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D-Galactose Uptake by Snail Intestine: Competitive Inhibition by Phlorizin and Sodium Dependence*

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The net entry of galactose into the tissue of snail everted intestinal rings with 2 or 15 minute long incubation periods has been measured. With 10^{-4} M phlorizin, the mediated transport is completely blocked while only the passive entry of sugar is produced. Lower concentrations of the glycoside partially inhibit transport according to competitive inhibition kinetics ($K_i = 10^{-7}$ M). The transport of galactose is Na^+ dependent. In the absence of Na^+, transport ceases and the sugar entry can be explained through simple diffusion. With 15 mM Na^+ (control 71,4 mM) transport diminishes and a marked increase in the apparent K_m with no changes in the V_{max} is observed. One mM harmaline completely blocks galactose (0.5 mM) transport. One mM ouabain also makes transport null, but only after tissue preincubation with the inhibitor on the serosal side.

The snail intestine of *Helix aspersa* has been shown in previous work carried out in our laboratory to actively transport sugars and amino acids (3, 6, 9, 13). With everted intestinal sacs the sugar transport system was observed to be Na^+ dependent (5), while phlorizin exerted a markedly inhibitory effect on the active transport of D-glucose, 3-O-methyl-glucose and D-galactose (3).

In the present work, both the influence of Na^+ and the inhibitory effect of phlorizin on the net entry of D-galactose into the intestinal tissue are analyzed in kinetic terms. Furthermore, a comparative study of the absorption capacity all along the intestine is presented.

Materials and Methods

Measurements of the net entry of D-galactose in everted intestine of snail *Helix aspersa* have been taken in a variety of work procedures. The 15 minute long experiments were carried out according to the technique described in previous works (6). To compare the transport capacity throughout the length of the intestine, this was divided into four

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portions of equal length and were then placed separately in erlenmeyers in groups of four corresponding to as many animals and to the same anatomic region.

In the two minute long experiments the everted intestine was incubated intact. Up to 4 intestines belonging to as many specimens were placed in each erlenmeyer with 10 ml of medium. At the end of the incubation period, the intestinal preparations were vacuum filtered quickly in a Buchner to separate them from the incubation liquid, they were then washed with cold physiological solution and dried on wet filter paper; finally the wet weight of each one of them was determined. Each intestine was introduced in 0.5 ml of 0.1 N NO_3H. After 24 h in cold storage, aliquot samples were taken to determine radioactivity, allowing to estimate the amount of D-galactose transferred to the tissue water by comparing it with the radioactivity of the samples in the incubation medium. The results are expressed as the rate of net entry of sugar in μmoles of D-galactose per unit of wet weight in the incubation time ($\bar{x} \pm$ S.E.).

The incubation medium (16) contained D-galactose in every case at the required concentration, which included the adequate proportion of marked substrate (^{14}C). In Na^+ substitution experiments, NaCl was replaced by either Tris-HCl concentrations of equivalent osmolarity for the same pH or, in some cases, by $CaCl_2$ concentrations. Phlorizin was adjusted to the desired final concentration by adding 20 μl of solution in ethanol to the 10 ml of the medium.

D-(1-^{14}C) galactose (3 mCi/mmol) was supplied by Amersham.

Results

Competitive character of transport inhibition by phlorizin. In snail intestinal sacs, the inhibitory effect of phlorizin on the active transport of monosaccharides had been shown (3). To characterize the type of inhibition, it was deemed preferable to measure the rate of sugar entry into the tissue during 2 minute long incubations at 30°C. Concentrations of 10^{-4} M phlorizin were used to completely block the transport, and of $5 \times \times 10^{-8}$ M to study the inhibition kinetics.

With 10^{-4} M phlorizin (fig. 1), galactose entry depends linearly on its concentration in the medium, which means that the transport system has been abolished and that the obtained values correspond apparently to a passive component of simple diffusion. The slope of the straight line, adjusted after the method of the minimum squares, allows us to estimate a coefficient of mass transference of 0.28 μmoles g w.w.$^{-1}$ 2 min^{-1} mM^{-1}.

The sugar entry under control conditions and in the presence of 5×10^{-8} M phlorizin tends to saturation, which indicates that it includes a saturable component of mediated transport. The difference between the total entry values and those corresponding to the passive component (obtained with 10^{-4} M phlorizin), allows us to deduce sugar penetration into the tissue by means of specific transport mechanisms. Its graphic representation by the double reciprocal method (fig. 2) is consistent with the competitive character of the phlorizin induced inhibition, which does not modify the saturation rate ($V_{max} = 1.25$ μmoles/g w.w./2 min), while it significantly increases the apparent constant for transport (from 8 mM in absence of the inhibitor to 12.5 mM with phlorizin). The K_i calculated for phlorizin yields a 10^{-7} mM value.

Na^+ dependence on galactose transport. Experiments with intestinal everted rings

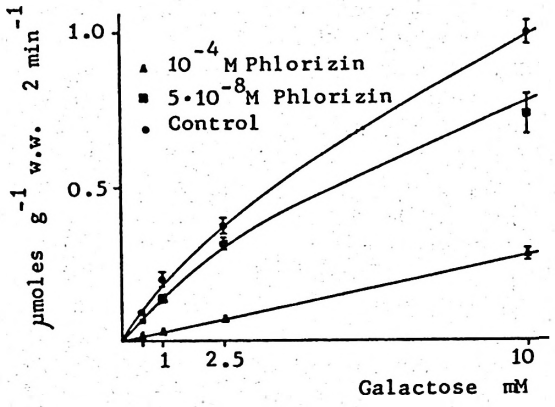

Fig. 1. *Effect of phlorizin on the kinetics of D-galactose entry in snail everted intestine.*

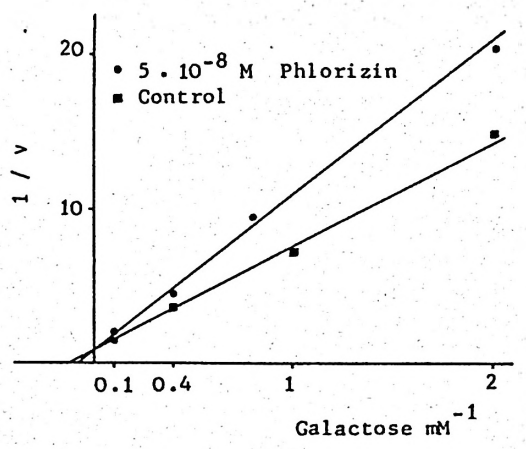

Fig. 2. *Competitive inhibition of phlorizin on the D-galactose transport.*

have been carried out to study the influence of varying Na^+ concentrations in the incubation medium on galactose entry into the tissue water during 15 min at 30°C.

The results obtained (fig. 3) reveal that the aforesaid entry does not vary significantly even when the cation concentration drops to 50 % (35.7 mM) with respect to that of the complete medium (71.4 mM). With 15 mM Na^+ in the medium, sugar transference is, however, significantly lessened. In complete absence of Na^+ or with 7.5 mM Na^+, galactose entry into the tissue is a linear function of its concentration in the medium and its value coincides with that of mannitol entry at the same concentra-

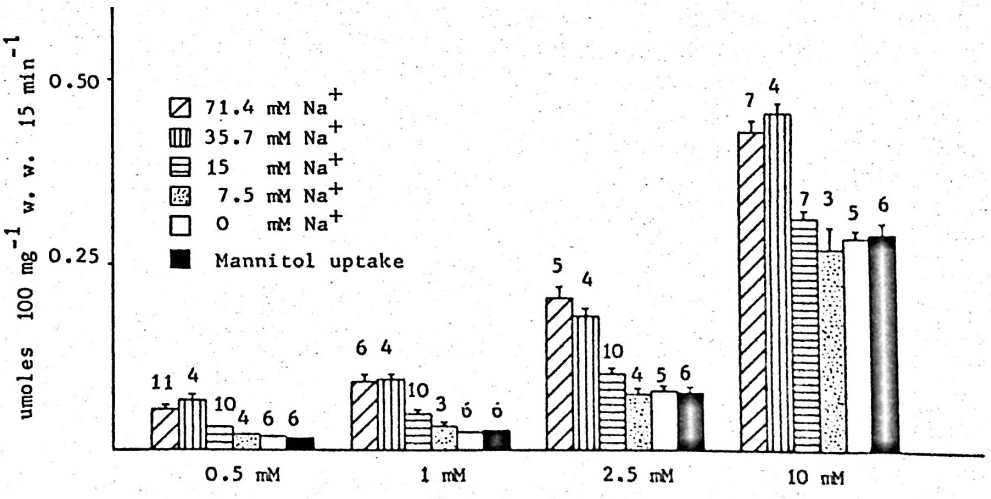

Fig. 3. *Net entry of D-galactose, at various Na^+ concentrations in the medium, and of D-mannitol.*
Number of experiments at the top of the bars.

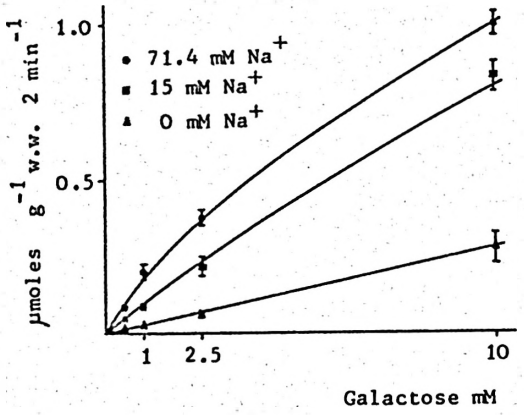

Fig. 4. *Effect of Na⁺ on the kinetics of D-galactose entry in snail everted intestine.*

tions. Therefore, under Na^+ deprivation the galactose transport ceases, the sugar entering the tissue only through simple diffusion.

In order to kinetically characterize the inhibition due to Na^+ deficiency, experiments of galactose entry were carried out in whole everted intestine, during 2 min periods, in normal medium with 15 mM Na^+ and in total absence of Na^+ (replaced by either Tris/HCl or Ca^{++}).

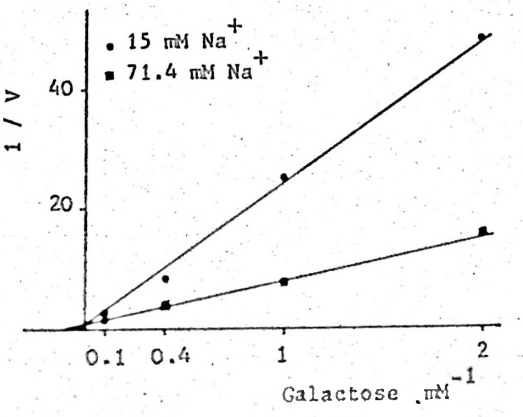

Fig. 5. *Inhibition of D-galactose transport from Na^+ deficiency.*

The results (fig. 4) confirm the total and specific dependence on Na^+ for galactose transport in snail intestine, since in the absence of Na^+ (regardless of the substitute used), the sugar entry kinetics is linear, as corresponds to a passive process of diffusion. Furthermore, the slope of the straight line coincides with that obtained in the experiments carried out in the presence of 10^{-4} M phlorizin, described in the precedent section.

With 15 mM Na^+, galactose entry is significantly inferior to that obtained in whole medium, although it still includes a saturable component. The graphic representation following the double reciprocal method of the corrected transport component (after substracting from the values of total entry those corresponding to diffusion) suggests that the inhibition from Na^+ deficiency is due to lesser affinity for the carrier, since it clearly increases the K_m (from 8 mM to 28.6 mM) without changes in the V_{max} (fig. 5).

Assays have been conducted to study the possible effect of ouabain and harmaline, well known inhibitors in other animal species (2, 14, 18, 20). In 2 min long incubations with 0.5 mM galactose (fig. 6), the presence of 1 mM harmaline

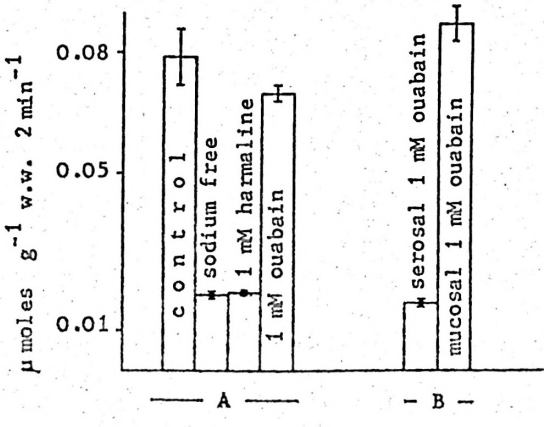

Fig. 6. *Effect of 1 mM harmaline and 1 mM ouabain on the net entry of 0.5 mM galactose.* A: 2 min incubation. B: 30 min preincubation and 2 min incubation both with ouabain.

makes galactose transport null, since the sugar influx does not differ from that measured in the absence of Na^+ or in the presence of 10^{-4} M phlorizin.

As regards 1 mM ouabain, no inhibition is observed when its presence occurs only during the 2 min long incubations, but the transport is abolished when the intestinal preparation has been preincubated with the glycoside for 30 min. When the preincubation was carried out so that the ouabain had direct access only to the mucosal surface of the tissue (by ligating both ends of the everted intestine), no effects were observed.

Absorption along the intestine. The entry of 1 mM galactose into the tissue has been measured in 15 min long experiments at 20°C. The intestine was divided in four portions of equal length. The results obtained from 16 animals are expressed in table I.

Table I. *Entry of 1 mM D-galactose throughout the length of snail intestine.*
The mean values correspond to four determinations, each one of which has been obtained from a set of four portions of intestine belonging to the same region.

Intestinal region (proximal to distal)	Wet weight (mg)	Galactose uptake (μmoles g^{-1} w.w.)
1st	28.8 ± 2.0	0.30 ± 0.01
2nd	19.8 ± 2.0	0.43 ± 0.02
3th	18.1 ± 3.2	0.56 ± 0.05
4th	12.9 ± 2.5	0.78 ± 0.06

The influx of galactose referred to intestine length does not change in the various regions (1st to 4th, proximal to distal). If it refers instead to the wet weight of the tissue, it increases progressively in distal direction.

Discussion

Previous results (3) showing the strong inhibition of phlorizin on the active transport of galactose in snail intestine have been confirmed. Galactose transport is totally blocked with 10^{-4} M phlorizin, and the sugar entry into the tissue becomes linear with its concentration in the medium. The transport of galactose is partially inhibited by 5×10^{-8} M phlorizin. If the galactose that has entered the tissue passively (with 10^{-4} M phlorizin) is subtracted from the total galactose entered, the values for the sugar entering the cells through transport are obtained, which makes possible to estimate the kinetic parameters of galactose transport in both the presence and absence of 5×10^{-8} M phlorizin. The V_{max} coincides in both cases (1.25 μmoles/g w.w./2 min), but the K_m increases in the presence of the inhibitor (from 8 to 12.5 mM). This shows that phlorizin, as described in other species studied (7, 11, 19, 22), competitively inhibits the intestinal transport of sugars in snail. The value obtained for K_i, 10^{-7} M, shows that the affinity for the common carrier is much higher for phlorizin than for galactose, as it has also been reported for mammal intestine (12, 23).

With sacs of everted intestine, the active transport of monosaccharides in snail (5) has been observed to be specifically dependent on Na^+. In the present experiments, the entry of galactose into the tissue in 15 min, is inhibited on lessening the Na^+ concentration in the medium, although higher than 50% decreases with respect to the normal medium (71.4 mM) are required to render it noticeable. In total absence of the cation as well as with 7.5 mM Na^+, galactose seems to enter the tissue by simple diffusion.

The experiments with 2 min long incubations confirm the previous data and make it possible to better analyze the ki-

netic aspects of Na^+ influence on the intestinal transport of galactose. The inhibition from Na^+ deficiency proceeds without variation in the V_{max}, while a clear rise in the K_m, from 8 mM (normal medium) to 28.6 mM (15 mM Na^+) is observed, as if it were due to a decrease of the carrier affinity for the sugar, which agrees with other authors' reports on various species (7).

By comparison with the results obtained in similar experiments with L-phenylalanine (13) and L-leucine (9), the active transport of sugars in snail intestine shows greater dependence on Na^+ than that of those neutral amino acids.

The presence of 1 mM harmaline in the medium during the 2 min long incubations determines the passive entry of galactose into the tissue. This seems to indicate that a blocking action on the sugar transport from the mucosal is taking place, which may be due to harmaline and Na^+ competing for their binding site in the carrier, as previously pointed out by other authors in other species (2, 14, 21).

Inhibition by 1 mM ouabain is not apparent with just 2 min long incubations; it requires prolong preincubations and access from the serosal side. It indicates that it has nothing to do with a direct action on the transport system itself in the luminal membrane. Works by other authors make reference to the relatively low permeability of the intestinal wall to ouabain (15, 17), which explains the high concentrations of the glycoside required to obtain an inhibition of the substrate transport when it is present only on the mucosal side (10). Since its inhibitory action on Na^+, K^+. ATP-ase in intestinal epithelium of other species (8, 18), located in the serosal membrane, is well known, it seems reasonable to attribute a similar effect on snail intestine. It would support the interpretation of the ouabain induced inhibition on the Na^+ dependent sugar transport in accordance with the general model, postulated by data gathered essentially from mammal species.

As regards the capacity of the galactose transport through the different intestinal regions, no differences are observed when these refer to length, but an increase is seen towards distal position when it refers to wet weight of tissue, although the latter phenomenon might be related to the decrease in weight. At any rate, structural studies conducted on the snail intestinal wall report that the relative proportion of absorbing cells of the epithelium increases in the proximal-distal sense (1).

Resumen

Se ha medido la entrada neta de galactosa al tejido en anillos de intestino evertido de caracol, con tiempos de incubación de 2 ó 15 minutos. Con florricina 10^{-4} M se impide totalmente el transporte mediado y sólo se produce entrada pasiva del azúcar. Concentraciones inferiores del glucósido inhiben parcialmente el transporte de acuerdo con una cinética de inhibición competitiva ($K_i = 10^{-7}$ M). El transporte de galactosa es dependiente del Na^+. En ausencia de Na^+ el transporte cesa y la entrada de azúcar es explicable por difusión simple. Con Na^+ 15 mM (control 71,4 mM) el transporte está disminuido y se aprecia un aumento notable de la K_m aparente sin cambio en V_{max}. La harmalina 1 mM bloquea completamente el transporte de galactosa (0,5 mM). La ouabaína 1 mM también anula el transporte, pero sólo después de preincubación del tejido con el inhibidor en el lado serosal.

References

1. ABAURREA, A. and VÁZQUEZ, J. J.: *Morfol. normal y patol.*, **3**, 403-417, 1979.
2. ALVARADO, F., BROT-LAROCHE, E., L'HERMINIER, M., MURER, H. and STRANGE, G.: *Pflügers Arch.*, **382**, 35-41, 1979.
3. BARBER, A., JORDANA, R. and PONZ, F.: *Rev. esp. Fisiol.*, **31**, 119-124, 1975.

4. BARBER, A., JORDANA, R. and PONZ, F.: *Rev. esp. Fisiol.*, **31**, 125-130, 1975.
5. BARBER, A., JORDANA, R. and PONZ, F.: *Rev. esp. Fisiol.*, **31**, 271-276, 1975.
6. BARBER, A., JORDANA, R. and PONZ, F.: *Rev. esp. Fisiol.*, **35**, 243-248, 1979.
7. BROT-LAROCHE, E. and ALVARADO, F.: In «Intestinal Transport», (M. Gilles-Baillien and R. Gilles, eds.). Springer-Verlag, Berlin, 1983, pp. 147-169.
8. CHEZ, R. A., PALMER, R. R., SCHULTZ, S. G. and CURRAN, P. F.: *J. Gen. Physiol.*, **50**, 2357-2375, 1967.
9. DEAN, J. I., BARBER, A. and PONZ, F.: *XX Congreso S.E.C.F.*, Murcia, 1984, A. 290.
10. FORTH, W. and RUMMEL, W.: *Helv. physiol. pharmacol. Acta*, **25**, 8-23, 1967.
11. GROOT, J. A., ALBUS, H., BAKKER, R., SIEGENBEEK VAN HEUKELOM, J. and ZUIDEMA, Th.: In «Intestinal Transport» (M. Gilles-Baillien and R. Gilles, eds.). Springer-Verlag, Berlin, 1983, pp. 321-340.
12. HOPFER, U. and GROSECLOSE, R.: *J. Biol. Chem.*, **255**, 4453-4462, 1980.
13. HUETO, M. N., MARTÍNEZ, A. M., BARBER, A. and PONZ, F.: *Rev. esp. Fisiol.*, **38**, 403-408, 1982.
14. IM, W. B., MISCH, D. W. and FAUST, R. G.: *Biochem. Pharmacol.*, **29**, 2307-2317, 1980.
15. LAUTERBACH, F., GERBER, Ch., NITZ, D. and PRESCHER, K.: *Naunyn-Schmiedebergers Arch. Pharmak. exp. Path.*, **257**, 432, 1967.
16. MENG, K.: *Zool. Jber.*, **68**, 539, 1960.
17. NADAL, J., PROUS, J. and PONZ, F.: *Rev. esp. Fisiol.*, **23**, 241-242, 1967.
18. ROBINSON, J. W. L.: *J. Physiol.*, **206**, 41-60, 1970.
19. SEMENZA, G.: In «Intestinal Transport» (M. Gilles-Baillien and R. Gilles, eds.). Springer-Verlag, Berlin, 1983, pp. 184-187.
20. SEPÚLVEDA, F. V., BUCLON, M. and ROBINSON, J. W. L.: *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **295**, 231-236, 1976.
21. SEPÚLVEDA, F. V. and ROBINSON, J. W. L.: *Biochim. Biophys. Acta*, **373**, 527-531, 1974.
22. TANNENBAUM, C., TOGGENBURGER, G., KESSLER, M. ROTHSTEIN, A. and SEMENZA, G.: *J. Supramolec. Struct.*, **6**, 519-533, 1977.
23. TOGGENBURGER, G., KESSLER, M. and SEMENZA, G.: *Biochim. Biophys. Acta*, **688**, 557-571, 1982.