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Experimental Methods

A simple in vitro perfusion system to measure intestinal nutrient uptake

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Measurement of intestinal nutrient uptake is a basic problem for nutritional biochemists. Various approaches have been followed, from the simple everted sac technique to the sophisticated in vivo *perfusion preparations. All of them offer advantages and withstand disadvantages. The major factor to consider in choosing any particular system should be the physiologic significance of gathered data. The closest approximation to true uptake values comes from perfusion experiments in animals with implanted microcannulas that do not interfere with mesenteric vascular flow. Nonetheless, this approach requires complex surgical procedures and equipment that make it unsuitable for plain laboratories. This paper describes a simple* in vitro *perfusion system of intestinal segments that expresses several features of* in vivo *perfusion and overcomes some drawbacks of the everted sac technique. A short everted intestinal segment, bathed by the luminal medium, is perfused continuously through the serosal side. Exiting fluid is collected in small fractions. Translocation of nutrients (glucose, alanine) to the serosal fluid follows a sigmoidal course and reaches a plateau that remains stable for up to 1 hr. Cumulative nutrient uptake was linear after a lag phase of 3 to 6 min. In contrast to everted sacs, the continuous serosal flow avoids solute accumulation in the tissue phase. Oxygen, a limiting substrate, can be supplied through both the luminal and serosal fluids, allowing an almost optimal tissue performance. The system described combines digestion and absorption activities and permits several measurements using different intestinal segments from the same animal.* (J. Nutr. Biochem. 9:52–57, 1998) © *Elsevier Science Inc. 1998*

Keywords: glucose; alanine; absorption; jejunum; ileum; perfusion

Introduction

A classical paper by R.B. Fisher and D.S. Parsons,[1] published in 1949, described one of the earliest in vitro preparations to study intestinal solute absorption. In this system an intestinal segment, with its natural orientation, was circularly perfused by the luminal side. Samples were drawn from the luminal and serosal reservoirs. This approach, used for a few years, was rapidly substituted by the simpler everted sac technique of Wilson and Wiseman.[2] Here, a short-everted-intestinal segment is tied at one end and filled with a small amount of media (serosal compartment) before closing the other end. Sacs are incubated in flasks (luminal compartment) for the desired periods of time. This simple and reliable technique is limited by the reduced volume and poor oxygenation of the serosal compartment that cannot be sampled throughout the incubation period.

Other in vitro preparations include the Ussing chambers, rings, sleeves, and membrane vesicles. In vivo studies are represented by in situ experiments in which intestinal loops are perfused simultaneously by the luminal and serosal routes.[3–7]

The current trend points toward studies with chronically catheterized rats that allow measurement of intestinal solute uptake in unanesthetized unrestrained animals.[8–12] Although this technique approaches truly physiologic conditions, the surgical procedures are difficult and sophisticated instrumentation is required.

The financial support of CONICIT (Venezuela), Grant S1-2753, is gratefully acknowledged.
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Received January 21, 1997; accepted June 12, 1997.

655 Avenue of the Americas, New York, NY 10010

0955-2863/98/$19.00
PII S0955-2863(97)00088-0

Therefore, there is still room for in vitro experimental approaches that, although remaining simple, would yield results that resemble those of the in vivo experiments. Recently, Tchercansky et al.[13] used an in vitro, nonrecirculating, procedure to luminally perfuse intestinal segments. In this approach, samples form the serosal compartment (15 mL) are taken at regular intervals. Alternatively, a system is described here that uses the nonrecirculating serosal perfusion of everted segments. These are placed in an incubation chamber, the large volume of which (100 mL) allows solute uptake without substantial changes in luminal concentration. In contrast to the everted sacs or segments perfused luminally, the continuous serosal flow avoids the accumulation of solutes in the tissue phase. An almost optimal tissue performance is achieved delivering oxygen through both the luminal and serosal fluids. The continuous collection of the serosal effluent permits the determination of precise translocation kinetics and monitor the establishment of the steady-state.

Methods and materials

Materials

NAD, NADH, glucose, maltose, alanine, glucose oxidase, peroxidase, alanine dehydrogenase, and *o*-dianisidine were from Sigma Chemical Company (St. Louis, MO, USA). Hydrazine hydrate was from Eastman Kodak Co. (Rochester, NJ, USA). Dowex AG 1-X8 (acetate salt) was from BioRad Laboratories (Richmond, CA, USA). U-[^{14}C]-D-glucose (270 μCi/mmol) was purchased from Amersham International (Amersham, UK).

Animals

Male Sprague-Dawley rats (0.18 to 0.25 kg) were obtained from Charles River Breeding Co., Wilmington, MA., USA, or from the colony kept at the Institute of Experimental Biology, Central University of Venezuela. They were housed individually in wire-bottom cages and had free access to either a nonpurified commercial diet (Purina, Venezuela) or to purified diets based on glucose or starch, the mass fractions of which were 0.65 and 0.73, respectively.[14]

The small intestine was excised from animals under ether anesthesia, placed in cold Krebs-Ringer buffer (pH 7.4)[15] and rinsed with the same buffer to remove intestinal contents. The tissue was everted on a glass rod and cut in pieces. Segments were kept in cold buffer, continuously oxygenated, until use for perfusion experiments.

Perfusion system

An everted intestinal segment, 10.5 cm in length, is fixed through cannulas (Insyte 18G/2 in; Deseret Medical Inc., Sandy, UT, USA; catheters cut to a length of 3 mm) to a plastic chamber (12 × 4 × 3 cm) provided with syringe-type connectors. This container is filled with 100 mL of Krebs-Ringer buffer (pH 7.4) (luminal solution) continuously bubbled with a 0.95 O_2–0.05 CO_2 mixture. Oxygen is supplied through a plastic tube, with evenly distributed holes, which is affixed to the bottom of the chamber just underneath the hanging sac. The same buffer, continuously gassed with oxygen, is used as serosal fluid. It is perfused, using a peristaltic pump, through the segment at a rate of 1 mL/min. Exiting fluid is collected in 3 mL fractions on 0.1 mL of 0.3 N $ZnSO_4$. Experiments are run in a closed chamber provided with a thermostat that kept the temperature at 36 ± 1°C. A diagram of the perfusion system is presented in *Figure 1*. After a 9-min equilibration period, solutes are added to the luminal solution and the perfusion is continued for up to 1 hr. Samples from the luminal medium are taken at the beginning and end of each run and mixed with 0.3 N $ZnSO_4$. At the end, the intestinal segment is rinsed, and its dry weight determined.

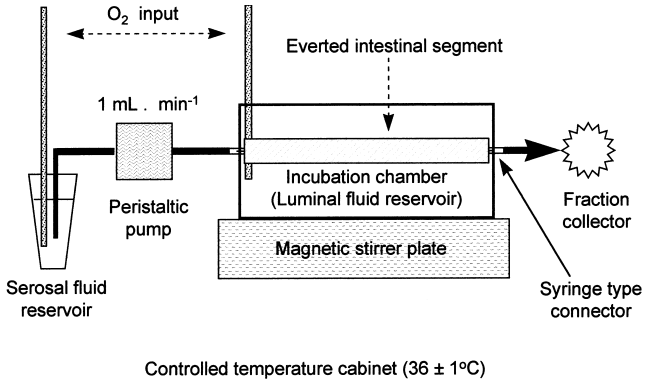

Figure 1 Diagram of the perfusion system. An everted intestinal segment is fixed through cannulas to a plastic chamber (12 × 4 × 3 cm) filled with 100 mL of Krebs-Ringer buffer (pH 7.4). The same buffer is perfused through the segment, using a peristaltic pump, at a rate of 1 mL/min. Luminal and serosal solutions are gassed continuously with a 0.95:0.05 O_2–CO_2 mixture.

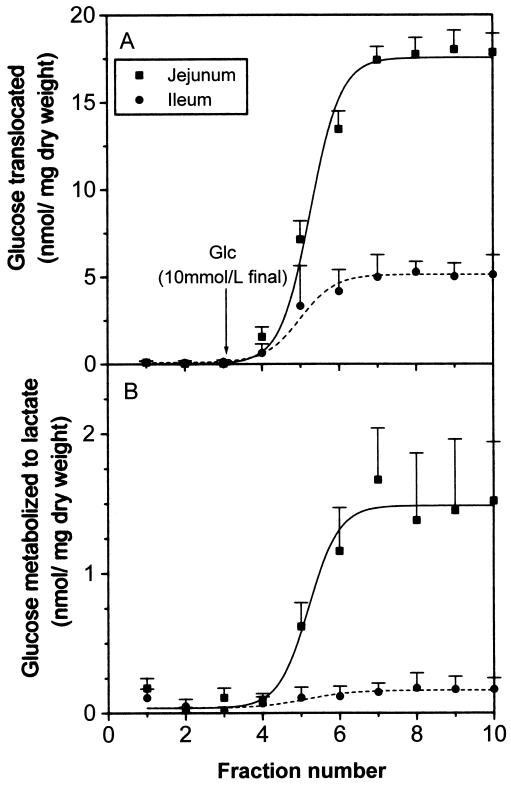

Figure 2 Glucose (*A*) and lactate (*B*) output by perfused everted intestinal segments. Perfusion was performed as indicated in Methods and materials using segments located between 24 and 34.5 cm (jejunum) and 84 to 94.5 cm away from pylorus (ileum). Mean dry weight of segments were 51.6 ± 04.18 and 48.26 ± 4.49 mg (n = 4) for the jejunal and ileal samples, respectively. Donor animals were fed the glucose diet.

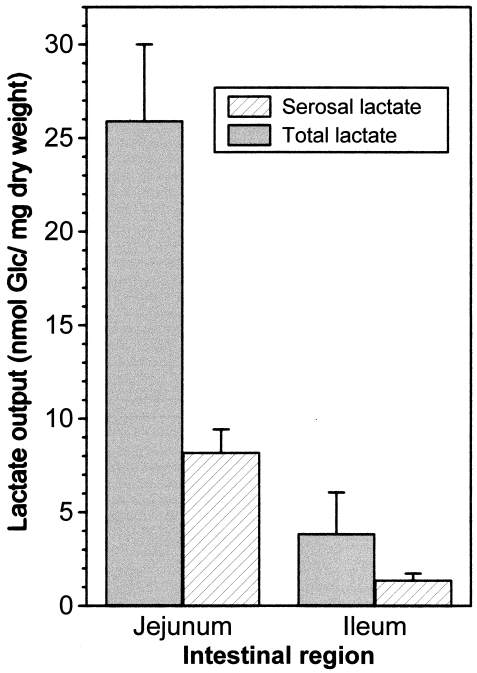

Figure 3 Serosal and total lactate output by perfused intestinal segments. Total lactate corresponds to the amount of metabolite recovered in the serosal effluent and in the luminal medium at the end of perfusion. Data from experiments presented in *Figure 1*.

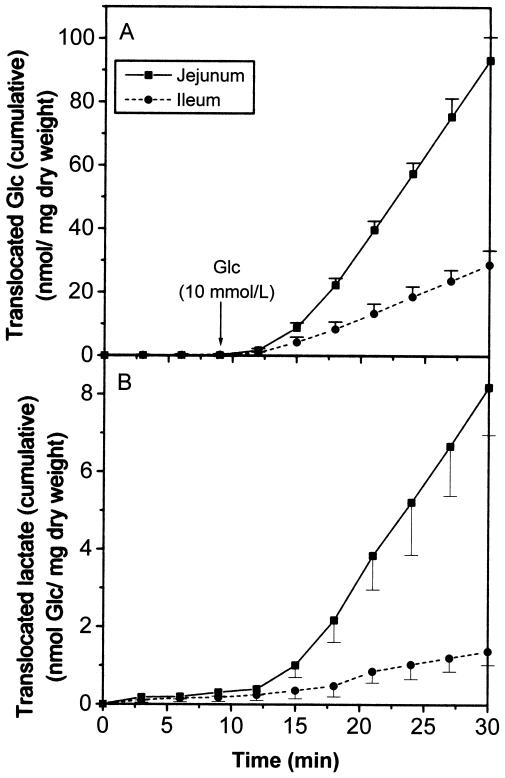

Figure 4 Cumulative glucose (*A*) and lactate (*B*) output by perfused intestinal segments. Data were taken from *Figure 1*.

Metabolite assays

Fractions were neutralized with 0.1 mL of 0.3 N $Ba(OH)_2$ and cleared by centrifugation. Samples were taken for glucose,[16] lactate,[17] and alanine[18] assays. Results are expressed as nmol of solute transported or metabolized/mg dry weight. Lactate values are depicted as glucose equivalents.

Results and discussion

1. Intestinal uptake of glucose by everted intestinal segments perfused in vitro

Addition of glucose (10 mmol/L) to the luminal fluid results in the rapid appearance of the sugar in the serosal eluate. *Figure 2A* compares the glucose output of perfused jejunal and ileal segments from glucose fed rats. Translocation followed a trend that was fitted to a sigmoidal curve. A stable plateau was reached after 12 min (fraction 7), amounting to 18 and 6 nmol/mg dry weight for the jejunal and ileal segments, respectively. This reflects the distribution of glucose uptake activity along the small intestine.[19]

This translocation pattern results from a three-step process: absorption at the luminal membrane, accumulation inside the enterocyte, and transport across the basolateral membrane.[20] A similar sigmoidal trend was reported for galactose uptake in intestinal segments perfused in vivo.[4] This finding proves that the in vitro preparation, although being more simple, behaves as the in vivo one, despite the interruption of normal blood flow.

2. Metabolic competence of perfused segments

Because the intestinal mucosa possesses the whole glycolytic machinery,[21] it would compete with the basolateral transport system (GLUT 2) for absorbed glucose.[22,23] At the beginning of perfusion (*Figure 2B*), small amounts of lactate appeared in the serosal fluid. It represents the washing of the metabolite accumulated in the segments before the establishment of perfusion. After glucose addition, lactate release to the serosal eluate increased rapidly, following a sigmoidal course delayed in comparison to glucose translocation. It proves the competition process, alluded to above, between transport and metabolism for absorbed glucose.

The ileal output of lactate was lower than that in the jejunal segments, amounting to only one-sixth of the jejunal lactate release. Most likely, the decrease in lactate output is determined by the low capability of the tissue to perform glycolysis.[3,24]

Lactate values shown in *Figure 2B* represent only the fraction released to the serosal fluid. Nonetheless, larger amounts of lactate were found in the luminal medium sampled at the end of the perfusion period (*Figure 3*). This may be an artifact of the preparation because, apparently, the luminal fluid acts as a lactate sink because of its large volume. High luminal lactate values have been reported in experiments with everted sacs[22] and with segments perfused in vivo.[6] Total lactate production of segments from the jejunal region was five times larger than that of the ileal segments. In both cases, the mass fraction of lactate translocated to the serosal fluid was 0.25.

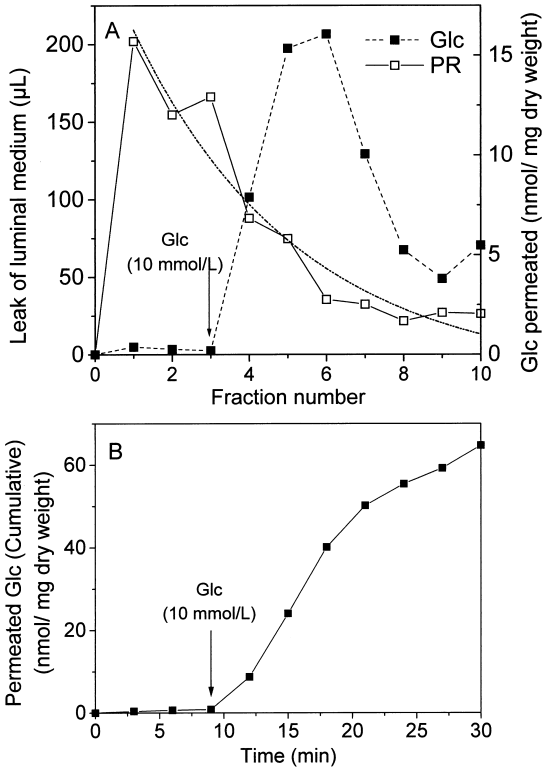

Figure 5 *A*, Time-course appearance of glucose and phenol red in the serosal effluent of a perfused leaking-segment. Phenol red (0.17 mmol/L) was present in the luminal medium from the beginning of perfusion. Leakage of luminal media was calculated measuring the absorbance of phenol red (560 nm) in the serosal effluent. It seems to follow a negative exponential curve (dotted line). Glucose (10 mmol/L final) was added after the collection of fraction 3. Glc, glucose; PR, phenol red. *B*, Cumulative glucose output.

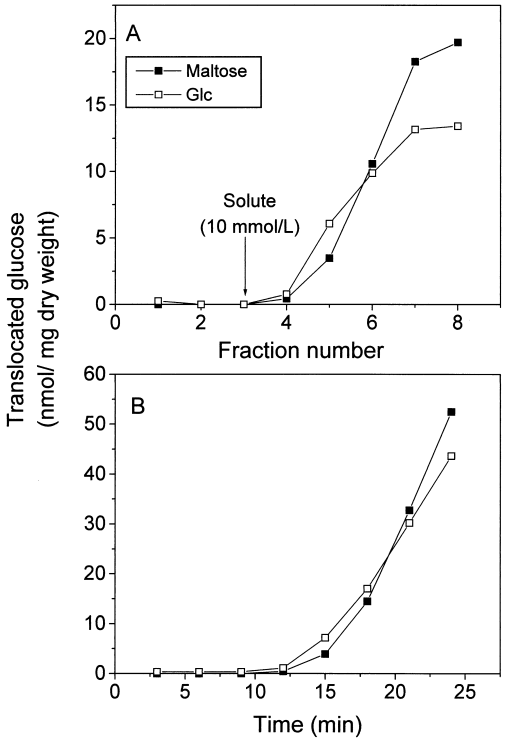

Figure 6 Per fraction (*A*) and cumulative (*B*) glucose output of jejunal segments from rats fed a starch diet incubated with either 10 mmol/L glucose or 10 mmol/L maltose. Sugars were added at the end of fraction 3. Dry weight of segments were 41.7 (maltose) and 39.6 mg (glucose). For further details see *Figure 1*.

3. Stability and performance of the preparation

The quality of an intestinal preparation can be assessed by its capability to reach and sustain a steady-state. Such condition, for glucose transport and metabolism, is proved if the time-course for cumulative solute output follows a linear trend after a lag phase. As shown in *Figure 4* (*A* and *B*) linear solute turnout was reached 6 min after glucose addition. Lag phases lasting between 5 and 15 min have been reported for everted sacs,[22] and segments perfused, both in vitro[25] and in vivo.[4] At the end of the experiment (30 min), the jejunal pieces had released over 90 nmol of glucose/mg dry weight. The ileal segments translocated less than 30 nmol/mg dry weight. After the steady-state was reached, rates of glucose output were 5.71 and 1.64 nmol/mg dry weight min^{-1} for the jejunal and ileal segments, respectively (*Figure 4A*). Lactate output rates amounted to 0.48 and 0.07 nmol Glc/mg dry weight min^{-1} for the jejunal and ileal samples, respectively (*Figure 4B*).

4. Detection of pin holes

A frequent problem while working with intestinal preparations is the occasional presence of pin holes, which may be difficult to detect, leading to false results. A usual practice to check for leaks is the addition to the luminal medium of impermeable substances, such as phenol red.[1,25] *Figure 5* (*A*) portrays the behavior of a perfused segment that had a tiny pin hole. Because of the leak, the dye appeared in the serosal fluid from the very beginning of perfusion, but its amount decreased after a negative exponential curve. This result suggests that the hole self-seals, at least partially, with time. After glucose addition, eluate sugar concentration increased abruptly leading to a transient peak that results from a combination of the leak and the physiologic translocation process. Therefore, the total amount of sugar translocated was larger than that because of the physiological transport process. Furthermore, the cumulative output changed from the linear trend shown in *Figure 2* to a sigmoidal curve (*Figure 5B*). Therefore, the continuous monitoring of solute output from segments, either on a per fraction or cumulative basis, precludes the use of impermeable dyes for pin hole detection.

5. Versatility

The features of the system described for glucose transport and metabolism were confirmed using other solutes such as the disaccharide maltose (*Figure 6*; *A* and *B*) or the amino acid alanine (*Figure 7*; *A* and *B*). In contrast to free glucose, maltose should be hydrolyzed before resulting glucose moieties could be absorbed. It was found that, after an initial delay, both per fraction and cumulative transport values were larger for maltose than for glucose. This reflects the so-called disaccharide advantage[26,27] according to which,

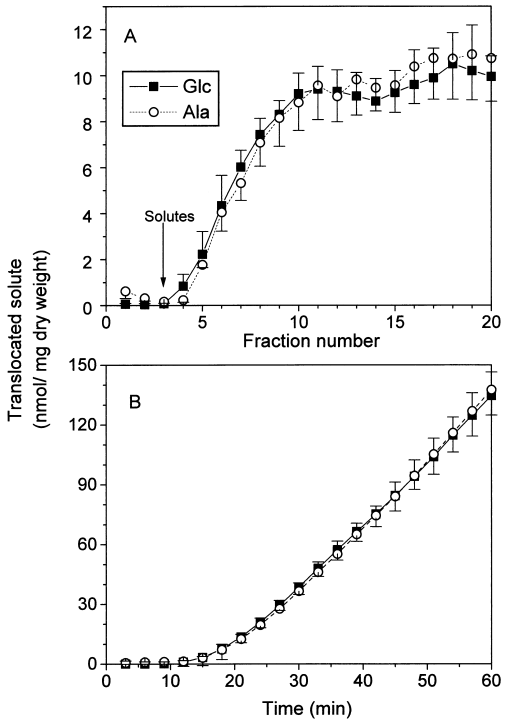

Figure 7 Per fraction (*A*) and cumulative (*B*) glucose and alanine output of jejunal segments (mean dry weight 91.2 ± 11.8 mg; $n = 3$) incubated simultaneously with 10 mmol/L glucose and 5 mmol/L alanine. Solutes were added at the end of fraction 3.

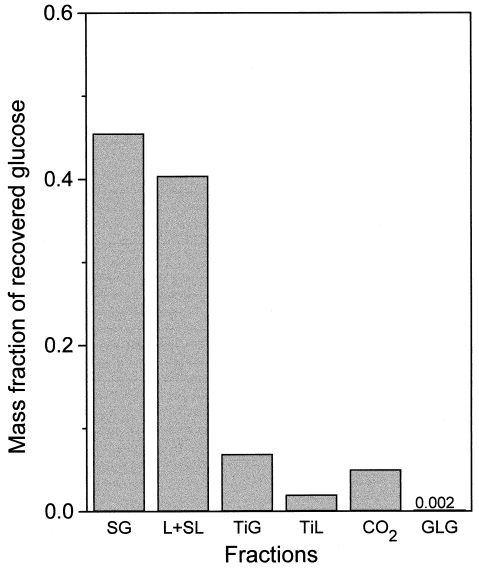

Figure 8 Partition of absorbed glucose in everted segments perfused with [^{14}C]-glucose. Segments from jejunum of rats fed a commercial diet (mean dry weight 83.13 ± 7.13 mg; $n = 3$) were incubated with [^{14}C]-glucose (10 mmol/L; 0.0005 μCi/μmol). Labeled glucose was separated from lactate by ion exchange in a (3 × 0.6 cm) Dowex AG 1-X8 (acetate salt) column. Lactate was eluted with 0.5M NaCl. Glycogen was isolated after digestion of tissue with 30% KOH. CO_2 mass fraction was estimated as 0.05 of absorbed glucose.[6,22] Fractions. SG, Serosal glucose; L + SL, Luminal + serosal lactate; TiG, Tissue glucose; TiL, Tissue Lactate; CO_2, Carbonic anhydride; GLG, Glycogen.

the hydrolysis of maltose and absorption of glucose are closely linked (*Figure 6, A* and *B*).

In the experiments of *Figure 7*, glucose (10 mmol/L) and alanine (5 mmol/L) were added simultaneously. Output kinetics for both solutes were essentially identical (*Figure 7, A* and *B*). These results confirm the capability of the intestinal preparation to handle a large solute load. The experiments portrayed were conducted for 1 hr, during which solute output was maintained. This shows the remarkable stability of the preparation to sustain steady-state rates for a long period.

6. Accumulation of solutes within the tissue phase

A major drawback of everted sacs is the accumulation of solutes and metabolites within the tissue phase. For instance, Pritchard y Porteus[22] found that the mass fractions of absorbed glucose and total lactate repressed within the sac walls were 0.3 and 0.23, respectively. In contrast, in intestinal segments perfused in vivo, the amount of glucose retained in the tissue was negligible.[6] In segments from rats fed a commercial diet perfused in vitro it was estimated, using [^{14}C]-glucose, that tissue glucose amounted only to 0.07 (*Figure 8*). Similarly, tissue lactate accounted for 0.02 of the total glucose mass fraction. *Figure 8* also shows that the incorporation of glucose into glycogen by the intestinal tissue was negligible. Therefore, the preparation reported here resembles more closely the behavior of the in vivo perfusion than that of everted sacs.

Findings presented in this study show that the in vitro perfusion of intestinal segments is a reliable technique that yields results similar to those of in vivo preparations. The major advantage of serosal perfusion comes from the possibility to continuously sample the serosal fluid and, therefore, obtain the kinetics of solute output. Moreover, translocation occurs without appreciable accumulation of metabolites within the tissue phase. The stability of the preparation and the attainment of a suitable steady-state allows a full range of perfusion experiments, like changing the composition of luminal and serosal media, introducing substrate or inhibitor pulses, etc. Because several perfusion experiments can be conducted simultaneously, the effect of different parameters could be tested on segments from the same animal.

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