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TRANSPORT OF 5-THIO-D-GLUCOSE IN HAMSTER SMALL INTESTINE

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SUMMARY

Segments of hamster small intestine have been used to study the interaction of 5-thio-D-glucose with the D-glucose transport system. By all current criteria, 5-thio-D-glucose is a substrate for the system. We have examined accumulation against a concentration gradient, substrate inhibition, phlorizin sensitivity, Na^+ dependence, energy dependence and counterflow. The estimated K_m value for 5-thio-D-glucose is of the order of 2.8 mM.

Two interesting differences between D-glucose and 5-thio-D-glucose have been found. 5-Thio-D-glucose is less effective than D-glucose in inhibiting the specific binding of D-[^{3}H]glucose to brush borders. 5-Thio-D-glucose is also a poor substrate for hexokinase. The effect of the sulphur substitution on possible ring conformation of the sugar is discussed with respect to the specificity of the transport system.

INTRODUCTION

The involvement of stereospecificity in the absorption of sugar from the intestine was first intimated by the finding that glucose was absorbed much more rapidly than other sugars of similar molecular size[1]. Studies were subsequently extended to many other sugars and their derivatives[2–4]. From a review of the literature available in 1960, CRANE[5] concluded that certain minimal requirements were necessary for intestinal active transport of sugars (Fig. 1a). More recently, however, CZAKY AND LASSEN[6] and ALVARADO[7], have shown xylose to be accumulated against a concentration gradient although poorly so, indicating that C-6 is not an absolute requirement. It seems to have been generally assumed that the sugar must be in the D-form[8]. However, it was predicted[8] and it has now been shown that L-glucose is actively transported[9,10] owing to the fact that L- and D-glucose can present the same conformation of the underlying tetrahydropyranose structure.

The specificity requirements for transport have recently been emphasized by BARNETT *et al.*[11,12] in terms of the ability of the hydroxyl groups to form hydrogen bonds with the carrier. They concluded that D-glucose possesses the ideal structure for active transport and that the removal or inversion of two or more hydroxyl groups results in the loss of the correct conformation for transport. Thus galactose, inverted at C-4, D-allose inverted at C-3, 6-deoxy-D-glucose and 1-deoxy-D-glucose are all ac-

Abbreviation: PD, potential difference.

tively accumulated by the intestine. Gulose inverted at C-3 and C-4, and 1,6-dideoxy-D-glucose are not accumulated[13,14]. However, the hydroxyl group at C-2 has major importance as 2-deoxy-D-glucose is not transported at all[13].

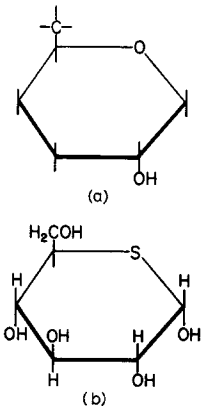

Fig. 1. a. Common structural features of actively absorbed compounds from CRANE[5]. b. Structure of 5-thio-D-glucose[15].

No studies have been reported of tests of the need for an oxygen atom in the pyranose ring. With the availability of 5-thio-D-glucose[15], a sugar containing a sulphur atom in the ring instead of oxygen (Fig. 1b) it became possible to test this particular parameter. Unless the oxygen atom is essential for the recognition and binding of the sugar molecule by the carrier, it might be expected that 5-thio-D-glucose would be actively transported. A further possibility was that the sulphur atom would interact with the membrane protein at or near the carrier possibly in an irreversible manner.

The purpose of this present paper is to report studies which show that 5-thio-D-glucose satisfies all the criteria for an actively transported substrate. It appears not to interact with the membrane in other detectable ways.

METHODS AND MATERIALS

Incubation technique

Tissue accumulation experiments were performed by incubation *in vitro* of rings of everted small intestine from fasted hamsters, as described by CRANE AND MANDELSTAM[16]. Approx. 100–200 mg wet wt. of tissue were placed in 25-ml Erlenmeyer flasks containing 5 ml of Krebs–Henseleit phosphate buffer[17] equilibrated at 37°. The buffer was gassed with 100 % O_2 for at least 45 min prior to incubation. Incubations were terminated by removal of the tissue, and the tissue and media were processed for subsequent assay as described by CRANE AND MANDELSTAM[16].

Calculation of results

In some experiments the concentration of the substance per ml tissue water is expressed as a percentage of the initial medium concentration, *i.e.* the percent filling. Results from kinetic studies are expressed as rates of entry in mmoles of substrate

accumulated per ml tissue water over a 5-min time period, assuming a water content of approx. 80% of tissue weight. All data are corrected for D-mannitol space.

Transmural potential (PD) measurements

PD measurements were made by the method of LYON AND CRANE[18], as recently modified by CASPARY *et al.*[19]. Cannulated everted sacs 5–6 cm in length were prepared from the mid-gut region of hamster small intestine. The sacs were incubated at 37° with Krebs–Henseleit bicarbonate buffer previously equilibrated with O_2–CO_2 (95:5, v/v)[17]. All additions were made to the mucosal fluid compartment. The PD across the intestine was measured using KCl–agar salt bridges connected, *via* a liquid junction, to Ag/AgCl electrodes. Voltage changes were detected with a Keithley 610B electrometer (Keithley Instruments, Cleveland, Ohio).

Glucose binding to isolated brush borders

The method used was that recently described by EICHHOLZ *et al.*[20]. Isolated brush borders were suspended in 50 mM Tris buffer–5 mM Mg^{2+} (pH 7.4) to a final concentration of exactly 0.5 mg protein/ml. 1 ml of the preparation was incubated with a 0.1 μM radioactive mixture of D-[^{3}H]glucose and L-[^{14}C]glucose at 37° for 15 min. The incubation was terminated by passing the sample through a previously wetted Millipore filter under suction. The filtrate was collected directly into a scintillation vial. A second vial was used to collect the effluent when the residue was washed with 1 ml of buffer. The washed filter was itself placed in the bottom of a third vial.

From the ratios of D-glucose/L-glucose in the sample and the reaction mixture, the amount of D-glucose non-specifically bound was calculated. By subtracting this result from total D-glucose bound, the amount of D-glucose specifically bound was calculated.

Incubation with yeast hexokinase

5-Thio-D-glucose was tested as a substrate for yeast hexokinase (Sigma Chemical Co.) by the method of CRANE AND SOLS[21]. The disappearance of free hexose from the medium was determined after precipitation of hexose 6-phosphate with barium–zinc[22].

Compounds

The following compounds were obtained from commercial sources. D-[^{14}C]-Galactose, L-[^{14}C]glucose, D-[^{3}H]glucose, D-[^{14}C]mannitol from New England Nuclear Corp.; 3-*O*-methyl-D-[^{14}C]glucose from Calbiochem. 6-Deoxy-D-[^{3}H]glucose was prepared in this laboratory. 5-Thio-D-glucose was kindly provided by Dr. R. L. Whistler, Department of Biochemistry, Purdue University, Lafayette, Ind.

Analytical methods

Radioactive materials were assayed with the Beckman liquid scintillation system using the method of PATTERSON AND GREEN[23] for scintillation counting. 5-Thio-D-glucose was estimated using the reducing sugar assay described by SOMOGYI[24].

RESULTS

In an initial experiment, rings of intestine were incubated with 3 mM 5-thio-D-glucose for various time intervals. As seen in Fig. 2, 5-thio-D-glucose was accumu-

lated against a concentration gradient. After as little as 2.5 min incubation, a tissue/medium ratio of greater than 1.0 was produced, which subsequently increased to greater than 6.0 after 30 min. This result is very similar to that found in previous studies with D-glucose and other actively transported sugars[8].

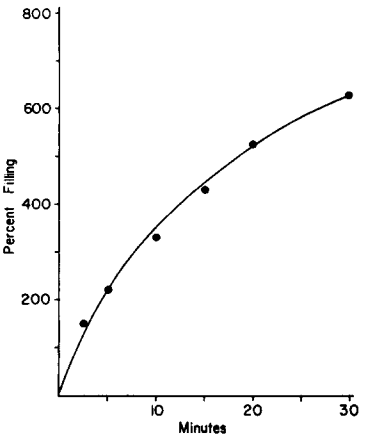

Fig. 2. Uptake of 5-thio-D-glucose by rings of hamster small intestine. The initial concentration of 5-thio-D-glucose was 3 mM.

5-Thio-D-glucose was therefore tested for further characteristics of sugar active transport such as Na^+ dependence, phlorizin sensitivity and energy dependence[8]. Table Ia shows that reducing the Na^+ concentration from 118 to 21 mM caused 45% inhibition of transport. The transport process was clearly energy dependent as it was inhibited by 60% in the presence of 0.5 mM 2,4-dinitrophenol (Table Ib).

TABLE I

UPTAKE OF 5-THIO-D-GLUCOSE BY HAMSTER SMALL INTESTINE

Incubation conditions were 10 min (Expt. a) and 15 min (Expt. b). Inhibition was calculated from the rates in the absence of inhibitors.

Expt.	*Conditions*	*Filling* (%)	*Inhibition* (%)
a	3 mM 5-thio-D-glucose		
	No addition; 139 mM Na^+	203	—
	Tris medium; 118 mM Tris, 21 mM Na^+	112	45.0
b	9 mM 5-thio-D-glucose		
	No addition	155	—
	0.5 mM 2,4-dinitrophenol	61	60.5

Transmural PD studies; phlorizin sensitivity

As has been demonstrated repeatedly, actively transported sugars induce a rise in transmural PD in the small intestine[18,19]. This is accounted for by the formulation of a bifunctional, Na^+-dependent carrier in the brush border membrane. In agreement with the demonstrated Na^+ dependency of 5-thio-D-glucose accumulation (Table Ia)

a PD of 2.8 mV over the resting potential was induced when 6 mM 5-thio-D-glucose was placed on the mucosal side of an everted sac of hamster small intestine. Phlorizin sensitivity was therefore tested in this system. The PD in response to the sugar was reduced by about 33 % on the addition of 1 μM phlorizin, and 66 % when the concentration was increased to 3 μM.

Inhibition of sugar transport

As 5-thio-D-glucose was not available in radioactive form, the effect of cold compound on the uptake of a number of ^{14}C- and ^{3}H-labelled actively transported sugars by tissue slices was investigated. In addition, the effect of preincubation of the tissue with 5-thio-D-glucose on the subsequent accumulation of 6-deoxy-D-glucose was determined. The accumulation of 3-*O*-methyl-D-glucose, L-glucose, D-galactose and 6-deoxy-D-glucose against their concentration gradient was inhibited in the presence of 5-thio-D-glucose (Table II). Preincubation with 3 mM 5-thio-D glucose for 20 min did not appear to significantly inhibit transport of 6-deoxy-D-glucose (Table III). The small variation in percent filling between preincubation with buffer

TABLE II

EFFECT OF 5-THIO-D-GLUCOSE ON THE UPTAKE OF CERTAIN ACTIVELY TRANSPORTED SUGARS

Because of varying affinities of the transported sugars for the transport system, and the limited amount of 5-thio-D-glucose available, incubation conditions were varied to optimize any inhibition. Expts. a and b, 5 min; Expts. c and d, 20 min.

Expt.	*Conditions*	*Filling* (%)	*Inhibition* (%)
a	0.6 mM D-galactose	498	—
	0.6 mM D-galactose + 12 mM 5-thio-D-glucose	211	57.8
b	1 mM 6-deoxy-D-glucose	669	—
	1 mM 6-deoxy-D-glucose + 9 mM 5-thio-D-glucose	267	60.2
c	12 mM 3-*O*-methyl-D-glucose	136	—
	12 mM 3-*O*-methyl-D-glucose + 12 mM 5-thio-D-glucose	46	66.2
d	0.6 mM L-glucose	130	—
	0.6 mM L-glucose + 3.0 mM 5-thio-D-glucose	66	49.0

TABLE III

EFFECT OF PREINCUBATION WITH 5-THIO-D-GLUCOSE ON ACTIVE TRANSPORT OF 6-DEOXY-D-GLUCOSE

Tissue slices were preincubated for 20 min with buffer (a), 3 mM 5-thio-D-glucose (b) and 3 mM D-glucose (c). They were then transferred to 0.6 mM 6-deoxy-D-[^{3}H]glucose for 10 min. At least three preparations were tested in each group.

Expt.	*Condition during preincubation*	*Filling* (%) *with 6-deoxy-D-glucose*	*Inhibition* (%)
a	Buffer	368	—
b	3 mM 5-thio-D-glucose	327	11.0
c	3 mM D-glucose	324	12.0

or 5-thio-D-glucose of about 10 % may be explained as competition by residual 5-thio-D-glucose adhering to the tissue and reentering the medium. Similar slight inhibition was found on preincubation with 3 mM D-glucose.

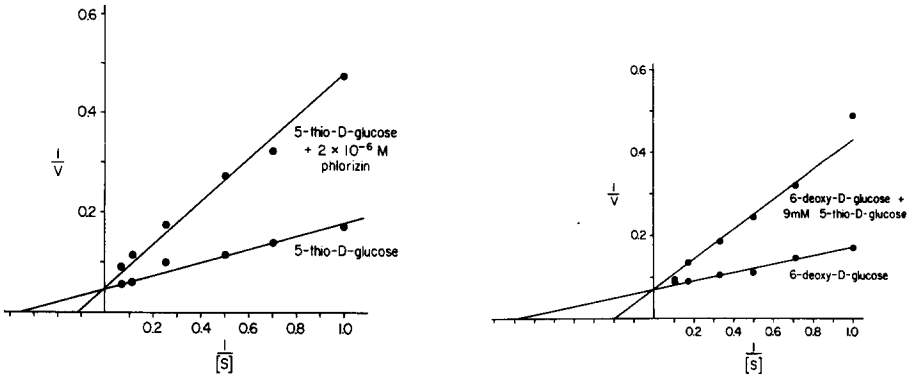

Fig. 3. LINEWEAVER–BURK plots[25] of rate of uptake *vs.* concentration of 5-thio-D-glucose. Also shown is the competitive inhibition by phlorizin. Each point represents the mean of at least three experiments.

Fig. 4. The effect of 9 mM 5-thio-D-glucose on the rate *vs.* concentration relationship of 6-deoxy-D-glucose. K_i for 5-thio-D-glucose was 3.5 mM. Each point represents the mean of at least three experiments.

Evaluation of K_m

As shown in Fig. 3, transport of 5-thio-D-glucose exhibited typical saturation kinetics with a K_m of the order of 2.8 mM. In the presence of phlorizin, accumulation of 5-thio-D-glucose was inhibited confirming the data obtained from PD measurements. The kinetics of the inhibition appear to be competitive.

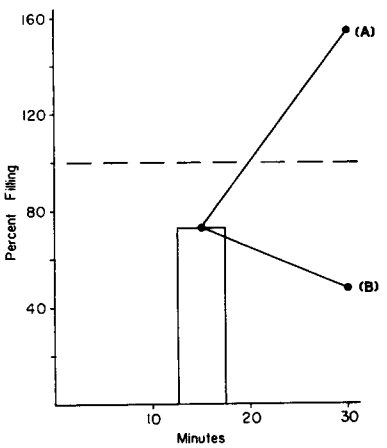

Fig. 5. Counterflow of L-[^{14}C]glucose induced by 5-thio-D-glucose. Rings of hamster small intestine were incubated for 15 min with 0.6 mM L-[^{14}C]glucose. The percent filling after this incubation is indicated by the bar. The tissue was then transferred to either (A) 0.6 mM L-[^{14}C]glucose or (B) a mixture of 0.6 mM L-[^{14}C]glucose and 21 mM 5-thio-D-glucose, and then incubation continued for a further 15 min. The points represent the mean of at least three experiments, the dotted line 100 % filling.

TABLE IV

COMPETITION FOR BINDING WITH D-[^{3}H]GLUCOSE

D-[^{3}H]Glucose concn., 0.1 μM.

Conditions	D-[^{3}H]*Glucose* (*counts/min*)	L-[^{14}C]*Glucose* (*counts/min*)	*Ratio* D-*glucose*/L-*glucose*	*Counts bound*	*Average*	*Activity* (%)
Control	23 689	358	66	22 679	23 203	100
	24 634	321	76	23 727	—	—
+ 0.01 M 5-thio-D-glucose	1 363	292	4.66	539	608	3
	1 403	257	5.45	678	—	—
+ 1 mM 5-thio-D-glucose	6 055	137	44	5 667	4 457	19
	4 131	313	13	3 247		—
+ 0.1 mM 5-thio-D-glucose	18 319	300	61	17 473	18 562	80
	20 468	289	70	19 652	—	—
+ 10 μM 5-thio-D-glucose	22 581	310	72	21 707	19 570	84
	18 237	285	63	17 433	—	—
+ 1 μM 5-thio-D-glucose	23 767	320	74	22 865	24 209	104
	26 414	305	86	25 553	—	—
+ 0.1 mM D-glucose	1 693	459	3.68	398	473	2
	1 567	361	4.33	548	—	—
+ 0.01 mM D-glucose	4 616	439	10	3 156	3 266	14
	4 349	267	16	3 376	—	—
+ 0.1 mM Phlorizin	4 479	382	11	3 594	3 498	15

Determination of K_t

Because of the lengthy procedure associated with estimating 5-thio-D-glucose, a study was undertaken in which the effect of 5-thio-D-glucose on the K_m and v_{max} of 6-deoxy-D-[^{3}H]glucose transport was determined. The results would suggest a direct competition between 5-thio-D-glucose and 6-deoxy-D-glucose for the transport system (Fig. 4). The concentration of 5-thio-D-glucose which resulted in 50% inhibition of 6-deoxy-D-glucose transport, K_t, should also be equivalent to the K_m of the compound. Determined by this indirect method, a value of the order of 3.5 mM was found, close to that determined by direct measurement.

Counterflow

All the data would suggest that 5-thio-D-glucose acts like other sugars in entering the cell through the Na^+ and energy-dependent pathway. 5-Thio-D-glucose should therefore be capable of causing counterflow, *i.e.* the outflow of a sugar from the cell into the medium against its concentration gradient. In order to demonstrate counterflow, the tissue was incubated for 15 min with 0.6 mM L-[^{14}C]glucose and then transferred to a second incubation flask containing the same substrate concentration *plus* 21 mM 5-thio-D-glucose. As can be seen in Fig. 5, the presence of 5-thio-D-glucose during the second incubation not only prevented further uptake of L-glucose but induced an outflow of L-glucose against a concentration gradient.

Effect of 5-thio-D-glucose on the binding of D-glucose to brush border

5-Thio-D-glucose markedly inhibited the specific binding of D-[^{3}H]glucose to brush borders, as did unlabelled D-glucose and phlorizin (Table IV). However, the concentration at which D-glucose and 5-thio-D-glucose were maximally effective differed by 100-fold. 0.1 mM D-glucose inhibited binding by 98% compared with 0.1 mM 5-thio-D-glucose which produced only 20% inhibition. 0.01 M 5-thio-D-glucose was needed to achieve a similar 97% inhibition on binding.

5-Thio-D-glucose as a substrate for hexokinase

In agreement with the results of HOFFMAN AND WHISTLER[26], 5-thio-D-glucose proved to be a very poor substrate for hexokinase when compared to glucose (Table V).

TABLE V

THE EXTENT OF UTILIZATION OF 5-THIO-D-GLUCOSE BY YEAST HEXOKINASE

Compound	*Amount added (μmoles/ml)*	*Enzyme (units/ml)*	*Length of incubation (h)*	*Utilization (%)*
D-Glucose	3.0	4.0	0.5	>95
5-Thio-D-glucose	3.0	4.0	0.5	0
	3.0	4.0	11.0	< 5

DISCUSSION

Two previous reports have been made on the effect of 5-thio-D-glucose in biological systems. SHANKLAND *et al.*[27] found it to interfere with the utilization of

D-glucose for development of *Drosophila melanogaster*. They suggested that 5-thio-D-glucose might interfere with enzymes involved in sugar metabolism and or the transport of glucose across cell membranes.

HOFFMAN AND WHISTLER[26] showed that intraperitoneal injection of 5-thio-D-glucose into rats caused glycosuria and hyperglycemia. Further tests *in vitro* indicated that the uptake of D-glucose by rat liver, kidney and diaphragm was inhibited by low concentrations of the thio-sugar. 5-Thio-D-glucose did not inhibit the overall metabolism of D-glucose in a kidney homogenate but did slightly inhibit glycolysis. However, HOFFMAN AND WHISTLER concluded that the main site of action, at least in kidney and diaphragm, was probably at the membrane transport level.

In the present study, the initial observations on the inhibition of sugar transport by 5-thio-D-glucose (Table II) gave no information as to the underlying mechanism of the inhibition. Possibilities were that (a) the carrier was inactivated by irreversible binding; (b) the energy supplying mechanisms of the cell were inhibited or (c) there was some type of competition for the carrier.

Inhibition through the first mechanism would show as an effect on maximal velocity of sugar transport because of a reduction in the number of carriers. Preincubation with 5-thio-D-glucose clearly did not lower the subsequent v_{max} for 6-deoxy-D-glucose transport thus eliminating inactivation of the carrier as the mechanism of 5-thio-D-glucose inhibition.

That the effects might be explained by an inhibition of 5-thio-D-glucose on metabolism is also unlikely for several reasons. The epithelial cells must still be capable of maintaining a Na^+ gradient after preincubation with the thio-sugar to enable subsequent unimpaired active accumulation of 6-deoxy-D-glucose. The possibility that 5-thio-D-glucose might cause inhibition when simultaneously present in the media with another sugar by competing for various metabolic steps is eliminated by the fact that all the test sugars used in the study were nonmetabolizable.

The third mechanism thus seems the most likely especially as kinetic studies indicate the inhibition of 5-thio-D-glucose on 6-deoxy-D-glucose accumulation is competitive in nature, being a change in K_m of the sugar rather than v_{max}.

In fact, 5-thio-D-glucose exhibits all the criteria needed for it to be accepted as a substrate for the D-glucose active transport system; namely, accumulation against a concentration gradient, energy and Na^+ dependence, counterflow, substrate inhibition and phlorizin sensitivity. It has a K_m of 2.8 mM which may not be significantly different from the affinity of D-glucose for the system, 1.5 mM.

There are two interesting differences between D-glucose and 5-thio-D-glucose. One is the larger concentration of the latter needed to inhibit binding of labelled D-glucose to brush borders. However, the significance of this result is difficult to judge since the binding of sugars appears to be different from that of sugar active transport[20].

Secondly, 5-thio-D-glucose is a very poor substrate for hexokinase in contrast to glucose, and it is therefore probable that it is non-metabolized. In their experiments, HOFFMAN AND WHISTLER[26] found they could recover 97 % of 5-thio-D-glucose injected into rats unaltered in the urine.

In a consideration of the conformational structure of these sugars, it is necessary to look at the ring structure of tetrahydropyran and the compound from which it is derived, cyclohexane. Cyclohexane is known to exist in a stable chair form in which the carbon atoms lie above and below the plane of the ring allowing attainment of

the strainless C–C–C bond angle of 109.5° (ref. 28). Basic considerations of the effect of substituting an oxygen atom in the cyclohexane ring to obtain tetrahydropyran leads one to conclude that there should be little distortion of the chair form. The C–O–C bond angle is near 109.5°, the average C–O bond length is 1.42 Å compared with a C–C bond length of 1.53 Å (ref. 29), and the effective atomic radius of oxygen, 0.60 Å, is somewhat less than that of carbon 0.77 Å. The fact the tetrahydropyran exists in a near perfect chair form has been confirmed in NMR studies both by BUYS[31] and LAMBERT *et al.*[32].

However, the substitution of carbon by sulphur as in thiane, introduces a C–S bond of average length 1.81 Å, and a C–S–C bond angle of about 99°. In addition, the effective atomic radius of sulphur is 1.06 (ref. 31). It would seem likely, therefore, that there would be some distortion or puckering of the chair. This has been confirmed experimentally[31,32]. It was, therefore, somewhat surprising to find that an NMR study of 5-thio-D-xylopyranoside indicated that the substitution caused no important alterations in ring conformation[33].

If this is true, then we have measured here the effect of an exchange of atoms in the ring without an important contribution from a conformational shift as might have been expected. The exchange of atoms has little effect. The oxygen atom in the ring is neither an absolute requirement for, nor an important determinant of transport.

In our continuing search for the probable transportable conformation of glucose, one may ask whether the introduction of a sulphur atom renders more improbable, for glucose, the attainment of skewed forms which formerly had to be seriously considered. Further physical studies of the conformational implications of the introduction of sulphur into the ring would thus be highly desirable.

ADDENDUM

Since the completion of this manuscript J. E. G. BARNETT has informed us of his parallel studies on 5-thio-D-glucose.

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