REVISTA ESPAÑOLA DE FISIOLOGIA, 47 (2), 69-74, 1991

Effect of DTNB on Rat Intestinal Galactose Transport *in vivo**

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(Received on December 17, 1990)

N. DIEZ, A. BARBER and F. PONZ. *Effect of DTNB on Rat Intestinal Galactose Transport* in vivo. Rev. esp. Fisiol., 47 (2), 69-74, 1991.

The effect of the non-penetrating reagent of -SH groups: acid 5,5'-dithiobis (2-nitrobenzoic), (DTNB), on 1 mM galactose absorption in rat intestine *in vivo* has been studied. DTNB inhibits sugar absorption in about 35 %, which is due to an action on the mediated transport component, but without affecting the diffusional passive one. Consequently it does not modify galactose absorption in the presence of 0.5 mM phlorizin or that of the non-transportable sugar 2-deoxy-glucose. Galactose transport inhibition appears after a not longer than 5 min preexposure period and it remains constant at least up to 30 min. The inhibitory effect does not vary between 0.1 and 1 mM DTNB and it reverses completely with 0.5 mM dithioerythritol. Protection by excess of substrate has not been observed. Results show that DTNB affects sulfhydryl groups very probably located at the luminal side and related to the proteins of the cotransport system.

Key words: DTNB, Intestinal absorption, Galactose, Transport, Rat.

Intestinal active transport of sugars implies a sequential translocation of the substrate across the luminal and basolateral membranes of the enterocytes. Penetration into the cytosol from lumen depends on a Na^+-sugar cotransport system asymmetrically located in the microvillous membrane. Previous works carried out in our laboratory have shown that the heavy metals Hg, Zn, Cd and Cu inhibit sugar and amino acid active transport both *in vivo* (11, 13-16) and *in vitro* (8, 9), which has been attributed to an interaction with -SH groups of transport proteins.

By using specific reagents of distinct penetrability with brush border membrane vesicles, the importance of the sulfhydryl groups for the cotransporter function has been shown and its likely spacial location in the membrane has been suggested (1, 7,

* Supported by a grant from the Spanish DGICYT (PB86-0407).

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18). However, vesicle preparation entails the loss of the epithelial structure as well as cellular disorganization with possible changes in the reactivity and accessibility of the aforesaid -SH groups. Therefore, the study of the effect of acid 5,5'-dithiobis (2-nitrobenzoic) (DTNB), a very slightly penetrating reagent which combines specifically with -SH groups (3, 4, 7) on sugar intestinal absorption in intact intestine *in vivo*, has been deemed of interest.

Materials and Methods

Intestinal absorption by the jejunum of white Wistar rats (180-225 g), fasted for 24 h, has been measured according to the *in vivo* luminal perfusion technique of PONZ *et al.* (12). Briefly, a proximal jejunum segment of about 20 cm was perfused *in situ*, without recirculation (single pass), at 5.6 ml/min rate (peristaltic pump), with NaCl 0.9 % solution buffered with Tris-HCl (7.4 pH) to which sugar or reagents to be studied were added.

Generally three successive absorption periods of two minute duration in equal experimental conditions were carried out in each animal. Between one condition and a different one, intermediate washings of the jejunal segment with saline solution for 2 or 5 min were performed and the residual content was emptied by pumping air for 2 min. The absorbed sugar, determined by measuring radioactivity, was estimated from the difference between the content in the perfusion fluid both before and after its passage through the intestine, and it is expressed in µmoles/cm/min. The sugars D-(1-^{14}C)-galactose (59.6 mCi/mmol) and 2-(1-^{14}C)-deoxy-D-glucose (58.0 mCi/mmol) were purchased by Radiochemical Dupont. Other products were D(+)-galactose (Merck), dithioerythritol (Aldrich, Chemie), 2-deoxy-D-glucose, phlorizin and DTNB (Sigma).

Oxygen uptake measurement in everted intestinal rings (2) have also been verified according to Warburg's direct method (19).

Results

The effect of 0.25 mM DTNB on 1 mM galactose absorption has been studied, discriminating the transport and passive components. Firstly, the total absorption was determined and thereafter the passive one (by blocking transport with 0.5 mM phlorizin) in control conditions. Subsequently the mucosa was preexposed to the reagent for 5 min by passing the perfusion solution containing 0.25 mM DTNB. Once the intestine had been emptied by air passage, the total and passive absorption was determined in the presence of the reagent. Results (fig. 1) show that DTNB inhibits sugar absorption in about 34.8 % and it does not significantly modify the passive component in the presence of phlorizin. Similar experiments with 1 mM 2-deoxy-D-glucose, a non-transportable sugar, showed that its passive absorption was not altered by the presence of 0.25 mM DTNB either. It is, therefore, deduced that the inhibition by DTNB on galactose absorption is exerted only on the

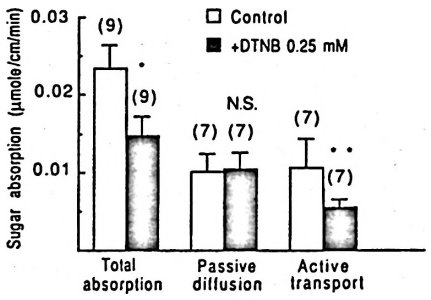

Fig. 1. *Effects of DTNB on the total absorption, passive diffusion in the presence of 0.5 mM phlorizin, and active transport of 1 mM D-galactose.* Vertical bars denote S.E. of the mean of the number of separate determinations given on the top of the histograms. *: $0.01 < p \leq 0.05$; **: $p \leq 0.01$.

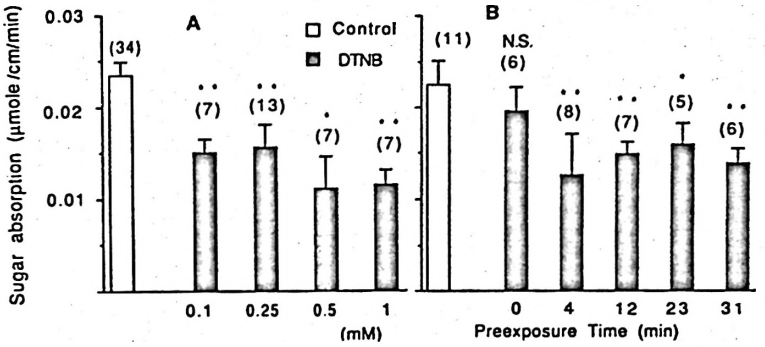

Fig. 2. *Influence of DTNB concentration (A) and preexposure time (B) on the inhibition of 1 mM galactose absorption.*
Statistical significance and symbols as in figure 1.

mediated transport component which decreases in somewhat more than 60 %.

As DTNB reactivity seems better at 8 pH (1, 3), experiments at this pH were also carried out. Results were not significantly different from those previously described at 7.4 pH.

Influence of DTNB concentration and exposure time. — The effect of various DTNB concentrations on 1 mM galactose absorption has been determined (fig. 2A). With as low as 0.1 mM DTNB the inhibition is manifest (35 %) and similar to the one obtained with 0.25 mM (38.8 %). At 0.5 mM and 1 mM concentrations, the inhibition seems somewhat higher although the differences are not statistically significant.

As to the time the reagent stays in contact with the mucosa, fig. 2B shows that without preexposure the inhibition is not appreciable and that preexposures between 4 and 31 min cause a similar inhibition.

Reversibility of the DTNB effect. — In order to evaluate the possible reversion of the inhibition by DTNB, sugar absorption has been measured after washing the intestine previously exposed to the reagent, with perfusion solution (PS) either alone or with 0.5 mM dithioerythritol (DTE), a reagent which specifically reduces the disulfide bridges (21). In previous experiments DTE at 0.5 mM concentration was proven not to modify the sugar absorption. Results (fig. 3) show that the washing of the intestinal segment with PS for 5 or even 15 min does not modify the inhibition exerted by DTNB on galactose absorption, while the inhibition

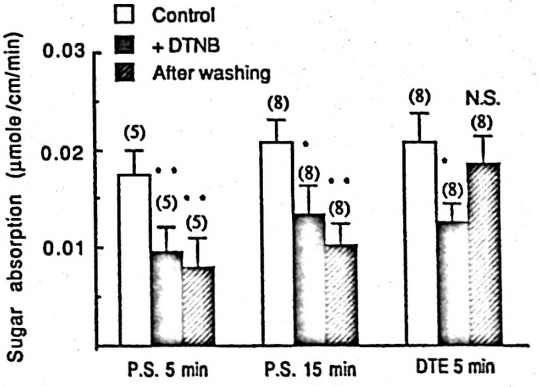

Fig. 3. *Inhibition of galactose absorption by 0.25 mM DTNB, absence of reversion by washing with physiological solution (P. S.) and reversion by 0.5 mM DTE.*
Statistical significance and symbols as in figure 1.

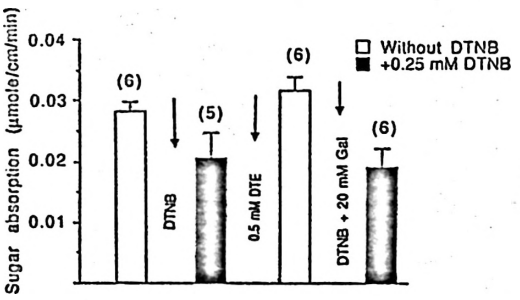

Fig. 4. *Non protection by substrate (20 mM galactose) against DTNB inhibition.* Arrows mean single pass during 5 min of the indicated reagents. Statistical significance and symbols as stated in figure 1.

disappears if the washings are carried out with 0.5 mM DTE (fig. 3).

Non protection by the substrate. — Although the -SH groups do not seem to be involved in the sugar binding site in the cotransporter, the possible protection by excess of substrate from the inhibition by DTNB has been studied. Results (fig. 4) indicate that 1 mM galactose absorption following the simultaneous preexposure of the mucosa to both 20 mM galactose and 0.25 mM DTNB does not significantly differ from the one obtained when the preexposure was in the presence of DTNB alone.

Oxygen uptake by the tissue. — Oxygen uptake by everted jejunal rings in the presence of 10 mM glucose is lineal during 60 min experimental time and it is not modified by 0.25 mM DTNB statistically.

Discussion

The present results show that DTNB inhibits galactose absorption in rat intestine *in vivo*. The DTNB action is exerted only on the active absorption component and not on the passive diffusional entry. The active transport is not totally blocked. Similar results were obtained with DTNB in vesicles (6, 7) the inhibition being attributed to its reaction with -SH groups located on the external side of the membrane.

In the range of 0.1 to 1 mM concentrations, transport inhibition by the reagent remains almost constant which suggests that all the -SH groups that could react with DTNB, have done so with the smallest of the concentrations.

As it had been observed in our laboratory on the influence of heavy metals in sugar absorption *in vivo* and *in vitro* (8, 14-16), a short preexposure period of the mucosa to the reagent is required for the inhibitory effect of DTNB on absorption to appear. This may be due to the possible difficulty of access to the -SH groups essential for sugar transport and to the required time for reaching the reaction equilibrium (5). With a 4 min preexposure, inhibition is already clear and does not appreciably increase with longer periods.

DTE has widely been used to reverse the inhibition due to -SH group reagents in *in vitro* (6, 7) and *in vivo* (14) systems. In the present work, the obtained reversion was complete. Since 0.5 mM DTE did not modify galactose absorption, our results confirm that inhibition by DTNB can be explained by oxidation of the membrane thiol groups; the disulfide bridges or the resulting intermediate compounds would again be reduced by DTE action (3, 21).

Sulfhydryl reagents often disturb cellular metabolism (10, 17, 20). Oxygen uptake experiments reveal that galactose transport inhibition by DTNB cannot be attributed to effects on oxidative metabolism, in keeping with the very low penetrating ability of this reagent. Relevant epithelial desquamations have not been observed either.

Protection by the substrate (sugar) has not been observed, which seems to indicate that the -SH groups reacting with

DTNB are not located within the sugar-binding site. Taking into account the low liposolubility of the reagent, the thiol groups affected by DTNB should be located at the outer surface of the membrane or more deeply but surrounded by a hydrophilic environment.

It may be concluded that DTNB inhibits galactose absorption by interaction with sylfhydryl groups accesible from the luminal side which belong to the proteins of the transport system hindering its function.

Resumen

Se estudia el efecto del reactivo de grupos −SH no penetrante: ácido 5,5'-ditiobis-(2-nitrobenzoico), (DTNB), sobre la absorción de galactosa 1 mM en intestino de rata *in vivo*. El DTNB inhibe en un 35 % la absorción del azúcar, lo que se debe a acción sobre el componente de transporte mediado, sin que se afecte el pasivo difusional. Así, no modifica ni la absorción de galactosa no sensible a florricina ni la del azúcar no transportable 2-d-glucosa. La inhibición del transporte de galactosa se manifiesta tras un período de preexposición no superior a 5 min y se mantiene constante al menos hasta los 30 min. El efecto inhibidor no varía entre 0,1 y 1 mM y se revierte por completo con DTE 0,5 mM. No se observa protección por exceso de sustrato. Los resultados indican que el DTNB afecta a grupos sulfhidrilo de proteínas de membrana, localizados probablemente en la cara luminal y relacionados con las moléculas implicadas en el sistema de cotransporte.

Palabras clave: DTNB, Absorción intestinal, Galactosa, Transporte, Rata.

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