Biochimica et Biophysica Acta, 455 (1976) 126–143

BBA 77484

TRANSPORT AND PHOSPHORYLATION OF 2-DEOXY-D-GALACTOSE IN RENAL CORTICAL CELLS

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(Received April 5th, 1976)

SUMMARY

The transport and phosphorylation of 2-deoxy-D-[^{3}H]galactose in rabbit renal cortical cells was studied.

1. The uptake of 2-deoxy-galactose by cortical slices is associated with an appearance of both free and phosphorylated sugar in the cells. At 1 mM external sugar the cells establish a steady-state gradient of free 2-deoxy-galactose of 3.97 ± 0.15 (23 animals).

2. The acid-labile sugar phosphate accumulated in the tissue has been identified by a combination of paper and radio-chromatography, as well as on the basis of some of its chemical properties, as 2-deoxy-D-galactose 1-phosphate. Ice-cold trichloroacetic acid produces a decomposition of this compound.

3. Increasing external pH (6–8) brings about a decrease in the steady-state levels of both free and phosphorylated sugar in slices. On the other hand, increasing pH activates the phosphorylation of 2-deoxy-D-galactose by a crude kinase in a tissue extract.

4. Sugar phosphate accumulated in the cells is dephosphorylated by the action of a Zn^{2+}-activated phosphatase.

5. The effiux of 2-deoxy-D-galactose from the cells is rather slow compared with that found for D-galactose. The efflux is associated with some dephosphorylation of cellular sugar phosphate, and some loss of 2-deoxy-galactose phosphate into the wash-out medium takes place.

6. An inhibition analysis of the uptake of 2-deoxy-D-galactose by the slices indicates that the transport site is shared by D-galactose. The following points of interaction between the sugar molecule and the carrier are identified: C_1-OH, C_3-OH and C_4-OH (both axial) and C_6-OH. A (pyranose) ring structure is also essential. A close packing between the substrate and the carrier in the vicinity of C_2 is indicated.

7. The data suggest that the above transport system is localized predominantly at the antiluminal (basolateral) face of the renal tubular cells. While the detailed

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Abbreviations: Gal, D-galactose; 2-d Gal, 2-deoxy-D-galactose; 2-d Gal-1-*P*, 2-deoxy-D-galatose 1-phosphate; 3-d Gal, 3-deoxy-D-galactose; 2-d Glc, 2-deoxy-D-glucose; 2-d Glc-6-*P*, 2 deoxy-D-glucose 6-phosphate; TES, *N*-tris (hydroxymethyl) methylaminoethane sulphonic acid.

mechanism of the actual transport step (i.e. active transport of the free sugar, or by the action of a phosphotransferase) is still unclear, the data present evidence that both galactokinase and a Zn^{2+}-activated phosphatase participate in the maintenance of an intracellular steady state of the transported sugar.

INTRODUCTION

2-Deoxy-D-galactose was shown to be actively accumulated in slices of rabbit and rat renal cortex [1, 2] by a Na^+-independent transport mechanism; D-Galactose competes for a shared transport site [3]. However, major discrepancies became apparent when the transport of these two related sugars was investigated in more detail, namely: (1) in spite of the competitive inhibition of 2-deoxy-D-galactose transport by galactose, kinetic data demonstrated a lack of agreement between the respective values of K_t and K_i [2, 3]; (2) D-glucose, a competitive inhibitor of the transport of D-galactose, failed to inhibit the transport of 2-deoxy-D-galactose even at a 50-fold molar excess [2]; (3) increasing the external pH produced opposite effects in the accumulation of 2-deoxy-D-galactose and D-galactose in the tissue, i.e. decreasing the former and activating the latter [6]. Such data led to the suggestion that several transport pathways with overlapping specificities are involved in the active transport of these sugars [2, 4]. Such conclusion was supported by more recent data when it was demonstrated by micropuncture that 2-deoxy-D-galactose was not reabsorbed from the proximal convoluted tubule of the rat kidney whereas D-galactose was readily taken up at the brush border of the tubular cells [5]; however, by the clearance technique a reabsorption of 2-deoxy-D-galactose from the glomerular filtrate was readily found [6].

The availability of 2-deoxy-D-[^{3}H]galactose [7] and of a relatively simple analytical procedure for the determination of free and phosphorylated sugars in tissue extracts [8] prompted a more detailed investigation of the transport properties of this hexose. It will be shown that the specificity pattern for the active transport of 2-deoxy-D-galactose, described here, is identical with that demonstrated for the transport of this sugar across the antiluminal face of the renal tubular cells of the flounder [9, 10]. A considerable portion of 2-deoxy-D-galactose entering the cells is present as 2-deoxy-D-galactosyl-1-*P*.

Some preliminary data have been presented [11].

METHODS AND MATERIALS

Experiments described here were carried out using the renal cortex of healthy adult rabbits.

Experimental

Slices. The procedure for the incubation of renal cortical slices was that used in such investigation in this laboratory [2, 8]. The uptake of 2-deoxy-D-galactose was studied as follows: Groups of slices were preincubated 45 min under standard conditions (O_2, 25 °C) in 50-ml conical flasks containing 8 ml of saline of the Krebs-Ringer type buffered at pH 7.2 with a Tris/TES buffer mixture (13 mM, final conc), thus

establishing a steady state of cellular solutes. Where required, the pH of the saline was varied by the adjustment of the proportions of both buffer constituents. The slices were then transferred into flasks containing salines with 1 mM 2-deoxy-D-[^{3}H]galactose (usually 0.1 μCi/ml), without (controls) or with other additions. In the blotted and weighed slices total, free and phosphorylated sugar was determined by the previously established method [8]. Evidence for the applicability of the analytical procedure will be presented in Results. The mean values for a group of slices are expressed in μmol sugar/g tissue wet weight $\pm$ S.E. In several instances the apparent intracellular concentration (S_i) of free and phosphorylated sugar were assessed after correction for the extracellular (inulin) space, 0.25 kg per kg tissue wet weight (see ref. 1). From these data and the final external sugar concentration (S_0) the apparent accumulation ratio of the free sugar, S_i/S_0 could be evaluated.

Efflux experiments were carried out as described previously [12, 8]. The values of tissue free and phosphorylated sugar were determined before and after the wash-out of the sugar, and the rate constants were evaluated.

The inhibition analysis was used to investigate the structural requirements for the interaction of 2-deoxy-D-galactose with the transport carrier. This approach is based on the assumption that an analog exhibiting an inhibition of the substrate uptake by the tissue shares the involved transport site; conversely, a lack of an inhibitory effect signals that the analog molecule lacks a specific structural group in the appropriate steric configuration. Kinetic data obtained with slices are affected by the complex geometry of the preparation. This applies particularly to short periods of incubation, thus preventing a meaningful measurement of the kinetic parameters. Therefore the tissue uptake of 1 mM 2-deoxy-D-galactose after 60 min incubation was followed without (controls) and with 5 mM analogs. The statistical significance of the difference of means for total, free and phosphorylated sugars was then evaluated. An interpretation of the data has to take into account two complicating aspects: (1) The polarity of renal epithelial cells appears to be characterized by anatomically separate transport pathways for a given substrate with differing specificities [2, 13, 10]. (2) As discussed below, in the case of 2-deoxy-D-galactose, transport and cellular metabolism of the substrate, and the effects of the studied analogs on both these processes, should be considered. In the absence of detailed information on the nature, specificity and kinetic parameters of the reactions involved in substrate metabolism subsequent to the actual transport step, the inhibitory effect of analogs on the total uptake of 2-deoxy-D-galactose by the tissue, i.e. on the rate-limiting process, appears to be most informative.

Substrate phosphorylation by tissue extracts. A membrane-free extract of renal cortex was employed. For preparation and assay conditions see ref. 8. In the deproteinized assay mixture phosphorylated 2-deoxy-D-galactose was determined after separation of this compound on a Dowex 1 column (see below). The results are expressed in μmol 2-deoxy-D-galactose phosphate formed per mg protein per min.

Materials

Sugars. The preparation and purification of 2-deoxy-D-[^{3}H]-galactose has been described elsewhere. Unless otherwise stated, sugars of the highest available quality were purchased commercially and their purity was checked by paper chromatography. Where required, the compounds were recrystallized. The authors are indebted for

generous gifts of the following sugars: 2-*O*-methyl-D-galactose, Dr. J. Chittenden, Nijmegen, The Netherlands; 3-deoxy-D-galactose, Dr. J. E. G. Barnett, Nottingham, England; 1,2;5,6-di-*O*-isopropylidene-D-gulose, Dr. K. N. Slessor, Burnaby, B. C., Canada; the free sugar was prepared from this substance by treating a solution with Amberlite IR-120 (H^+) [14].

Hog intestinal phosphatase (Type IV, approx. 1 unit/mg) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other materials and reagents were commercial preparations.

RESULTS

The uptake and phosphorylation of 2-deoxy-D-galactose in renal cortical cells

Fischer and Weidemann [15] have shown that 2-deoxy-D-galactose is metabolized in yeast and an acid-labile 2-deoxy-D-galactose 1-phosphate is formed. Preliminary experiments indicated major discrepancies in the amounts of the free and phosphorylated sugar found in renal cortical slices when comparing two methods of extraction and separation of free and phosphorylated tissue sugar (see ref. 8). The validity of the employed analytical procedure, i.e. (i) extraction of total (free plus phosphorylated) tissue sugar with boiling water, (ii) determination of free sugar after removal of sugar phosphate by the $ZnSO_4$ plus $Ba(OH)_2$ treatment, and (iii) calculation of the latter as the difference between total and free tissue sugar, was established as follows: Four groups of slices (6 per flask) were incubated aerobically (O_2) for 60 min at 25 °C in saline containing 1 mM 2-deoxy-D-[^{3}H]galactose (0.1 μCi/ml). In each blotted and weighed slice (25–75 mg wet wt.) total, free and phosphorylated sugar was determined by one of the following procedures.

(a) Each slice was extracted with 3 ml boiling water for 10 min. The tissue was then homogenized, the suspension was centrifuged and in the supernatant the activity corresponding to total (free plus phosphorylated) tissue sugar was determined. 2 ml of the aqueous extract was then treated with 1 ml 0.15 M $ZnSO_4$ and 0.15 M $Ba(OH)_2$, thus removing phosphorylated sugar; this allowed the assay of free tissue sugar in the supernatant [8]. The difference between total and free tissue sugar (in μmol/g tissue wet wt.) corresponds to phosphorylated substrate.

(b) The slices were extracted at 0 °C with 5 % (w/v) trichloroacetic acid for 30 min. After exhaustive extraction of trichloroacetic acid with ethyl ether the activity corresponding to total tissue sugar was determined. 2 ml of the extract were then passed through a 5×50 mm column of Dowex 1-X8 (Cl^- form, 100–200 mesh) and the column was washed twice with 1 ml water. In the combined eluate free 2-deoxy-galactose was assayed. The acidic sugar product was then eluted from the column with 2 ml 2 M HCl, followed by 2 ml water, and was determined in the combined eluate. The mean values ± S.E. are given in Table I.

Whereas the two extraction techniques yielded identical values for total tissue sugar, major discrepancies appeared when comparing values for free and phosphorylated sugar: Practically no phosphorylated 2-deoxy-D-galactose was detected in the trichloroacetic acid extract, and the values for free sugar were close to those of total. In contrast, phosphorylated sugar represented approx. 40 % of total tissue sugar when the tissue was extracted with boiling water.

Evidence that this discrepancy was due to a breakdown of an acid-labile

TABLE I

COMPARISON OF ANALYTICAL PROCEDURES FOR THE DETERMINATION OF FREE AND PHOSPHORYLATED 2-DEOXY-D-GALACTOSE IN RENAL CORTICAL SLICES

Four groups of slices (6 per flask) were incubated 60 min aerobically (O_2) at 25 °C in saline containing 1 mM 2-deoxy-D-[^{3}H]galactose (0.1 μCi/ml). For details of the extraction and separation procedures a–d for the determination of total, free and phosphorylated 2-dGal, see Methods and Materials. Values (in μmol/g tissue wet wt.) are given for the mean ±S.E. of data obtained from each slice.

Procedure	Extraction	Separation	Tissue sugar		
			Total (μmol/g)	Free (μmol/g)	Phosphorylated (μmol/g)
a	water, 100 °C	$ZnSO_4$+$Ba(OH)_2$	4.37±0.15	2.54±0.21	1.76±0.27
b	5 % trichloroacetic acid, 0 °C	Dowex 1	4.08±0.22	4.00±0.28	0.09±0.01
c	water, 100 °C	Dowex 1	4.17±0.08	2.56±0.05	1.75±0.05
d	water, 100 °C then 5 % trichloroacetic acid, 0 °C	Dowex 1	4.06±0.16	4.49±0.21	0.15±0.01

anionic metabolic product of 2-deoxy-D-galactose in the course of its extraction from the tissue with ice-cold trichloroacetic was provided as follows:

(c) A further group of slices was extracted with boiling water, as above, and a portion of the turbid extract was used for the separation of free and phosphorylated sugar on a Dowex 1 column (see procedure b).

(d) A group of slices was again extracted with boiling water. A portion of the ice-cold extract was then acidified with trichloroacetic acid (final concentration 5 %) and maintained on ice for 30 min. The precipitated proteins were spun off and the supernatant, after removal of the acid with ether, was used for the separation of free and phosphorylated sugar on a Dowex column (see b). As shown in Table I, values obtained by procedure c were in reasonable agreement with those yielded by the $ZnSO_4$-$Ba(OH)_2$ method, proving that the breakdown of the anionic sugar product was not produced by the ion-exchange resin. Procedure d then demonstrated that acidification of the aqueous extract led to the breakdown of the metabolic production of 2-deoxy-D-galactose.

The above experiment indicates that the use of ice-cold trichloroacetic acid for the extraction of tissue sugars and their metabolic products may lead to erroneous results when acid-labile metabolites are present.

The nature of the acid-labile metabolite of 2-deoxy-D-galactose

Evidence that 2-deoxy-D-galactose 1-phosphate is the metabolic product of 2-deoxy-D-galactose in renal cortical cells was provided as follows: Slices (approx. 200 mg) were first incubated aerobically (O_2) for 45 min at 25 °C in saline labelled with $^{33}PO_4^{2-}$ (0.2 μCi/ml) in order to assure an equilibration of the specific activities of phosphates in the medium and the cells. The tissue was then transferred into fresh saline of the same composition containing also 2-deoxy-D-galactose [^{3}H] (2 μCi/ml) and the flask was incubated aerobically for 60 min. After blotting and weighing the slices were placed into 4 ml boiling water for 10 min; the tube contents were homogenized and centrifuged. The supernatant was used for further tests. On each of two 12-cm wide strips of Whatman paper No. 1 the following spots were placed: (1) 0.05 μmol non-labelled 2-deoxy-D-galactose; (2) 25 μl of the tissue extract; (3) μl tissue extract which had previously been treated for 30 min at 37 °C with 0.5 mg (i.e. 0.5 unit) of intestinal alkaline phosphatase; the action of the enzyme was stopped by placing the mixture for 10 min into boiling water. The chromatograms were then developed overnight (descending technique) using two solvent systems [15, 16]: (a) *n*-propanol/NH_4OH/ water (6 : 3 : 1, v/v); (b) 95 % ethanol 1 M ammonium acetate (pH 5.0) (7 : 3, v/v). After drying the papers were cut lengthwise to separate the chromatograms corresponding to the three spots. The location of the spot of 2-deoxy-D-galactose was detected [17] with alkaline $AgNO_3$. The activity on the other two chromatograms was determined in 1 cm strips and the results were expressed as percent of the total cpm spotted on the paper. Identical results were obtained with both solvent systems.

Fig. 1 shows two distinct peaks of ^{3}H activity: The larger peak with the smaller R_f (0.33) corresponded to that of ^{33}P, suggesting the presence of a phosphate ester of 2-deoxy-D-galactose. The larger peak (R_f 0.67) coincided with the spot of authentic 2-deoxy-D-galactose and thus represents free sugar. Treatment with alkaline phosphatase eliminated ^{3}H from the first peak and a corresponding increase in the

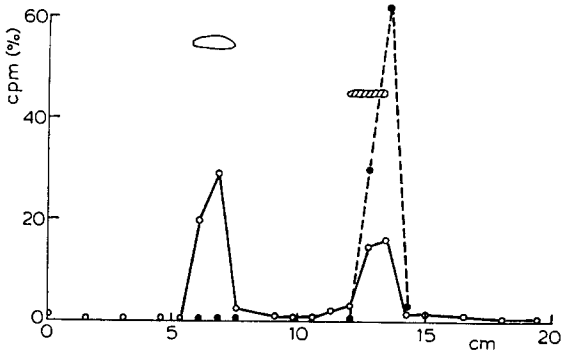


Fig. 1. Radio-chromatographic identification of the acid-labile metabolic product of 2-deoxy-D-galactose. Slices were incubated 60 min aerobically (O_2) in saline containing 1.3 mM $^{33}PO_4^{2-}$ (0.2 μCi/ml) and 1 mM 2-deoxy-D-[^{3}H]galactose (2 μCi/ml). Samples of aqueous tissue extracts were spotted on Whatman paper No. 1 and were developed by the descending technique using a propanol/NH_4OH/water solvent mixture. For experimental details see text. Abscissa: cm from start. Ordinate: cpm ^{3}H (in percent of total activity placed on the paper). ○—○, tissue extract; ●—●, tissue extract after treatment with alkaline phosphatase. Spots: clear, activity of ^{33}P on chromatogram; shaded, authentic 2-deoxy-D-galactose.

peak of free 2-deoxy-D-galactose occurred. This result thus confirms the presence of 2-deoxy-galactosyl-phosphate in the tissue extract. With the above solvent system the peaks of inorganic and sugar phosphates are so close that no distinction could be made as to the size of the ^{33}P peak of the sugar phosphate before and after phosphatase treatment. No evidence for the presence in the extract of fluorescent-labelled components (indicative of UDP-2-deoxy-galactose) or of 2-deoxy-D-glucose was obtained (cf. ref 15).

Experiments not given here in detail showed that treatment of the tissue extract with 5 % trichloroacetic acid had the same effect as phosphatase. This observation thus confirmed data obtained in the course of verifying the employed analytical technique (see Table I above).

Further information concerning the structure of the phosphate ester of 2-deoxy-D-galactose was provided by a comparison of the hydrolysis of sugar phosphates by acids. As opposed to the relatively acid-stable hexose 6-phosphates, hexose 1-phosphates are readily hydrolyzed by 1 M HCl (at 100 °C) (see, e.g. ref 16). The acid lability of the above compound (see also ref. 15) contrasts with the relative stability of synthetic 2-deoxy-D-galactose 6-phosphate to hydrolysis in 0.05 M H_2SO_4 at 100 °C [18]. The present data thus indicate that 2-deoxy-D-galactose 1-phosphate is the metabolic product of 2-deoxy-D-galactose in renal tissue. The great acid lability, comparable to that found for 2-deoxy-D-ribose 1-phosphate [19] as well as for methyl-glycosides of 2-deoxy-sugars [20] suggests the possibility that the reactivity of both C_1 derivatives of deoxy-sugars is due to a removal of a large inductive effect of C_2-OH (see ref. 20).

Active transport of 2-deoxy-D-galactose in renal cortical cells

The time-curve of sugar uptake presented in Fig. 2 shows that at $S_0 = 1$ mM the cells established a concentration gradient of the free sugar within 15 min of

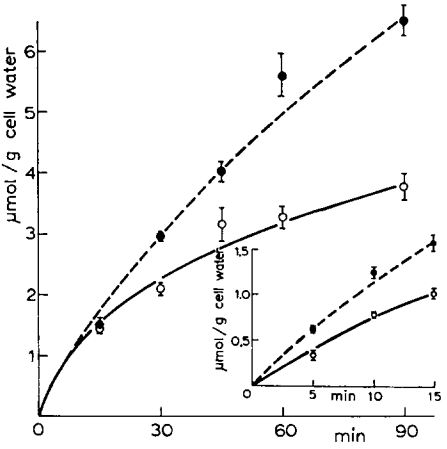


Fig. 2. Uptake of 2-deoxy-D-galactose by renal cortical cells. Groups of slices (5–6 per flask) were incubated under standard conditions (O_2, 25 °C) for varying periods of time in saline containing 1 mM 2-deoxy-D-[^{3}H]galactose (0.05 µCi/ml). Apparent intracellular concentrations (in µmol/g cell water, ±S.E.) of free (○) and phosphorylated (●) sugar are given. Insert: Results of an experiment with shorter times of incubation.

incubation; after 60 min, the concentration gradient approached a steady-state level. The mean value of S_i/S_0 of 3.97±0.14 (23 animals) at 60 min was somewhat lower than that previous reported. The difference can be accounted for by the action of a tissue phosphatase (see below). The apparent cellular concentration of 2-deoxy-D-galactose phosphate increased practically linearly during the 90 min of incubation. The insert on Fig. 2 shows that even at short incubation periods (5–15 min) the cellular level of phosphorylated sugar was higher than that of free 2-deoxy-D-galactose.

Eeffct of pH on transport and phosphorylation of 2-deoxy-D-galactose

It has been shown previously that as opposed to some other sugars such as

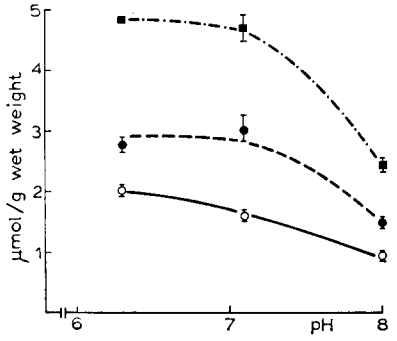


Fig. 3. pH effect on the accumulation of 2-deoxy-D-galactose in renal cortical cells. Groups of slices (5 per flask) were first incubated 45 min aerobically (O_2) at 25 °C in salines at pH (initial values) 6.18, 7.18 and 8.25. The pH was varied using appropriate mixtures of 0.308 M Tris and TES; the final concentration of the buffer mixture was 13 mM. The tissue was then incubated under identical conditions for 60 min in salines containing 1 mM 2-deoxy-D-[^{3}H]galactose (0.1 µCi/ml). Mean values of total (●), free (○) and phosphorylated (■) tissue sugar (in µmol/g tissue wet wt. ±S.E.) are given, as well as final pH values of the media.

TABLE II

EFFECT OF pH ON THE PHOSPHORYLATION OF 2-DEOXY-D-GALACTOSE BY A MEMBRANE-FREE TISSUE EXTRACT

The reaction mixture contained in a final volume of 2 ml the following (final concentration): 0.15 M KCl; 1 mM $MgCl_2$; 1 mM Na_2-EDTA; 1 mM dithiothreitol; 2.5 mM ATP; 2.5 mM 2-deoxy-D-[^{3}H]-galactose (0.1 μCi/ml); 30 mM Tris/TES buffer mixture of appropriate pH, tissue extract (1 mg protein). Incubation: 37 °C for 10 min. Values: μmol sugar phosphate formed per mg protein per min.

pH of assay mixture (final)	Sugar phosphate (μmol/mg protein per min)
6.02	0.020
6.62	0.085
7.24	0.163
7.86	0.148

2-deoxy-D-glucose or D-galactose, increasing saline pH (and a concomitant increase in cellular pH) depressed the cellular accumulation of 2-deoxy-D-galactose [4]. Information on the pH effect on cellular levels of free and phosphorylated sugar might shed some light on the mechanism of such pH effect.

Fig. 3 shows that increasing saline pH from 6.18 to 8.25 (values measured before incubation of the tissue) produced a decrease in free tissue sugar. The accumulation ratio, S_i/S_o, decreased from 4.3 to 1.55. A significant decrease in the tissue level of 2-deoxy-D-galactose 1-phosphate was observed only when the external pH was increased above 7.2. The experimental data are consistent with the view that external pH affects the actual transport mechanism. When the effect of pH on the activity of a crude kinase in a cell-free extract of the renal cortex was tested (Table II) an increased phosphorylation of 2-deoxy-D-galactose was found. A similar pH effect was observed when D-galactose served as substrate (see ref. 8, Fig. 6). The rate of phosphorylation of 2-deoxy-D-galactose was only 25–33 % of that found for D-galactose as substrate.

The dephosphorylation of cellular 2-deoxy-D-galactose 1-phosphate

Evidence that intracellular 2-deoxy-D-galactose phosphate is dephosphorylated by the action of a (Zn^{2+}-activated) phosphatase was obtained as follows: At room temperature the slices were allowed to be in contact with 0.05 M $ZnSO_4$ for 60 min prior to the precipitation of proteins and sugar phosphate by the addition of $Ba(OH)_2$. Under these conditions the values of free tissue sugar were nearly twice as high as those obtained by the present analytical procedure (Table III). Thus, an intracellular enzyme produced a dephosphorylation of 2-deoxy-D-galactose. It may be assumed that the involved enzyme is a Zn^{2+}-activated alkaline phosphatase shown elsewhere [8] to produce the dephosphorylation of intracellular galactose 1-phosphate.

The efflux of 2-deoxy-D-galactose from slices

Experiments were performed in order to enquire whether the wash-out of sugar from the cells is associated with a dephosphorylation of cellular 2-deoxy-D-galactose 1-phosphate; such process has been shown to take place with D-galactose as substrate [8]. Data given in Table IV established that in the course of the sugar efflux, a net decrease of both free and phosphorylated tissue sugar occurred. The amount of free

TABLE III

TISSUE 2-DEOXY-D-GALACTOSE: EFFECT OF A Zn-ACTIVATED CELLULAR PHOSPHATASE

Groups of slices (6 per flask) were loaded with 2-deoxy-D-[^{3}H]galactose by aerobic incubation of the tissue (O_2) for 60 min in saline containing 1 mM sugar. In the blotted and weighed tissue the sugar was determined by two procedures: (A) the slices were placed for 10 min in 3 ml boiling water and the extract was treated with $ZnSO_4$ plus $Ba(OH)_2$ (see Methods and Materials). Values of free and phosphorylated sugar are given. (B) The tissue was placed for 60 min into 3 ml 0.05 M $ZnSO_4$, then homogenized and the proteins plus sugar phosphate were precipitated by the addition of 1 ml 0.15 M $Ba(OH)_2$. Free tissue 2-deoxy-D-galactose was assayed in the supernatant. Mean values ± S.E. are given.

Procedure	Tissue sugar (μmol/g)	
	Free	Phosphorylated
A	1.84±0.08	2.82±0.18
B	3.41±0.13	—

TABLE IV

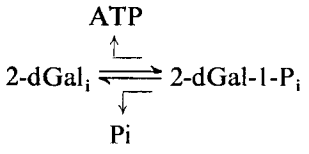
BALANCE SHEET OF TISSUE 2-DEOXY-D-GALACTOSE DURING AN EFFLUX EXPERIMENT

16 slices were loaded with 2-deoxy-D-[^{3}H]galactose by 60 min aerobic incubation at 25 °C in saline containing 1 mM sugar (0.2 μCi/ml). Eight slices were taken for the determination fo initial tissue sugar. The remaining tissue was used to follow the wash-out of the sugar at 25 °C into a series of tubes, each containing 10 ml sugar-free saline. The final value of tissue sugar was then determined. Total, free and phosphorylated 2-deoxy-D-galactose in the wash-out media was also analyzed using both the $ZnSO_4$ plus $Ba(OH)_2$, and the ion-exchange techniques. Normalized data in μmol/g tissue wet wt. ±S.E. are given.

	2-Deoxy-D-[^{3}H]galactose (μmol/g)		
	Total	Free	Phosphorylated
Tissue: initial	4.49±0.04	1.58±0.05	2.89±0.06
Tissue: final	3.15±0.05	1.38±0.08	1.76±0.05
Efflux media	1.83	1.25	0.58

2-deoxy-D-galactose found in the efflux media in the course of 90 min, i.e. 1.25 μmol/g tissue, exceeded by a factor of five the observed decrease in free tissue sugar and this excess could have originated only from the sugar phosphate. It should be noted that, as opposed to data found for D-galactose, the renal tubular cells appear to be somewhat leaky for 2-deoxy-D-galactose phosphate: About 50 % of the loss of tissue sugar phosphate could be accounted for by the appearance of an anionic derivative of 2-deoxy-D-galactose. This compound was systematically found in the wash-out media at each time interval of the experiment by both the $ZnSO_4$ plus $Ba(OH)_2$, and the ion-exchange techniques for the separation of free sugar from its phosphate. The recovery of counts was within the limits of error for such experiments: The total amount of sugar in the efflux media plus that remaining in the tissue, i.e. 4.98 μmol, represent 111 % of that initially present in the tissue.

The above observations, taken in conjunction with the established cellular phosphorylation of 2-deoxy-D-galactose (Table II above) provide evidence that both a galactokinase and a Zn^{2+}-activated (alkaline) phosphatase participate in the regulation of the intracellular levels of free and phosphorylated 2-deoxy-D-galactose. This relationship is expressed by the scheme:



where 2-dGal stands for the sugar, and the subscript i denotes an intracellular compartment; P_i, inorganic phosphate.

The exsitence of these reactions, as well as the leakiness of the cell membrane for the sugar phosphate, prevent the derivation of a meaningful rate constant for the efflux of 2-deoxy-D-galactose renal cells. The point of presenting the efflux curve in Fig. 4 is merely in a comparison with such a curve for D-galactose, obtained under comparable conditions (see ref. 8). The efflux of 2-deoxy-D-galactose appeared to originate from two compartments: The faster component corresponds satisfactorily to a wash-out of the sugar from the extracellular compartment, (see ref. 21 for the assessment). The efflux of 2-deoxy-D-galactose from the cellular compartment was systematically found to be considerably slower than that for D-galactose. This fact may contribute to the higher accumulation ratio obtained for 2-deoxy-D-galactose (mean S_i/S_o values for the free sugar: 3.97 ± 0.15 ($n = 23$), as compared with D-galactose (S_i/S_o: 1.45 ± 0.09, $n = 17$).

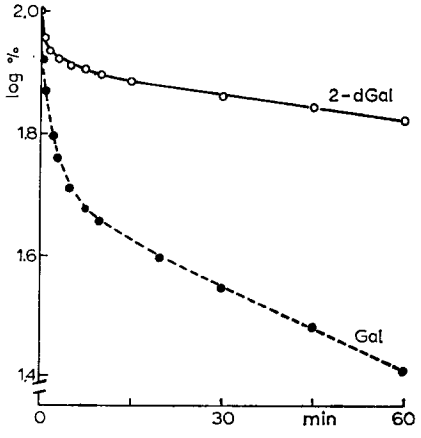


Fig. 4. The efflux of 2-deoxy-D-galactose and D-galactose from renal cortical slices. Slices were first loaded with the sugars by aerobic incubation for 60 min at 25 °C in salines containing 1 mM 2-deoxy-D-[^{3}H]galactose (○) and D-[1-^{14}C]galactose (●), respectively, and the efflux of these sugars from the tissue was followed. For further details, see legend to Table IV. Ordinate: log % of initial activity.

TABLE V

EFFECT OF STRUCTURAL ANALOGS ON THE TRANSPORT OF 2-DEOXY-D-GALACTOSE BY RENAL CORTICAL SLICES

Groups of slices (5–6 per flask) were brought to a steady state of tissue solutes by aerobic (O_2) preincubation for 45 min at 25 °C in standard saline. The tissue was then transferred to flasks with the same medium plus 1 mM 2-deoxy-D-[^{3}H]galactose (0.1 μCi/ml) without (control) and with 5 mM analog, and was incubated under identical conditions for 60 min. In the blotted slices total, free and phosphorylated sugar was determined. Means ± S.E. of free and phosphorylated tissue sugar (μmol/g tissue wet wt.) are given. The inhibition of 2-deoxy-D-galactose uptake by the analogs is based on values of total tissue sugar (control without inhibitor = 100 %).

Inhibitor (5 mM)	Control		With inhibitor		Percent of control	
	Tissue sugar (μmol/g)		Tissue sugar (μmol/g)			
	Free	Phosphorylated	Free	Phosphorylated		
2-Deoxy-D-galactose	4.26±0.22	2.07±0.12	1.48±0.04	0.25±0.03	27	*
D-Galactose	1.93±0.05	3.72±0.09	0.75±0.02	0.21±0.02	17	*
Methyl-α-2-deoxy-D-galactoside	3.48±0.19	4.19±0.05	4.35±0.18	3.74±0.04	100	n.s.
Methyl-α-D-galactoside	2.23±0.13	2.82±0.06	1.71±0.09	2.87±0.08	91	n.s.
Methyl-β-D-galactoside	2.23±0.13	2.82±0.06	1.70±0.08	3.04±0.12	94	n.s.
D-Talose	2.13±0.07	3.58±0.09	1.91±0.05	2.73±0.09	82	**
2-*O*-Methyl-D-galactose	1.96±0.06	2.59±0.09	1.96±0.06	2.72±0.17	105	n.s.
2-Amino-2-deoxy-D-galactose	1.96±0.06	2.59±0.09	1.56±0.03	2.29±0.05	87	**
3-Deoxy-D-galactose	3.14±0.15	2.56±0.16	2.44±0.15	2.59±0.17	94	n.s.
D-Gulose	2.15±0.06	2.67±0.07	2.11±0.09	2.91±0.09	104	n.s.
D-Glucose	1.17±0.06	3.25±0.13	1.39±0.07	3.19±0.12	108	n.s.
L-Glucose	3.14±0.15	2.56±0.16	2.37±0.08	1.61±0.06	70	*
D-Galactitol	1.93±0.07	3.73±0.09	2.09±0.06	3.58±0.13	102	n.s.
D-Tagatose	1.96±0.06	2.59±0.09	1.68±0.06	2.38±0.04	92	n.s.
6-Deoxy-D-galactose	1.93±0.07	3.73±0.09	1.54±0.03	3.61±0.05	91	n.s.
L-Arabinose	1.87±0.07	3.44±0.17	1.93±0.09	3.08±0.13	89	**

Significance of inhibition: *, $P < 0.01$; **, $P < 0.05$; n.s., not significant.

The structural specificity of the active transport system for 2-deoxy-D-galactose

Previous limited data concerning the structural specificity of the transport of 2-deoxy-D-galactose revealed that D-galactose shared the transport carrier [1, 2]; on the other hand, a lack of inhibition by methyl-α-2-deoxy-D-galactoside [7] and by D-glucose [2] indicated requirements for a free C_1-OH and for C_4-OH in the axial configuration. The structural requirements for the interaction of 2-deoxy-D-galactose with the transport site were now investigated in detail by an inhibition analysis, using 18 structural analogs.

An inspection of data given in Table V reveal that D-galactose is the preferred substrate for the investigated transport system since it inhibited the total tissue uptake of 1 mM 2-deoxy-D-galactose even more (i.e. 83 %) than the 2-deoxy-hexose itself (78 %). If at all significantly inhibitory, the other tested sugars showed a low affinity for interaction with the carrier involved in the transport of 2-deoxy-D-galactose. It may also be seen from the data that of all the sugar analogs tested only the substrate and D-galactose affected the tissue level of 2-deoxy-D-galactose phosphate more (88 and 94 %, respectively) than that of the free sugar (65 and 69 %, respectively). Such results suggest that only galactose interferes with both the actual energy mechanism and the intracellular metabolism of the substrate.

The fact that conversion of D-galactose and 2-deoxy-D-galactose to their respective methyl-glycosides abolished the inhibitory action on 2-deoxy-D-galactose transport indicates a requirement for C_1-OH for transport, as suggested earlier [7]. Since 2-deoxy-D-galactose and D-galactose appear to be transported by the same carrier, C_2-OH in the equatorial configuration is not a structural requirement. However, both D-talose (the C_2-epimer of D-galactose) and 2-*O*-methyl-D-galactose were at best marginally effective as inhibitors suggesting that a close packing between the substrate and the carrier does not accomodate a bulky methyl group on C_2 or an axial -OH. 2-Deoxy-2-amino-D-galactose was marginally inhibitory (87 % of control). A structural requirement for C_3-OH in the axial configuration follows from the fact that 3-deoxy-D-galactose and also D-gulose (the C_3-epimer of D-galactose) were not inhibitory. The requirement for C_4-OH in the axial configuration is supported not only by the lack of an inhibitory effect of D-glucose and of 2-deoxy-D-glucose (see Table VI), but also by the slight but significant inhibitory effect (70 % of control, $P \ll 0.01$) of L-glucose (with its C_4-OH in the axial configuration). A (pyranose) ring

TABLE VI

EFFECT OF VARYING CONCENTRATIONS OF 2-DEOXY-D-GLUCOSE ON THE UPTAKE OF 2-DEOXY-D-GALACTOSE BY RENAL CORTICAL SLICES

For conditions of the experiment see legend to Table V.

2-Deoxy-D-glucose	Tissue 2-deoxy-D-galactose (μmol/g)	
(mM)	Free	Phosphorylated
0 (control)	1.99±0.07	2.78±0.09
1	1.85±0.04	2.66±0.08
5	1.78±0.07	3.22±0.06
10	1.75±0.05	2.68±0.11

structure appears to be essential in view of the ineffectiveness of D-galactitol as inhibitor. Such view is supported by the observation that D-tagatose, the ketofuranose corresponding to D-galactose, was also not effective. A strict requirement for C_6-OH is apparent from the fact that 6-deoxy-D-galactose as well as the respective pentoses L-arabinose and D-xylose were ineffective as inhibitors.

DISCUSSION

The experiments reported above are consistent with previous data that renal cortical slices effect an active (i.e. metabolically dependent up-hill) uptake of 2-deoxy-D-galactose. In the course of this transport, both free and phosphorylated sugar accumulate in the cells (Fig. 1). Evidence for the involvement of the following processes in the transport and phosphorylation of this sugar has been presented here: (a) the actual transport step across the cell membrane (Fig. 1); (b) phosphorylation of 2-deoxy-D-galactose by a cellular kinase (Table II); (c) dephosphorylation of the formed 2-deoxy-D-galactose 1-phosphate by a (Zn^{2+}-activated) phosphatase (Table III); and (d) efflux of 2-deoxy-D-galactose, and to some extent of 2-deoxy-galactose phosphate, from the cells (Fig. 4, Table IV). The properties of these processes will now be discussed.

Transport of 2-deoxy-D-galactose into renal cells. Previous experiments established the following properties of the metabolically dependent active uptake of 2-deoxy-D-galactose into renal cortical cells: (1) Na^+ independence (and insensitivity to ouabain) [1, 3, 4]; (2) Compared to the transport of other sugars, e.g. methyl-α-D-glucoside, a relative insensitivity to phlorizin (apparent K_i 0.9 mM) and sensitivity to phloretin (K_i 0.5 mM) [22]; (3) inhibition by external pH higher than 7.2 (ref. 4 and Fig. 3). While the first two properties are also shared with Na^+-independent transport systems for D-galactose [4] and 2-deoxy-D-glucose [1, 3, 4], a decreased accumulation in response to an increase of external pH distinguish the transport pathway of 2-deoxy-D-galactose from that involved in the transport and phosphorylation of the other mentioned hexoses [4].

The specificity pattern for the uptake of 2-deoxy-D-galactose (Table V) shows that the transport system is also shared by D-galactose. A similar inhibition analysis of the uptake of D-galactose by renal tissue did not yield such clear answer; in fact, 2-deoxy-D-galactose is a rather poor inhibitor of the galactose uptake [1, 2, 3]. It is suggested that such data do not invalidate the view that both sugars compete for a shared transport site. The reason for the discrepancy in the respective K_m and K_i values appears to reside in the fact that several systems with differing specificities are involved in the transport of D-galactose, only one of which is shared with the deoxy-sugar. Thus, galactose is accumulated in the cells by simultaneously operative Na^+-dependent and Na^+-independent transport systems [7, 6]. The former is probably shared with D-glucose and the methyl-glycosides of both hexoses, and appears to be localized at the luminal (brush-border) face of the proximal tubular cells [5, 13, 23, 24]. Such view is supported by direct observations of the properties of the absorption of D-galactose on the luminal (brush-border) face of the cells by double-perfusion micropuncture [5, 23], multiple indicator dilution technique [13] and the use of vesicles of microvilli [24, 25] as well as by the demonstration that D-galactose also interacts with the antiluminal membrane of tubular cells [13].

From the fact that substitutions of hydroxyls and changes of the steric configurations on all carbon atoms of the (pyranose) ring of galactose, with the exception of C_2-OH, decreased or abolished the inhibitory action on the uptake of 2-deoxy-D-galactose, suggest the following points of interaction between both sugars and the carrier: C_1-OH, C_3-OH and C_4-OH (both axial), and C_6-OH. A close packing between the carrier and the substrate in the vicinity of C_2 is indicated. Consistent with the transport specificities of all other sugars (see, e.g. refs. 26 and 27), a ring structure also appears to be essential. The requirement for C_1-OH may reflect the formation of a covalent bond (see below). The possibility of hydrogen bonding between the oxygens at C_3, C_4 and C_6, and the carrier has to be considered in the light of studies of galactose transport in the intestine [27]. The present investigation should therefore be extended using structural analogs with halogens replacing hydroxyls. An elucidation of some, apparently minor, inconsistencies arising from data in Table V would be desireable. Thus, the fact that L-glucose significantly inhibited the transport of 2-deoxy-D-galactose is difficult to reconcile with the above analysis. Also, the above analysis tacitly ignores possible effects of the α- and β-anomers on the various conformations a pyranose ringe can assume. Future studies may have to deal with this added complexity.

The lack of an effect of 2-deoxy-D-glucose on the transport of 2-deoxy-D-galactose (Tables V and VI) deserves further comment. It has been shown previously that 2-deoxy-D-galactose competitively inhibited the cellular transport of the glucose derivative [2] and a reasonable agreement between the K_m for the transport of 2-deoxy-galactose and the K_i of its inhibitory effect was found. The fact that 2-deoxy-D-glucose did not significantly affect the cellular uptake of the galactose derivative cannot be due to major differences in the respective affinities for a shared transport site since the apparent K_m values for the transport of both sugars were close. The alternative view, i.e. two transport pathways for 2-deoxy-D-galactose, only one of which is shared with 2-deoxy-glucose, appears to be consistent with observed data. In this context it should be noted that D-glucose, which is a competitive inhibitor of the transport of 2-deoxy-glucose [2] also had no effect on the transport of 2-deoxy-galactose (Table V). The above findings suggest that 2-deoxy-D-glucose is not a universal metabolic inhibitor acting either as an intracellular trap for ATP or by an inhibitory effect of its metabolic product, 2-deoxy-D-glucose 6-phosphate on the glycolytic cycle (see, e.g. ref. 28). No effect of 2-D-deoxy-glucose on the cellular levels of adenine nucleotides in renal cortical cells was found (unpublished data). It is of interest to note here that Kipnis and Cori [29] did not observe an effect of intracellular 2-deoxy-D-glucose 6-phosphate on the (facilitated) entry of 2-deoxy-D-galactose into muscle fibers of the diaphragm.

The described model of the possible interaction between the transported sugars and the carrier is in all details identical with that found by an inhibition analysis of the transport of D-galactose and 2-deoxy-D-galactose at the antiluminal face of flounder renal cells. [9]. The preparation employed there (teased tubules) permitted to study the transport processes occurring predominantly at the basolateral (antiluminal) face of the renal cells. In this preparation, galactose and 2-deoxy-galactose mutually competed for the transport site. If a generalization is permissible, it may be inferred that the investigated transport of 2-deoxy-D-galactose in renal slices took place at the antiluminal face of the tubular cells.

The impossibility of employing zero-time kinetics to investigate in slices the inhibition of 2-deoxy-D-galactose transport by structural analogs may introduce an additional complexity in the interpretation of experimental data. It could be visualized that a structural analog might affect the cellular metabolism of the substrate independently of its possible action on the actual transport step. One of the ways to check this possibility is to compare inhibitory data on total, free and phosphorylated sugar. Any major discrepancy between the data might indicate an inhibitory effect additional to that exerted on the actual entry mechanism for the substrate. No such discrepancy has been noted with the exception of the effects of D-galactose and increased concentrations of the substrate (see Results).

Data obtained using flounder renal tubules [9] suggested the possibility that the formation of 2-deoxy-D-galactose phosphate may represent the first step in the transfer of the sugar across the antiluminal membrane. Indications for the involvement of a phosphotransferase in the galactose uptake in rabbit renal cortical slices have also been obtained [8]. The present data per se did not provide further clues in this respect. The time-curve of 2-deoxy-D-galactose uptake (Fig. 2), demonstrating that at every time interval the cellular level of the phosphorylated sugar was identical or even higher than that of the free sugar is not inconsistent with such view. The mandatory requirement of a free C_1-OH (and/or C_6-OH) for transport would be a logical consequence of a transport system involving a phosphotransferase.

It would be desirable to complete the above study by investigating the transport specificity of 2-deoxy-D-galactose using vesicles derived from both the brush-broder and basolateral membranes of renal tubular cells. Such preparations could also shed some light on the actual transport mechanism. In this way, possible arguments arising from the differences in the pH response of the cellular uptake of D-galactose (Fig. 3) and 2-deoxy-D-galactose [8] might also be resolved.

Intracellular phosphorylation and dephosphorylation. The membrane-free tissue extract has been shown here to contain a kinase which phosphorylates 2-deoxy-D-galactose. Since the pH dependence of this reaction was identical with that found when D-galactose was the substrate [8] it may be inferred that galactokinase (ATP: D-galactose-1-phosphotransferase, EC 2.7.1.6) is the enzyme involved. A pH optimum of 8 for liver galactokinase has been found by Walker and Khan [30]. 2-Deoxy-D-galactose has been previously reported to serve as a poor substrate for liver [30] and yeast galactokinase [31, 15]. In the light of the differences in the response of the galactokinase and the transport system to variations of pH (compare Table II and Fig. 3) it may be assumed that galactokinase could not participate in the hypothetical phosphotransferase step at the cell membrane.

The activity of a (Zn^{2+}-activated, alkaline) phosphatase in the cells is documented by data in Tables III and IV.

More detailed information as to the properties of both these enzymes, particularly on their specificities, is desirable.

Possible models of 2-deoxy-D-galactose transport at the antiluminal face of the nephron. Data given above do not yet allow us to decide whether the sugar is actively transported across the cell membrane as free sugar, to be phosphorylated by the galactokinase within the cells or, alternatively, that a phosphotransferase is involved in the up-hill transport of the sugar as 2-deoxy-D-galactosyl-phosphate, and both the phosphatase and galactokinase participate in maintaining a steady-state cellular level

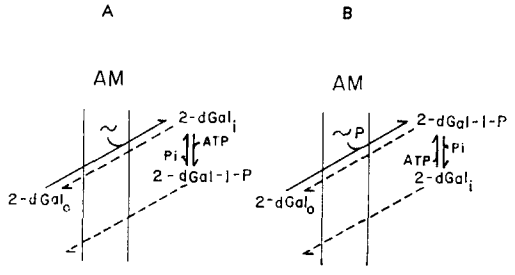


Fig. 5. Two possible models for the transport of 2-deoxy-D-galactose at the antiluminal membrane of renal tubular cells and the intracellular metabolism of the sugar. A, active transport system; B, phosphotransferase system. Symbols: AM, antiluminal membrane; 2-dGal, 2-deoxy-galactose; subscripts i and o denote the intracellular and extracellular space, respectively. Broken line, efflux mechanism(s).

of the sugar. Both these alternative tentative models are shown in Figs. 5A and 5B, respectively. The broken lines indicate the efflux mechanism(s) for both the free and phosphorylated sugar.

The localization of the transport system for 2-deoxy-D-galactose in the renal tubule. Any model of the renal transport and metabolism of 2-deoxy-D-galactose has to take account of events recorded under in vivo conditions. Two observations are pertinent here: Ullrich et al. [5] did not find an absorption of this sugar from the convoluted portion of the proximal tubule of rats. On the other hand, clearance data (ref. 32 and unpublished results of Dr. J. Bolton from this laboratory) clearly demonstrated a reabsorption of 2-deoxy-D-galactose in the rat nephron, with clearance ratios consistently of the order of 0.6–0.7. This transport process was associated with an apparent cellular accumulation of free sugar, judging from tissue/plasma ratios higher than 1.0 after correction for the tissue/plasma ratio of the employed marker of the glomerular filtration rate. This last observation would place the up-hill transport system for the sugar at the luminal face of the tubular cells. The involved transport process was not sensitive to phlorizin. These data suggest that the reabsorption of 2-deoxy-D-galactose from the renal tubule takes place in a portion of the nephron which was not explored by the double-perfusion micropuncture technique. Obviously, a further study of the transport specificity for sugars along the functionally different portions of the nephron will be required to elucidate the apparent discrepancies.

ACKNOWLEDGEMENTS

This investigation was supported in part by an U.S.P.H.S. N.I.H. Grant AM 12169. The authors gratefully acknowledge generous gifts of the following rare sugars: 2-*O*-methyl-D-galactose, Dr. J. Chittenden, University of Nijmegen, Nijmegen, The Netherlands; 3-deoxy-D-galactose, Dr. J. E. G. Barnett, Nottingham, England; 1,2; 5,6-di-*O*-isopropylidene-D-gulose, Dr. K. N. Slessor, Simon Fraser University, Burnaby, B. C., Canada. The authors are indebted to Dr. Bayard T. Storey for valuable comments.

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