and for the donation of spinach from SABA Fresh. We thank Rita Wallén (Department of Biology, Lund University) for taking the electron micrographs.

C. M. and K. G. contributed equally to the planning of the study, performed all experiments and analyses, and wrote the paper. B. W. planned the study, assisted in performing the experiments and in writing the paper. P.-Å. A. assisted in part of the analyses and in writing the paper. S. C. E. assisted in the preparation of thylakoid membranes. M. R. assisted in the statistical analyses. C. E.-A. planned the study and assisted in writing the paper.

There are no conflicts of interest.

### References

- Ujcic-Voortman JK, Schram MT, Jacobs-van der Bruggen MA, *et al.* (2009) Diabetes prevalence and risk factors among ethnic minorities. *Eur J Public Health* **19**, 511–515.
- Taufa T & Benjamin AL (2001) Diabetes: the by-product of westernization in Papua New Guinea. *PNG Med J* **44**, 108–110.
- Dyer J, Wood IS, Palejwala A, *et al.* (2002) Expression of monosaccharide transporters in intestine of diabetic humans. *Am J Physiol Gastrointest Liver Physiol* **282**, G241–G248.
- Albertsson PA, Kohnke R, Emek SC, *et al.* (2007) Chloroplast membranes retard fat digestion and induce satiety: effect of biological membranes on pancreatic lipase/co-lipase. *Biochem J* **401**, 727–733.
- Kohnke R, Lindqvist A, Goransson N, *et al.* (2009) Thylakoids suppress appetite by increasing cholecystokinin resulting in lower food intake and body weight in high-fat fed mice. *Phytother Res* **23**, 1778–1783.
- Kohnke R, Lindbo A, Larsson T, *et al.* (2009) Thylakoids promote release of the satiety hormone cholecystokinin while reducing insulin in healthy humans. *Scand J Gastroenterol* **44**, 712–719.
- Helliwell PA, Richardson M, Affleck J, *et al.* (2000) Regulation of GLUT5, GLUT2 and intestinal brush-border fructose absorption by the extracellular signal-regulated kinase, p38 mitogen-activated kinase and phosphatidylinositol 3-kinase intracellular signalling pathways: implications for adaptation to diabetes. *Biochem J* **350**, 163–169.
- Hirsh AJ & Cheeseman CI (1998) Cholecystokinin decreases intestinal hexose absorption by a parallel reduction in SGLT1 abundance in the brush-border membrane. *J Biol Chem* **273**, 14545–14549.
- Ducroc R, Guilmeau S, Akasbi K, *et al.* (2005) Luminal leptin induces rapid inhibition of active intestinal absorption of glucose mediated by sodium-glucose cotransporter 1. *Diabetes* **54**, 348–354.
- Krimi RB, Letteron P, Chedid P, *et al.* (2009) Resistin-like molecule- $\beta$  inhibits SGLT-1 activity and enhances GLUT2-dependent jejunal glucose transport. *Diabetes* **58**, 2032–2038.
- Ussing HH & Zerahn K (1951) Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol Scand* **23**, 110–127.
- Mangell P, Nejdfor P, Wang M, *et al.* (2002) *Lactobacillus plantarum* 299v inhibits *Escherichia coli*-induced intestinal permeability. *Digest Dis Sci* **47**, 511–516.
- Emek SC, Szilagyi A, Akerlund HE, *et al.* (2010) A large scale method for preparation of plant thylakoids for use in body weight regulation. *Prep Biochem Biotech* **40**, 13–27.
- Porra RJ, Thompson WA & Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* **975**, 384–394.
- Flores S, Graan T & Ort DR (1983) Measurement of the permeability of the chloroplast thylakoid membrane to amine buffers. *Photobiochem Photobiophys* **6**, 293–304.
- Emek SC, Akerlund H-E, Clausén M, *et al.* (2010) Pigments protect the light harvesting proteins of chloroplast thylakoid membranes against digestion by gastrointestinal proteases. *Food Hydrocolloid* (epublication ahead of print version 30 December 2010).
- Grass GM & Sweetana SA (1988) Measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharm Res* **5**, 372–376.
- Pantzar N, Westrom BR, Luts A, *et al.* (1993) Regional small-intestinal permeability *in vitro* to different-sized dextrans and proteins in the rat. *Scand J Gastroenterol* **28**, 205–211.
- Laurell CB (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal Biochem* **15**, 45–52.
- Dekker JP & Boekema EJ (2005) Supramolecular organization of thylakoid membrane proteins in green plants. *Biochim Biophys Acta* **1706**, 12–39.
- Albertsson PA (2001) A quantitative model of the domain structure of the photosynthetic membrane. *Trends Plant Sci* **6**, 349–358.
- Eberhard S, Finazzi G & Wollman FA (2008) The dynamics of photosynthesis. *Annu Rev Genet* **42**, 463–515.
- Nelson N & Ben-Shem A (2004) The complex architecture of oxygenic photosynthesis. *Nat Rev Mol Cell Biol* **5**, 971–982.
- Juhler RK, Andreasson E, Yu SG, *et al.* (1993) Composition of photosynthetic pigments in thylakoid membrane-vesicles from spinach. *Photosynth Res* **35**, 171–178.
- Schmid V (2008) Light-harvesting complexes of vascular plants. *Cell Mol Life Sci* **65**, 3619–3639.
- Duchêne S & Siegenthaler PA (2000) Do glycerolipids display lateral heterogeneity in the thylakoid membrane? *Lipids* **35**, 739–744.
- Akerlund HE, Andersson B, Persson A, *et al.* (1979) Isoelectric points of spinach thylakoid membrane surfaces

- as determined by cross partition. *Biochim Biophys Acta* **552**, 238–246.
28. Rayner M, Ljusberg H, Emek SC, *et al.* (2010) Chloroplast thylakoid membrane-stabilised emulsions. *J Sci Food Agr* **91**, 315–321.
  29. Shaw AB, Anderson MM & McCarty RE (1976) Role of galactolipids in spinach chloroplast lamellar membranes: II. Effects of galactolipid depletion on phosphorylation and electron flow. *Plant Physiol* **57**, 724–729.
  30. Onning G, Wang Q, Westrom BR, *et al.* (1996) Influence of oat saponins on intestinal permeability *in vitro* and *in vivo* in the rat. *Br J Nutr* **76**, 141–151.
  31. Nejdfors P, Wang Q, Ekelund M, *et al.* (1998) Increased colonic permeability in patients with ulcerative colitis: an *in vitro* study. *Scand J Gastroenterol* **33**, 749–753.
  32. Söderholm JD, Peterson KH, Olaison G, *et al.* (1999) Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? *Gastroenterology* **117**, 65–72.
  33. Vieira MM, Paik J, Blaner WS, *et al.* (2008) Carotenoids, retinol, and intestinal barrier function in children from northeastern Brazil. *J Pediatr Gastr Nutr* **47**, 652–659.
  34. Maciel AA, Oria RB, Braga-Neto MB, *et al.* (2007) Role of retinol in protecting epithelial cell damage induced by *Clostridium difficile* toxin A. *Toxicon* **50**, 1027–1040.