J. Physiol. Biochem., 53 (3), 281-288, 1997
Revista española de Fisiología

# Some technical precisions to a method for *in vivo* intestinal absorption studies

M. M. Pérez del Castillo, M. P. Lostao, A. Barber and F. Ponz

Departamento de Fisiología y Nutrición, Universidad de Navarra, 31080 Pamplona (Spain)

(Received on May 6, 1997)

M. M. PÉREZ DEL CASTILLO, M. P. LOSTAO, A. BARBER and F. PONZ. *Some technical precisions to a method for* in vivo *intestinal absorption studies.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 53 (3), 281-288, 1997.

Some modifications to the method of PONZ *et al.* for *in vivo* intestinal absorption studies using an *in situ* perfused segment of small intestine, under anesthesia, are described. They improve its accuracy and applicability, especially when slowly absorbed substrates are used or when volume flux measurements are desired. Calculations for the absorbed substrate and net fluid volume change determinations are reported. Several aspects of the use of the method under a single pass or a recirculation perfusion system are discussed.

Key words: Intestinal absorption method, Intestinal perfusion, Intestinal volume flux measurements.

In spite of some criticisms (5), the methods for the study of intestinal absorption based on the perfusion of an *in situ* cannulated segment of small intestine, in anesthetized animals, continue to be used (1, 3, 7) as they offer conditions not too far from the physiological ones and maintain the integrity of both the transcellular and the paracellular pathways across the epithelium.

One of the first reported methods (2, 4) has been used at this laboratory for a long time. From that experience, some modifications to improve its reliability are being incorporated and reported here. They are related to a better definition of the beginning and the end of each absorption period time, to the impossibility of a total elimination of a little fluid fraction retained by the intestinal mucosa, and to the facilities provided by volume markers.

## Materials and Methods

The animals, the procedure to prepare the *in situ* cannulated intestinal segment,

Correspondence to F. Ponz (e-mail: fponz@unav.es).

the components of the perfusion system, as well as the measurement of the segment length are the same as in the original description (2). Anesthesia can be carried out with urethane (125 mg/100 g), pentobarbital (60 mg/kg) or any other anesthetic of prolonged action.

In this study, Wistar rats were used, reared and handled under G.L.P. conditions. The $^{14}C$ or $^{3}H$ labeled substrates were determined by radioactivity counting. Fluid volume measurements were usually made by weight. Saline Krebs-Henseleit-Bicarbonate or Krebs-Ringer-Phosphate solutions, buffered at pH 7.4, (6) were used as washing and perfusion solutions. Gassifying these solutions was found to be unnecessary.

## CARRYING OUT THE EXPERIMENTS

*On single pass perfusion.–* The entry and exit cannulae, fixed at both ends of the intestinal segment, are connected to the perfusion system, and 40-50 mL of saline solution are pumped to wash and drag luminal residues. Air is then pumped to drain all the perfusion system. Then, the perfusion solution containing the substrate at a Sps concentration under the desired experimental conditions, is pumped at the selected rate, v. After discarding the first 15-20 mL, several fractions of the effluent, corresponding to the exactly measured periods of absorption, are consecutive and separately collected. The volume, Vf, and the substrate concentration, Sf, of each one of these effluent fractions are then determined.

To compare the absorption between different experimental conditions, the perfusion system including the intestine segment is washed by pumping 20-30 mL of substrate-free washing solution, then air is pumped to drain this solution, and the desired new solution is perfused. After discarding, as previously, the first 15-20 mL of effluent, the fractions corresponding to a series of consecutive absorption periods under the new condition are collected as before.

If the time of each absorption period is t min, the segment length is d cm, the solution volume entering the segment along that period is Vi mL and the substrate concentrations in the perfusion solution and in the effluent fraction are respectively Sps and Sf ($\mu mol \cdot mL^{-1}$), the absorbed substrate, $\Delta S$, and the absorption rate, Js, can be calculated as

$$\Delta S\ (\mu mol) = Vi \cdot Sps - Vf \cdot Sf,$$

$$Js\ (\mu mol \cdot cm^{-1} \cdot min^{-1}) = \Delta S \cdot d^{-1} \cdot t^{-1}$$

The net fluid volume change along the period, $\Delta V$, will be Vi - Vf. However, Vi cannot be exactly measured. It can be estimated as $v \cdot t$, but it must be taken into account that, with ordinary perfusion pumps, v is not strictly constant. Using perfusion rates in the range from 0.55 to 7.5 mL/min and intestinal segments of about 20 cm, standard errors in pumped volume may reach up to 3 % of the mean (table I). When $\Delta S$ and $\Delta V$ are very small related to the initial values of S and V, these errors may become significant. Under this last circumstance, an ideal volume marker is useless, because the differences in marker concentration would be also scarcely or non measurable. Therefore, single pass perfusion is not appropriate to measure the very small volume changes.

Assuming that $\Delta V$ is negligible, Vi = Vf, and the absorbed substrate will be

$$\Delta S = Vf\ (Sps - Sf)$$

*On recirculation perfusion.–* The connection of both cannulae to the perfusion system, the washing of the intestinal segment and the fluid draining by air pumping are the same as for single pass perfusion. Recirculation is then achieved by

Table I. *Steadiness in effluent volume collection (mL) under different perfusion rates.* With the peristaltic pump running at the selected perfusion rate, six effluent fractions were collected at 0, 10, 20, 30, 40 and 50 min after starting perfusion time during the indicated collecting period. Mean ± SEM, in mL. A) Perfusion of a 20 cm long rubber tube. B) Perfusion of an about 20 cm long jejunal segment, *in situ*.

| Perfusion rate ($mL \cdot min^{-1}$): | 0.55 | 2 | 5.6 | 7.5 |
|---|---|---|---|---|
| Collecting period (min): | 5 | 2 | 1 | 1 |
| A) | 2.77 ± 0.22 | 3.99 ± 0.02 | 5.64 ± 0.02 | 7.42 ± 0.09 |
| B) | 2.82 ± 0.13 | 4.07 ± 0.10 | 5.71 ± 0.10 | 7.65 ± 0.24 |

collecting the effluent in the same reservoir from which the perfusion solution is being sucked.

A volume of perfusion solution, Vi, with the substrate at the desired concentration, Sps, is put in the reservoir. Once the pump is running, the length of the absorption period is measured from the moment of sinking the suction tube into the perfusion solution, until that of its withdrawal and beginning of air pumping. This air passage forces the solution towards the reservoir, where a fluid volume, Vf, with a substrate concentration, Sf, is collected. As a small residual fluid volume, Vrf, containing the substrate at the same Sf concentration remains retained by the mucosa, an immediate washing with 15-20 mL of substrate-free solution followed by air pumping has to be done to drag and collect all that residual substrate. There are three different procedures to calculate ΔS and ΔV.

*Procedure A*: The effluent corresponding to this last washing is collected separately, and its volume, Vw, and substrate concentration, Sw, are determined. The absorbed substrate, ΔS, will be,

$$\Delta S = Vi \cdot Sps - Vf \cdot Sf - Vw \cdot Sw$$

Precise calculation of net change in fluid volume, ΔV, is difficult because the actual initial volume, V'i, is Vi plus a substrate-free residual fluid volume, Vri, previously retained by the mucosa. Likely, the actual final fluid volume, V'f, is Vf plus the substrate containing residual fluid volume retained by the mucosa at the end of the absorption period, Vrf. However, as results show (table II), when working with the same intestinal segment the differences between the residual volumes in the consecutive periods are very similar (SE ≤ 10 % of the mean), and can be assumed that Vri = Vrf. Under this assumption,

$$\Delta V = V'i - V'f = (Vi + Vri) - (Vf + Vrf) = \\ = Vi - Vf$$

*Procedure B*: Only ΔS can be calculated. The effluent of the last washing is collected together with Vf, and the whole volume, Vt, and the substrate concentration, St, are determined. Therefore,

$$\Delta S = Vi \cdot Sps - Vt \cdot St$$

*Procedure C*: An ideal volume marker, M, is used. After washing the intestine with substrate- and marker-free solution, a volume Vi of perfusion solution with substrate and marker (Sps and Mps concentration, respectively) is recirculated for some time, depending on the perfusion rate, to achieve distribution equilibrium of S and M in the whole Vi + Vri volume. Samples are now taken from the reservoir to determine the substrate and marker concentrations, So and Mo, and this

moment is taken as the beginning of the absorption period. After *t* min, other samples are taken to determine the respective final concentrations, Sf and Mf. Vo and Vf are the actual fluid volume at the beginning and the end of the absorption period, Vo = Vi(Mps/Mo), and Vf = Vi(Mps/Mf). Thus, the absorbed substrate will be

$$\Delta S = Vo \cdot So - Vf \cdot Sf = Vi \cdot Mps\ [(So/Mo) - (Sf/Mf)]$$

and the net change in fluid volume,

$$\Delta V = Vo\text{-}Vf = Vi \cdot Mps\ [(1/Mo) - (1/Mf)]$$

Nevertheless, these calculations must be corrected for the fluid volume (Vm), the substrate (So • Vm), and the marker (Mo • Vm) which have been lost with the samples taken for determination of So and Mo, whenever these losses were not negligible. After these corrections,

$$\Delta S = Vi \cdot Mps\ [(So/Mo)\text{–}(Sf/Mf)] - - Vm\ [(So - Sf\ (Mo/Mf)],$$

and

$$\Delta V = Vi \cdot Mps\ [(1/Mo) - (1/Mf)] - - Vm\ [1\text{–}(Mo/Mf)]$$

With A and B procedures, successive absorption periods, in the same or different experimental conditions, can be carried out by appropriately changing the reservoir and the solution to be recirculated. Under procedure C, enough washing and air pumping to drain the perfusion circuit from fluid has to be made, before starting the next absorption period.

## Results and Discussion

*Fluid retention by the mucosa in spite of air passing.*– If single pass perfusion is carried out as in the original description (2), the collected effluent includes a small fraction of substrate-free washing solution retained by the mucosa, so that a little dilution of the substrate not ascribable to its absorption is produced. Thus, substrate absorption values somewhat higher than the real ones are obtained. The error will be more significant on working with slow absorbed substrates and with short absorption periods. The modification that has been introduced, i.e. discarding the first 15-20 mL of substrate containing effluent before the beginning of the consecutive absorption periods, prevents that error source.

Values of fluid volume retained by the mucosa have been estimated from recirculation perfusion experiments (procedure A). As at the end of the absorption period, that volume, Vrf, contains substrate at Sf concentration, Vrf = Vw (Sw/Sf). With about six week old rats, weighing 150-200 g, and using about 20 cm long jejunal segments, a washing with 15-20 mL of substrate-free solution is enough to drag practically all the residual substrate contained in Vrf. As fig 1 shows, the radioactivity counting of the 5 ml effluent fractions corresponding to 15-20 and 20-25 mL washings, is quite negligible. Obviously, as the intestine segment length increases, a higher volume of washing solution has to be used.

Table II shows the values of Vrf obtained, as it has been indicated, from a series of determinations with the same intestine segment or with different segments and rats. In the first case, the Vrf values were found to be very similar, a fact that allows the assumption of taking Vri = Vrf. In the second case, with animals of similar age and weight (about 6 weeks old and 150-200 g body weight) the Vrf values were found to be roughly related to the intestinal segment length. With a perfusion rate of 5.6 mL • $min^{-1}$, the mean of the obtained Vrf/d values was 50.96 ± 1.6

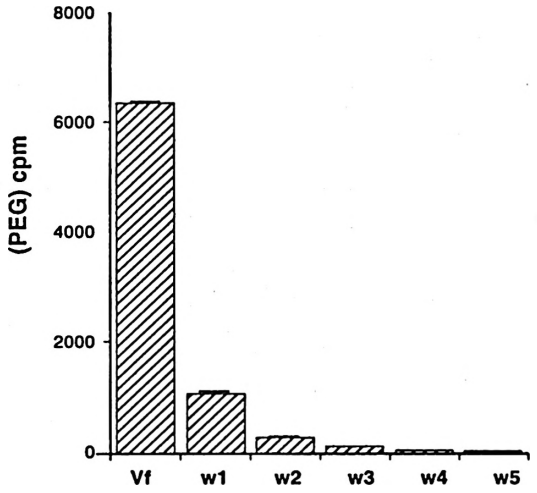


Fig. 1. *Washing solution volume required to drain all the substrate remaining in the fluid retained by the mucosa.*
After a perfusion of $^3$H-labelled PEG-4000, the fluid (Vf) was drained from the perfusion system by air pumping. Then, washing solution was perfused and consecutive 5 mL fractions of effluent (w1-w5) were separately collected and their radioactivity counted.

$\mu L \cdot cm^{-1}$, which represents about 1/4 - 1/5 of the fluid volume per cm that is filling the intestinal segment during perfusion.

In recirculation experiments, therefore, the mucosal fluid retention prevents the calculation of the absorbed substrate as $\Delta S = Vi \cdot Sps - Vf \cdot Sf$.

*Time elapsed on filling the intestinal segment.*– When perfusion starts, the front of the pumped solution takes up some time in covering the segment length, a time in which the total mucosal surface is not being used for absorption. Obviously, this time depends on the perfusion rate and on the segment length. In table III, the filling times at different perfusion rates are shown. This source of error, despite its small significance, is quite avoided by the modification of single pass perfusion procedure consisting in discarding the first 15-20 mL of effluent.

On recirculation perfusion, taking the suction tube immersion in the reservory solution and the withdrawal from the same solution as the beginning and the end of the absorption time, as it has been described, this little error becomes eliminated, since the time used in filling up the intestine is approximately the same as that used in dragging it by air pumping.

*Preferences for single pass versus recirculation perfusion.*– Single pass perfusion allows to maintain a steady concentration of the substrate during the whole absorp-

Table II. *Volumes of the fluid retained by the mucosa after air pumping.*
Retained fluid volume calculated (recirculation procedure A) as Vrf = Vw·(Sw/Sf), using ($^{14}$C) 2-deoxy-D-glucose as marker. Perfusion rate, 5.6 mL/min. Mean ± SEM. Number of data in parenthesis.

| Rat weight (g) | Intestine length (d) | Vrf (mL) | | Vrf/d (µl/cm) |
|---|---|---|---|---|
| 181 | 19.2 | 0.95 ± 0.09 | (4) | 49.42 ± 4.79 |
| 178 | 18.6 | 0.89 ± 0.06 | (6) | 48.15 ± 3.31 |
| 170 | 16.8 | 0.93 ± 0.07 | (5) | 55.63 ± 4.12 |
| 167 | 13.2 | 0.69 ± 0.11 | (3) | 52.05 ± 8.15 |
| 181 | 19.2 | 0.87 ± 0.02 | (3) | 45.25 ± 0.91 |
| 180 | 13.2 | 0.61 ± 0.05 | (4) | 46.88 ± 3.47 |
| 181 | 13.2 | 0.68 ± 0.11 | (4) | 51.69 ± 8.23 |
| 170 | 16.8 | 0.98 ± 0.02 | (4) | 58.62 ± 0.93 |
| | | | (33) | 50.96 ± 1.59 |

Table III. *Dimensions and filling times of jejune segments.*
Length measured as in (1); exterior iameter during perfusion, by micrometer gauge; interior volume, with PEG as marker. Filling time was considered as the time elapsed by the fluid front to cover the jejune segment.

| | Rat 1 | Rat 2 | | |
|---|---|---|---|---|
| *Dimensions* | | | | |
| length (cm) | 18 | 19.2 | | |
| exterior diameter (mm) | 6.20 | 6.15 | | |
| interior volume (mL) | 3.16 | 3.57 | | |
| interior volume ($mL \cdot cm^{-1}$) | 0.175 | 0.185 | | |
| *Filling time* | | | | |
| perfusion rate ($mL \cdot min^{-1}$) | 0.58 | 2 | 5.6 | 7.5 |
| filling time | 3-4 min | 45-60 s | 18-22 s | 10-12 s |

tion time, so that it may be more suitable for kinetics studies. Nevertheless, a well measurable change in substrate concentration is more difficult to obtain as the substrate absorption rate is lower, the intestinal segment length is shorter or the perfusion rate is higher. In experiments with about 20 cm segment length, 2.7 mL/min perfusion rate and 2 or 5 minute absorption period, the absorption of 1, 5 and 20 mmol/L D-galactose is well measured, with standard errors lower than 10 % of the mean. Instead, under the same conditions, the passive absorption of galactose, by adding 2 mmol/L phlorizin, is measured with 3-4 fold higher errors. Similar high errors were obtained when mannitol or non transported sugars are used as substrates. Single pass perfusion is, therefore, unsuitable to measure the absorption of slow absorbed substrates. This procedure must also be ruled out when measurement of net fluid volume change is desired.

On the other hand, recirculation perfusion may be used to measure absorption of fast or slow absorbed substrates, allowing also to measure fluid volume changes. However, the selected absorption periods are to be long enough to yield well measurable concentration and fluid volume differences, without improperly affecting kinetics. Absorption of 2 mmol/L galactose can be measured in 5 min periods with standard errors lower than 5 % of the mean. With 10 mmol/L 2-deoxy-D-glucose, or with other slow absorbed substrates, at least 15 min periods are recommended to yield acceptable measurements. Also 15 or more min periods are preferred to measure net fluid volume changes.

M. M. PÉREZ DEL CASTILLO, M. P. LOSTAO, A. BARBER y F. PONZ. *Precisiones técnicas a un método* in vivo *para estudios de absorción intestinal.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 53 (3), 281-288, 1997.

Se describen algunas modificaciones al método de PONZ *et al.* para estudios de absorción intestinal con perfusión de segmentos de intestino *in situ*. Las modificaciones mejoran su precisión y aplicabilidad, especialmente cuando se utilizan sustratos lentamente absorbibles o se desean medidas de flujos líquidos. Se aportan los cálculos para las determinaciones de la absorción del sustrato y del cambio de volumen líquido. Se discuten diversos aspectos acerca de la utilización del método con o sin recirculación de la solución de perfusión.

Palabras clave: Método de absorción intestinal, Perfusión intestinal en rata, Medidas de flujo líquido intestinal.

## References

1. Lundin, P. D. P., Weström, B. R., Pantzar, N. and Karlsson, B. W. (1997): *Dig. Dis. Sci.*, 42, 677-683.
2. Ponz, F., Ilundain, A. and Lluch, M. (1979): *Rev. esp. Fisiol.*, 35, 97-104.
3. Shi, X. and Gisolfi, C. V. (1996): *Am. J. Med. Sci.*, 311, 107-112.
4. Sols, A. and Ponz, F. (1947): *Rev. esp. Fisiol.*, 3, 207-211.
5. Uhing, M. A. and Kimura, R. E. (1995): *J. Clin. Invest.*, 95, 2790-2805.
6. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1951): Manometric techniques and tissue metabolism. Burgess Publ. Co. Minneapolis.
7. Wapnir, R. A., Sia, M. C. and Fisher, S. E. (1996): *J. Appl. Physiol.*, 81, 2523-2527.