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NONELECTROLYTE DISTRIBUTION IN MOUSE DIAPHRAGM MUSCLE

II. CELL VOLUME, D-XYLOSE DISTRIBUTION, AND THE EFFECT OF INSULIN IN HYPERTONIC SOLUTIONS

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SUMMARY

The D-xylose equilibrium distribution ratio (r value) and the relative cell volume are measured in the mouse diaphragm muscle at 34 °C in incubation media made hypertonic with varying concentrations of D-xylose. Under basal conditions, r increases with external xylose concentration, and the muscle fiber shrinks. The xylose r value does not rise if the muscles are shrunken by hypertonic addition of NaCl or mannitol instead of xylose. In the presence of insulin, both r and the cell volume remain constant as external xylose is increased. All observations conform quantitatively to the a priori predictions of the compartmentation theory of sugar exclusion and insulin action. The data contradict predictions of the association–induction hypothesis. The results indicate strongly that intracellular water behaves like normal water of aqueous solution and that no significant amount of intracellular xylose is adsorbed. The results also suggest that sugars are excluded from intracellular compartments via an active extrusion mechanism and that insulin causes inhibition of this mechanism.

INTRODUCTION

This report deals with the equilibrium behavior in mouse diaphragm muscle of D-xylose, a nonmetabolized pentose which is partially excluded from the cell [1]. When the muscle is bathed in solution containing this sugar, the xylose equilibrium distribution ratio between the cell water and the external solution is 0.36 at 34 °C; insulin increases this to 1.00.

The data of the previous paper in this series [1] seem to favor the idea that the sugar exclusion is due to intracellular compartments with which xylose cannot equilibrate; insulin allows equilibration to take place. If this picture is valid, it must be further assumed [1, 2] that the mechanism by which sugars are kept out of the intracellular compartments is not membrane impermeability, but rather active transport.

However, active transport would not have to be postulated if the insulin-

sensitive sugar were assumed to be adsorbed in the cytoplasm [1], as proposed by the association–induction hypothesis of Ling [3, 4]. This theory also postulates that all the cell water is tightly oriented in polarized multilayers [5, 6] and is therefore a poor solvent for sugars and other solutes.

These two opposing theories can be distinguished by a study of the xylose distribution ratio (r) and the cell water content under hypertonic conditions, as is shown below. The compartment theory makes necessary, quantitative predictions, while the association–induction hypothesis makes qualitative, but different, predictions.

METHODS

Intact mouse hemidiaphragm muscles were incubated at 34 °C as already described [1]. Incubation solutions were Krebs–Ringer media made hypertonic with varying concentrations of D-xylose or other components. In experiments with insulin, a maximal dose, 100 units/l, was always used. Tissue analysis of xylose, ions, and water were performed as in the previous paper [1]. Incubations lasted 3-5 h, which is a sufficient time to reach equilibrium [2] (except for high xylose concentrations in the presence of insulin). Extracellular water was measured on all samples by adding [^{14}C]sucrose to the incubation medium 5–15 min before the muscles were removed for analysis. It has been shown [1] that this short-term sucrose space gives a workable value for the true extracellular space.

Cell water content was calculated from tissue water content by correction for extracellular water as well as for solids trapped in the extracellular space. All xylose concentrations are expressed in millimolal (m$\tilde{\text{M}}$).

THEORY

In the experiments to be described, the muscles were exposed to solutions made hypertonic by addition of D-xylose, with or without insulin present. The xylose r value and relative cell water content (or shrinkage factor) were measured. In this section we will consider how these two observables are expected to vary with xylose concentration by the two opposing theories outlined above.

Compartmentation theory

Since this theory is based on the assumption that all the intracellular xylose is in aqueous solution, we can apply a simple osmotic treatment. We assume that the cell is divided functionally into two compartments, one of them accessible to the sugar under basal conditions, the other inaccessible. Using the two basic conditions of electroneutrality and osmotic balance, as developed by Boyle and Conway [7], we can derive the expression for the cell water content relative to its value at zero xylose concentration (isotonicity). This relative cell volume $v(x)$ is given by [2]:

$$v(x) = \left(1+\frac{r(0)x}{S}\right)\Big/\left(1+\frac{x}{S}\right), \tag{1}$$

where x is the xylose concentration, $r(0)$ is the xylose distribution coefficient at osmo-

tically negligible xylose concentrations, and S is the total activity of effectively impermeant solutes in a normal isotonic medium.

The expression shows that for $r(0) < 1$, the cell will shrink as xylose is added hypertonically to the solution. The shrinkage comes about because the inaccessible compartment must adjust its volume to maintain osmotic balance while the accessible one retains its isotonic volume always. The parameter $r(0)$ is crucial in determining the magnitude of the shrinkage; the data of the previous paper [1] assign this parameter a value of 0.36.

Eqn 1 shows that if $r(0) = 1$, which is the case for D-xylose in the presence of insulin, no shrinkage should occur regardless of hypertonic sugar concentration. This is an obvious result of the theory's claim that insulin (at 34 °C) makes all the cell water accessible to the sugar.

The treatment can be pursued to arrive at the dependence of xylose r value upon external xylose concentration. This is given by [2]:

$$r(x) = r(0)\frac{1+x/S}{1+r(0)x/S} \tag{2}$$

Therefore, under basal conditions, when $r(0) = 0.36$, the xylose r value will rise as external xylose is increased. This is because as xylose is added to the medium, the inaccessible compartment alone shrinks, thus rendering the accessible compartment a larger fraction of the total cell volume, and consequently causing the r value to rise. In the presence of insulin $r(0) = 1$, and so $r(x) = r(0) = 1$ always, as would be expected if both compartments become available to xylose in response to insulin.

Ordered water and binding theory

Ling [5, 6] considers that virtually all the cell water is adsorbed to intracellular protein sites and is arranged in multilayers. Water molecules closest to the proteins are bound most tightly, with the strength of binding decreasing in the outer layers of water farther from the protein sites. According to this theory, the cell water content will depend only upon the external activity of water, a result derived by Cope [8]. In addition, Cope has developed an expression for the expected cell shrinking behavior in hypertonic solutions. As explained in detail elsewhere [2] two conclusions can be derived which address the specific problem under consideration.

(1) Under basal conditions, the cell should shrink in response to hypertonic xylose in an approximately hyperbolic fashion.

(2) In the presence of insulin, assuming that the hormone causes xylose binding in the cytoplasm, hypertonic xylose should cause at least as much shrinkage as it does under basal conditions.

Besides making predictions of the cell water content, the association–induction hypothesis also predicts the dependence of xylose r value upon the tonicity of the medium [2]. The observed r value of a sugar is an ensemble-average of the r values in the individual layers of ordered water; in the outer, less tightly bound layers, the r values should be higher than in the inner, more tightly oriented layers. As the cell shrinks with increasing tonicity, the outer layers are stripped away first [9, 10], leaving behind the tightly bound inner layers. Thus, as the cell shrinks with increasing xylose concentration, the sugar's r value should decrease, since the remaining water

is on the average more structured than at isotonicity. It is not possible to quantitate this effect; a more thorough discussion of this subject is given elsewhere [2].

In the presence of insulin, the intracellular xylose is postulated to be in two states: free, dissolved in the cell water, and bound to intracellular sites. We have already seen that the free fraction should give rise to a distribution ratio which decreases with solute concentration. Since the bound fraction must be a saturable function of concentration, the sum of the two fractions should yield a measured r value which also decreases with external xylose concentration.

TABLE I

COMPARISON OF EQUILIBRIUM BEHAVIOR EXPECTED BY TWO OPPOSING THEORIES

Predictions of the compartmentation theory and association-induction hypothesis for the xylose r value (equilibrium distribution coefficient) and the cell shrinkage factor, v, as the incubation medium is made hypertonic by the addition of D-xylose, with insulin present or absent in the medium.

Observable parameter	Expected direction of change as xylose is added to medium	
	Compartmentation	Association–induction hypothesis
v, no insulin	Decreasing	Decreasing
r, no insulin	Increasing	Decreasing
v, insulin	Constant at 1.0	Decreasing
r, insulin	Constant at 1.0	Decreasing

Table I shows the behavior expected for the two parameters r and v as xylose is added into the incubation medium. The table demonstrates that the theories clash in three out of four predictions. The results below do indeed differentiate the theories.

RESULTS

The raw data from which the results were calculated is reported elsewhere [2]. Fig. 1 plots the relative cell volume, or shrinkage factor, v, as a function of external xylose concentration. In the absence of insulin, the cell shrinks as quantitatively expected by any theory which asserts that the intracellular pentose is in normal aqueous solution. With insulin present, the cell remains at its original volume up to 200 m$\tilde{\text{M}}$ xylose, again as predicted by the osmotic theory. Thus, the data conform to the model in which all of the internal xylose, including the insulin-dependent part, is osmotically active. This result entails serious evidence against the theory that insulin-sensitive xylose is adsorbed.

Fig. 2 plots the xylose r value as a function of external pentose. In the absence of insulin, the r value rises with concentration; the data follow rather well the a priori prediction of the compartmentation theory. In the presence of insulin, up to 100 m$\tilde{\text{M}}$, this theory is also followed; above 100 m$\tilde{\text{M}}$, we do see a departure of about 10% from the predicted r value of unity, but it is under just these conditions (high sugar and insulin) that we cannot be certain that equilibrium has truly been established [2].

A further experiment was done to test the two theories. The muscle was shrunken in medium made hypertonic by addition of NaCl (50 m$\tilde{\text{M}}$) or mannitiol (100 m$\tilde{\text{M}}$).

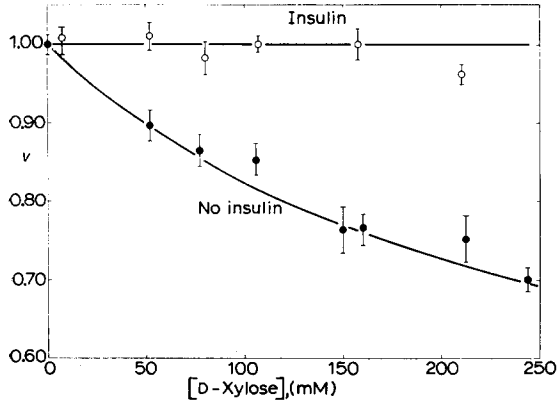

Fig. 1. Shrinkage of mouse diaphragm in hypertonic xylose. The equilibrium shrinkage factor v, as defined in the text, is plotted as a function of xylose concentration added hypertonically to Krebs–Ringer medium, in the absence (filled circles) and presence (open circles) of insulin. The solid curves are the predictions of the osmotic theory, Eqn 1. A value of 270 mM is used for the parameter S. (This is a measured, not a fitted, parameter.) Each point represents the mean and S.E. of 6 muscles.

The r value of a low concentration (29 m$\tilde{M}$) of xylose was then measured. The ordered water theory would again predict the xylose r value to be lower than at isotonicity, again because of the cell shrinkage. The compartment theory should now expect the r value to be the same as in isotonic solution; this is because NaCl and mannitol are effectively inaccessible to both compartments, and so both should shrink to the same extent. This will preserve the ratio of the xylose-accessible compartment to the total cell volume, and therefore the r value should be the same as in the non-shrunken cell. Table II shows that under these conditions of homogeneous shrinkage, the r value of xylose is indeed unchanged; only under conditions of differential shrinkage, when one compartment shrinks and the other does not, is the rise in r seen. The table also

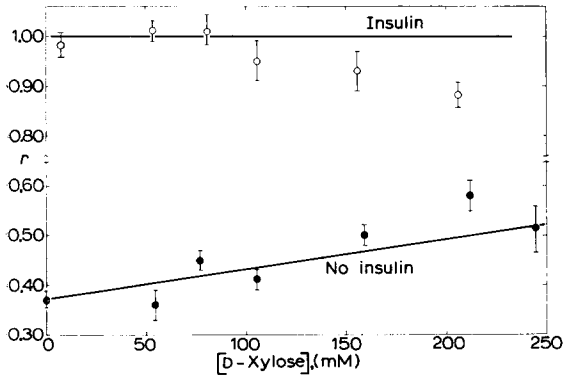

Fig. 2. Xylose distribution in hypertonic xylose. The equilibrium distribution coefficient r of D-xylose, measured on the same muscles used for data of Fig. 1, is plotted against the external xylose concentration, in the absence (filled circles) and presence (open circles) of insulin. The solid curves are predictions of the compartmentation theory, Eqn 2, using the same parameters as in Fig. 1. Each point represents 6 muscles.

TABLE II

DISTRIBUTION OF D-XYLOSE AND Na^+ UNDER HYPERTONIC CONDITIONS

Xylose, Na^+, and water content were measured in muscles shrunken by hypertonic addition of different agents. Results present mean and standard error. Other details are described in the text.

Shrinking agent	Shrinkage factor	r value	
		Xylose	Na^+
None	1.00±0.01 (4)	0.36±0.01*	0.25±0.03
Xylose, 160 mM̃ (differential shrinkage)	0.77±0.02 (6)	0.50±0.02	—
Xylose, 250 mM̃ (differential shrinkage)	0.71±0.02 (4)	0.51±0.05	0.25±0.03
NaCl, 50 mM̃ (homogeneous shrinkage)	0.76±0.03 (4)	0.35±0.05**	0.22±0.02
Mannitol, 100 mM̃ (homogeneous shrinkage)	0.76±0.03 (4)	0.35±0.03**	0.28±0.02

* Value of $r(0)$, measured in range of 0.2–30 mM external xylose. Mean and standard error of 20 muscles.

** Measured by including 29 mM xylose in the medium.

shows that under all conditions the Na^+ distribution ratio is the same. This fact rules out the possibility that the rise in xylose r value is an artifact due to partial destructuring of the cell water by the drastic hypertonic conditions employed, a possibility which proponents of the ordered water theory might raise [2].

DISCUSSION

These experiments have been directed at two questions:

(1) Is D-xylose (and by inference other sugars) excluded from the cell because of compartmentation or ordered water?

(2) Is the xylose taken up due to the action of insulin in free solution or adsorbed in the cell interior?

The first question was attacked by studying the equilibrium distribution of the sugar under hypertonic conditions. Two basic results were found. When the cell is shrunken with xylose, the xylose r value rises under basal conditions, but when it is shrunken with NaCl or mannitol, the xylose distribution is unchanged. These results strongly contradict the association–induction hypothesis predictions; they also provide rather good evidence in favor of a nonuniform distribution of intracellular xylose, i.e. for the compartmentation theory.

The second question above was attacked by comparing the cell shrinkage produced by hypertonic xylose under basal and insulinized conditions. The proposition that the insulin-sensitive xylose is bound is ruled out by the fact that it shows full osmotic activity, even up to 200 mM̃ external sugar.

These results as well as those of the preceding paper [1] cast grave doubt on the validity of the theory of Ling of water structure. In addition, the theory that insulin-

dependent sugar is bound in the muscle has been made less attractive. However, if this fraction of xylose is, in fact, free in the cytoplasm, the insulin-reversal experiments already reported [1] force us to the conclusion that the mechanism of sugar exclusion in mammalian muscle is an insulin-inhibitable active extrusion process. The implications of such an unexpected conclusion are speculated upon elsewhere [2].

Finally, it should be pointed out that although the results of Fig. 2 as well as the pattern of nonelectrolyte distribution already reported [1] favor the idea that a compartmental effect is involved in the sugar exclusion, the data before us do not clearly distinguish whether the active sugar transport mechanism is located in membranes of intracellular compartments or in the plasma membrane. If the latter possibilities were accepted, there would be no strict need to postulate intracellular compartmentation. In a previous report [2], these different possibilities are considered at length.

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