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NONELECTROLYTE DISTRIBUTION IN MOUSE DIAPHRAGM MUSCLE

I. THE PATTERN OF NONELECTROLYTE DISTRIBUTION AND REVERSAL OF THE INSULIN EFFECT

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SUMMARY

The steady-level distribution coefficients (r values) between the cell water and the incubation solution are measured for a variety of small, hydrophilic, nonmetabolized nonelectrolytes in a red muscle preparation, the mouse diaphragm. Four distinct groups of solutes are discerned: large sugars, with $r = 0.08$; many monosaccharides, with $r = 0.36$; some sugars in the presence of insulin at 20 °C, and D-ribose, with $r = 0.80$; and all compounds tested with four carbons or less, and some sugars in the presence of insulin at 34 °C, with $r = 1.00$. The results can be interpreted in terms of a four-compartment model of the muscle fiber. The model requires that 64% of the cell volume be noncytoplasmic .The results are considered difficult to interpret in terms of the association–induction hypothesis of Ling.

Special conditions are found under which D-xylose moves out of the muscle fiber against its concentration gradient. This observation requires either that insulin-sensitive xylose be adsorbed in the cytoplasm or that an active extrusion mechanism for sugars exists in the muscle.

INTRODUCTION

It has long been known [1–9] that muscle cells immersed in solution containing a nonmetabolized glucose analogue exclude the sugar at equilibrium; that is, the final steady-level concentration of the sugar in the muscle fiber water is less than the external concentration. Since in muscle the transport of sugars is generally believed to be mediated by a passive facilitated diffusion mechanism [10], this phenomenon has not often been explained by postulating outward active transport of sugars. Instead, most workers in the field have attributed the exclusion to the existence of intracellular compartments to which the sugars cannot gain entry. The solutes are able to equilibrate with only part of the muscle cell water.

On the other hand, the association–induction hypothesis of Ling postulates that sugars, as well as other solutes like Na^+, are not as soluble in the cell water as in normal water of aqueous solution [11–14]. The intracellular water is considered to be highly structured in "polarized multilayers" adsorbed to cytoplasmic protein sites. The theory states that solutes such as sugars are distributed in all the cell water, but that the water is a poor solvent for hydrophilic, polyatomic solutes. According to this theory [13], the degree of a solute's exclusion should depend on its size and its H-bonding capacity; as either of these chemical parameters is increased, the solute's solubility in the cell water should decrease.

It is further known that insulin can increase the "sugar space" of muscle [2, 3, 5, 15]; the effect appears to be stereospecific. The compartmentation theory claims that the hormone acts by rendering the intracellular compartments accessible to the sugars by activating a specific transport mechanism in the compartmental membranes. The association–induction hypothesis, however, postulates that additional sugar can enter the cell in response to insulin because the hormone activates cytoplasmic binding sites which selectively adsorb D-glucose and its analogues [16].

These two explanations of sugar exclusion and insulin action represent such fundamentally different views of cell water and cytoplasmic structure that an attempt should be made to test them experimentally. Therefore, a study was undertaken in a mammalian muscle to examine the steady-level behavior of small, nonmetabolized, hydrophilic solutes which are not generally considered actively transported in the tissue. This paper presents a survey of the equilibrium distribution coefficients between the cell water and external solution of such a series of solutes. Insulin is used as a tool to alter the distribution properties of some of these solutes in hopes of differentiating the opposing theories before us.

METHODS

Supplies

Chemicals used were reagent or biological grade, obtained from Sigma, Pfanstiehl, or Eastman Organic Chemicals. Bovine insulin was either crystalline Type II from Sigma or Iletin U-80 from the Pennsylvania Hospital Pharmacy. Radioactive compounds were obtained from New England Nuclear or Amersham Searle.

Adult male albino mice weighing 30-40 g, designation HTF(SW), were ordered weekly from Huntingdon Farms, Conshohocken, Pa. They were given Purina Lab Chow and water ad libidum.

Choice of tissue

The mouse diaphragm muscle was used in all experiments. This tissue was chosen rather than the more popular rat diaphragm because of its high degree of fiber homogeneity [17]; whereas the rat muscle is a mixed population of three fiber types, the mouse diaphragm fibers are nearly all red.

All incubations used the intact diaphragm or hemidiaphragm preparation [1], in which the rib cage is incubated along with the muscle. This technique prevents the muscle fibers from being cut. The time elapsed from the killing of the mouse (by breaking its neck) until complete dissection of the muscle was about 2 min.

Long-term incubation in vitro

Since equilibrium behavior is being studied, it is imperative to be able to keep the tissue alive in vitro for long periods of time. An incubation technique was developed which could achieve at least 30 h of survival time at 20 °C. Muscles were continuously shaken in a Krebs–Ringer solution modified with pyruvate, amino acids, and vitamins. These extra components were found helpful in the maintenance of long-term survival, but they did not affect the nonelectrolyte behavior to be described. The normal bicarbonate-buffered medium (pH 7.4) is given in Table I.

TABLE I

COMPOSITION OF NORMAL BICARBONATE-BUFFERED MEDIUM

Component	Concn (mM)	Component	Concn (mM)
Na^+	142.8	Cl^-	109.4
K^+	5.5	HCO_3^-	26.0
Ca^{2+}	2.5	P_i	1.9
Mg^{2+}	1.1	SO_4^{2-}	1.1
Glucose	5.5	Pyruvate$^-$	15.0

Sodium penicillin and streptomycin sulphate (50 mg/l) were included in the medium. A chemically defined tissue culture medium, the composition of which is reported elsewhere [18], was included in the final solution at 8% full strength. This supplement contained micromolar concentrations of amino acids, vitamins, and substrate organic acids, but no proteins or undefined components. When test nonelectrolytes were added to the solution, an osmotically equivalent amount of NaCl was removed. When the medium contained insulin, a maximal dose, 25–100 units/l, was used.

All incubations were carried out in Erlenmeyer flasks immersed in a shaker bath maintained at 20 ± 0.5 °C, except where otherwise noted. The volume of solution was so large with respect to the amount of tissue that the external solute concentrations remained constant throughout an incubation.

The medium was continuously bubbled with O_2–N_2–CO_2 (50:47.5:2.5, by vol.). At 20 °C, this mixture gave a much longer survival time than the more frequently used O_2–CO_2 (95:5, by vol.). At 34 °C, however, it was found necessary to use the latter mixture, probably because of diffusional limitation of oxygen [19] at the higher temperature.

As is shown elsewhere [20], tissue contents of Na^+, K^+, ATP, and creatine phosphate, as well as tissue water content, remain constant for at least 25 h at 20 °C under the incubation conditions described.

Tissue analysis

A hemidiaphragm muscle weighing 20–35 mg was removed from solution, excised from the rib cage, and cut into two pieces, which were then blotted and weighed on an analytical balance. The large piece (15–25 mg) was extracted for several hours in 0.5–3.0 ml of 1% trichloroacetic acid or 0.1 M HCl for nonelectrolyte analysis. The smaller piece (5–10 mg) was used for determination of water content (by drying overnight at 98 °C) and of tissue ion contents, which were routinely checked in long-term experiments.

A solute's equilibrium distribution coefficient, called r, is defined as the ratio of internal concentration (moles/kg intracellular water) to the external concentration (molal).

Metabolism of solutes

It is important to be sure that the solutes used are not metabolized in the tissue, especially the glucose analogues. It is known that D-xylose [21], 3-*O*-methylglucose [22], and 2-deoxy-D-galactose [23] are not metabolized appreciably in mammalian muscle. This conclusion has been confirmed for other pentoses [20].

Chemical assays

Pentoses [24], ketoses [25], 2-deoxy sugars [26], and vicinal diols [27] were assayed colorimetrically by standard procedures. Glucose was assayed enzymatically using the Glucostat kit (Worthington Biochemical Corp.). ATP, creatine phosphate, and free creatine were determined by methods developed in the laboratory of Davies [28].

Background tissue blanks were determined for all colorimetrically measured solutes. In general, they were small and reproducible. All colorimetric assays were read on a Klett colorimeter or a Beckman DU spectrophotometer.

^{14}C- or ^{3}H-labelled solutes were counted in Bray's solution by a Packard liquid scintillation counter. Relative quenching was determined by the channels-ratio method or the internal standard method; this correction was never greater than 5%.

RESULTS

Extracellular space

In order to estimate intracellular concentrations, it is necessary to know the volume of extracellular space, which in this work will be reported in units of g extracellular water/g total tissue water, and will be called σ. Three independent methods are used to arrive at a working value for σ.

Influx profile of sucrose or raffinose. It is generally realized that the classical extracellular markers sucrose and mannitol can enter the muscle fiber to some extent [29, 30]. Therefore, it is not correct to measure the equilibrium sucrose space to determine σ. However, if the marker enters the fiber slowly with respect to its entry into the true extracellular space, we can hope to separate the two fractions kinetically. For the tissue used here, the time to reach 95% equilibration with the extracellular space has been calculated to be in the order of 10 min for sucrose [20].

Figs 1a and 1b show the influxes of sucrose and of the trisaccharide raffinose. They are both biphasic curves, each with a fast fraction and a slow one. For sucrose, the fast fraction is complete in about 10 min, and the slow in about 30–60 min; the sucrose space does not change at all between 5 and 21 h. The raffinose influx shows a better separation of the fast and slow components. Here, the fast fraction is complete in about 30 min; the second fraction enters very slowly, not being apparent in the first two hours of the influx. By 21 h, however, this slow fraction is also complete. Thus, the 5–15 min sucrose space yields a working value for the extracellular space; likewise, the 1–2 h space of raffinose gives a good estimate for σ. The least-squares retropolated values for the fast fraction of the sucrose influx gives $\sigma = 0.200 \pm 0.005$ (S.E. of 14 muscles); the corresponding raffinose fraction gives $\sigma = 0.21 \pm 0.01$ (20).

It should be noted that the final equilibrium spaces for sucrose and raffinose are identical, and higher than the true extracellular space. The significance of this extra space, amounting to about 8% of the fiber water, will be discussed later.

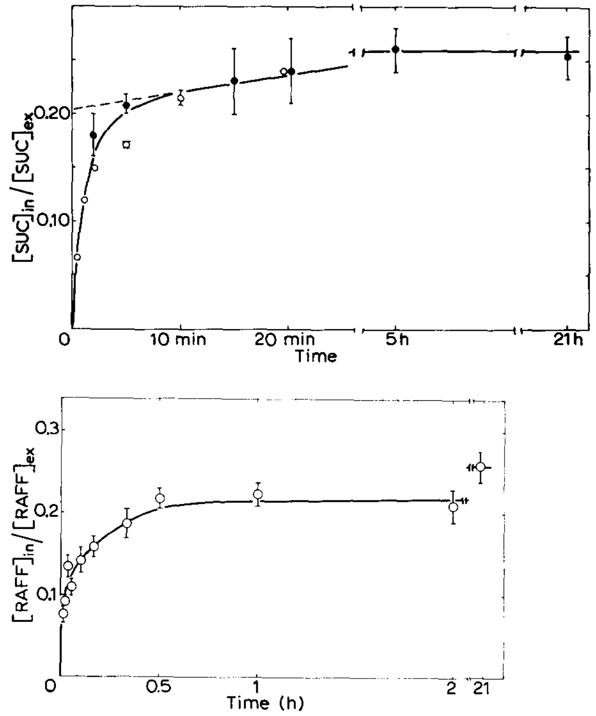

Fig. 1. Uptake of extracellular markers, 20 °C. a. Sucrose, external concn 50 mM. Open circles: influx begun immediately after dissection. Closed circles: influx begun after 6 h of sucrose-free incubation. b. Raffinose, external concn 50 mM. Details as in sucrose uptake. Each point with standard error bars represents 4 muscles. Points without bars represent single muscles.

Inulin space. Inulin, a large polysaccharide, has often been used as an extracellular marker, even though Ogston [31] showed that the molecule does not equilibrate with all the water in a dilute hyaluronic acid gel, a model connective tissue system. Assuming that inulin does not enter the fiber, its equilibrium will give a lower limit for σ. Muscles were incubated in a medium containing 0.5% inulin, and the tissue inulin concentration was measured. Eight muscles gave a value of 0.18 ± 0.01 for the extracellular parameter.

Glucose space. The mouse diaphragm, being a red muscle, has a high metabolic rate [17]. It has been suggested that in the noninsulinized rat diaphragm the transport of glucose is the rate-limiting step in its metabolism [2, 23, 32, 33]. Therefore, the intracellular free glucose should be close to zero; in addition, the concentration of glucose in the sarcoplasmic reticulum may also be expected to be low, since there is evidence that this compartment contains hexokinase and other glycolytic enzymes [34]. Thus, any glucose measured in the tissue should be located in the extracellular space. The tissue glucose content was measured in media of varying glucose concentrations, and the extracellular parameter was found to be 0.20 ± 0.01 (8) [20].

Thus, we have three different methods which give the same value for σ within a few percent. In all data to be presented, a value of 0.21 will be used. This value is independent of temperature and is not changed significantly by insulin [20].

Uptake of D-*xylose*

Fig. 2a shows the time course of D-xylose (as well as L-arabinose) uptake at 20 °C. The sugar enters quickly, attaining equilibrium in less than 4 h, and remains at the same internal concentration for at least 25 h. It is shown elsewhere [20] that if the muscle is first incubated in 65 mM xylose and then transferred to 27 mM xylose, the internal sugar comes to the same final concentration as it does when the muscle is simply incubated in 27 mM xylose medium. This concentration-shift experiment demonstrates that the distribution ratio represents a true steady level; it is not an artifact due to slow net permeability of the sugar.

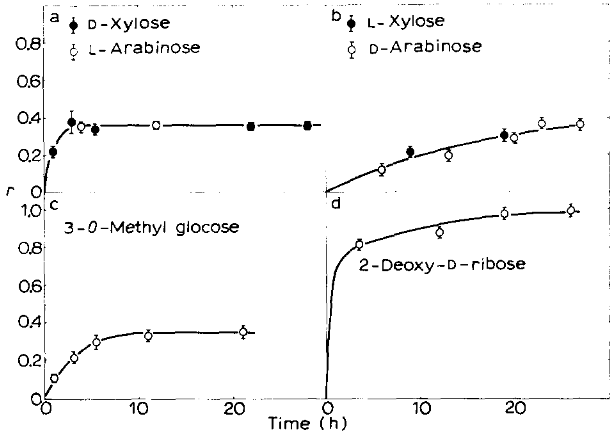

Fig. 2. Sugar uptake in mouse diaphragm. a. D-Xylose and L-arabinose, external concn 30 mM, b. L-Xylose and D-arabinose, 20 mM external concn. c. [^{14}C]3-*O*-Methylglucose, 10 mM external concn. d. 2-Deoxy-D-ribose, 45 mM external concn. Each point represents 4 muscles.

It should be immediately noticed that the *r* value is not 1.0, which might be expected for a passively transported solute, but 0.36; the sugar is excluded from the cell at equilibrium. Fig. 3, a plot of internal versus external xylose concentration at equilibrium, shows that *r* is concentration-independent up to 80 mM at least. Data in Table II show that *r* is not changed by raising the temperature to 34 °C.

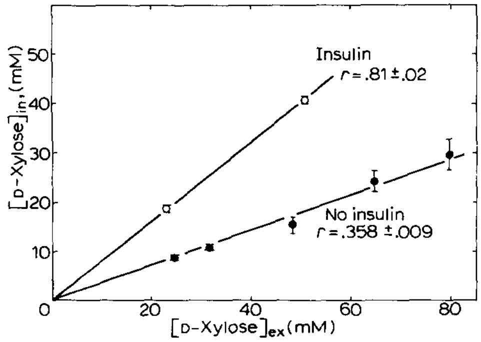

Fig. 3. D-Xylose isotherm in mouse diaphragm, 20 °C. Concentration of D-xylose in muscle fiber water at equilibrium is plotted against external concentration. Lower line, no insulin present (basal conditions); Upper line, insulin (50 units/l) added to medium. Each point represents 4–16 muscles.

TABLE II

THE PATTERN OF NONELECTROLYTE DISTRIBUTION IN MOUSE DIAPHRAGM

Solute	Incubation conditions	External concn (mM)	r±S.E.	(N)
D-Xylose	Normal	0.2–80	0.358±0.009	(38)
	20 °C+insulin	25–50	0.81 ±0.02	(10)
	34 °C	16–50	0.34 ±0.01	(16)
	34 °C+insulin	16–50	1.01 ±0.02	(10)
L-Xylose	Normal	20	0.39 ±0.08	(8)
D-Arabinose	Normal	20	0.35 ±0.02	(7)
L-Arabinose	Normal	0.2–45	0.36 ±0.02	(19)
D-Lyxose	Normal	30	0.38 ±0.03	(4)
D-Ribose	Normal	24	0.81 ±0.03	(8)
	20 °C+insulin	24	0.80 ±0.02	(8)
3-*O*-Methyl-D-glucose	Normal	5–10	0.38 ±0.02	(7)
2-Deoxy-D-galactose	Normal	17	0.36 ±0.01	(8)
	20 °C+insulin	13	0.78 ±0.02	(16)
2-Deoxy-D-ribose	Normal	4–45	0.99 ±0.01	(16)
	20 °C+insulin	44	1.00 ±0.01	(4)
Sucrose	Normal	50	0.08 ±0.03	(8)
Raffinose	Normal	50	0.07 ±0.03	(4)
Methanol	Normal	3	1.01 ±0.03	(8)
Ethylene glycol	Normal	2–10	0.98 ±0.04	(8)
Glycerol	Normal	50	0.98 ±0.04	(11)
2-Deoxyerythritol	Normal	50	1.00 ±0.03	(11)
Erythritol	Normal	50	> 0.90	
Xylitol	Normal	50	> 0.65	

A detailed analysis given elsewhere [20] shows that such a low *r* value might well be expected on the basis of simple facilitated-diffusion carrier theory. The analysis shows that if glucose and xylose share the same carrier, and if internal glucose concentration is kept low by metabolism, then xylose should have a distribution coefficient in the range 0.3–0.5 in a normal medium. A necessary prediction of this glucose gradient theory is that the xylose *r* value should depend on the external glucose concentration and must approach unity as external glucose goes to zero. Fig. 4 presents the

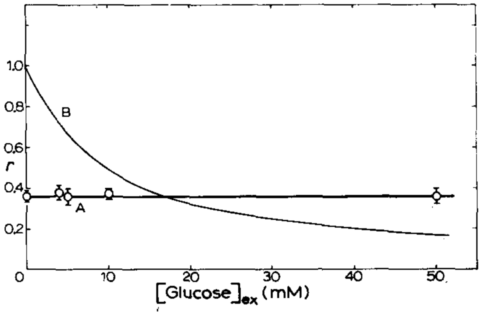

Fig. 4. Xylose *r*-value as a function of external glucose concentration. Mouse diaphragm muscles were equilibrated with solutions containing D-xylose (25–40 mM) at varying external glucose concns at 20 °C. Line A, best line through data points. Line B, behavior expected by "glucose gradient" theory, as explained in [20].

xylose r value as a function of external glucose concentration. The parameter is independent of external glucose in the range 0–50 mM (Curve A); the dependence demanded by the theory (Curve B) is not observed. Therefore, this trivial explanation of the low xylose r value is ruled out.

Fig. 5 shows the D-xylose uptake and concentration-shift experiment in the presence of insulin. The r value is not 0.36, but 0.81. The top curve of Fig. 3 shows that the insulinized r value is independent of xylose concentration up to at least 50 mM. It is important to realize that at 20 °C the sugar is still excluded from the cell to some extent; at 34 °C, however, the insulinized r value for D-xylose is 1.0, as reported in Table II.

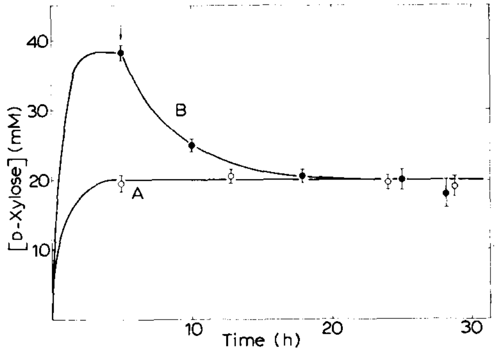

Fig. 5. D-Xylose uptake and concentration-shift in the presence of insulin. Curve A, time course of D-xylose uptake in mouse diaphragm incubated in medium containing 25 mM xylose, 20 °C. Curve B: muscles were first equilibrated in 48 mM xylose medium; at the arrow, they were transferred to 25 mM xylose medium. Each point represents 4 muscles.

Other sugars

The influxes of a number of different sugars under basal (i.e. noninsulinized) conditions are graphed in Fig. 2. Fig. 2a shows the fast-moving pentoses D-xylose and L-arabinose (D-lyxose is also in this group). These pentoses equilibrate rapidly, and all reach a final r value of 0.36. All three are insulin-sensitive, although accurate insulinized r values have been measured only for D-xylose. In contrast, two slow-moving pentoses, L-xylose and D-arabinose, are shown in Fig. 2b. These enter much more slowly than their optical isomers, though they do seem to approach the same final level. The uptake is largely unaffected by insulin [20]. Fig. 2c shows the uptake of 3-*O*-methyl-D-glucose, a seven -carbon glucose analogue. It is taken up to the same final distribution ratio as the pentoses and is also insulin-sensitive (data not shown). Another fast-moving, insulin-sensitive sugar not shown in the figure is 2-deoxy-D-galactose. Table II demonstrates that it follows the same pattern as D-xylose; its basal r value is 0.36, and insulin raises this to 0.78.

In Fig. 2d the uptake of the slightly smaller 2-deoxy-D-ribose is graphed. The influx is biphasic, with a fast initial uptake to a concentration ratio of about 0.80, followed by a slower rise to $r = 1.0$. As reported in Table II, insulin has no effect upon the r value of this sugar (nor does it affect the rate of uptake).

D-*Ribose*

Fig. 6 presents the uptake of the pentose D-ribose in the presence and absence of insulin. The basal uptake is slow compared to that of D-xylose, and it attains a higher steady level; the r value is not 0.36, but 0.81, as is also shown in a concentration-shift experiment [20]. Insulin increases the rate of uptake but not the final level. This sugar, then, is anomalous among the pentoses in its equilibrium behavior. This anomaly will be discussed later.

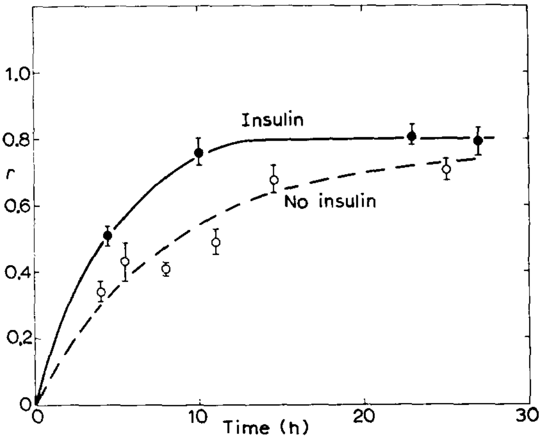

Fig. 6. D-Ribose uptake in mouse diaphragm. External ribose concentration was 24 mM in both the basal (lower curve) and insulinized (upper curve) uptakes. Each point represents 4 muscles.

A homologous series: the polyhydric alcohols

Ling [12, 13] has predicted from his theory of cell water that the r values of a homologous series of hydrophilic compounds, the polyhydric alcohols, should gradually decrease as the series progresses from methanol to the pentitols. Each additional member of the series is formed by adding a CHOH group onto the preceding member, thus increasing its size and hydrophilicity, and if Ling is correct, decreasing its affinity for the cell water.

Fig. 7 shows the influxes of several polyols, glycerol (C_3), erythritol (C_4), and

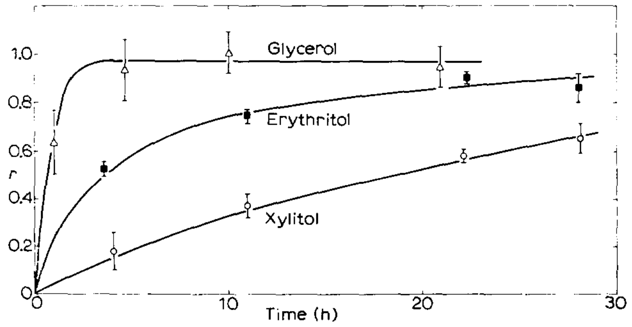

Fig. 7. Uptake of polyhydric alcohols in mouse diaphragm. Concentration of polyol in the medium was 50 mM in all cases. Each point represents 4 muscles.

xylitol (C_5). The last two of these are only slowly permeant, and a true equilibrium is not established even after 28 h. It is clear, though, that the *r* values of these two are considerably higher than that of the typical pentoses, 0.36; erythritol shows indications of leveling off between 0.9 and 1.0. The *r* values for the entire series, C_1 to C_5, are given in Table II. The lower polyols, methanol, ethylene glycol, glycerol, and 2-deoxyerythritol all enter the cell quickly (in 0.5–3 h), and *r* is within 2% of unity for all of them. We do not see a gradual decrease in *r* as the series progresses, as the ordered water theory had predicted.

Reversal of the insulin effect

The data presented above show that certain sugars are excluded from the muscle fiber water and that insulin removes this exclusion, either partially or totally, depending on the temperature. A further experiment was done to gain insight into the insulin effect. Muscles were allowed to take up D-xylose in the presence of insulin; they were then transferred to an insulin-free medium with the same xylose concentration. Since it has been shown [20] that several hours of insulin-free washing are sufficient to remove insulin from the muscle, it is of interest to examine the behavior of the insulin-sensitive xylose in the muscle as the hormone is washed away.

Figs 8a and 8b show two such experiments. After the muscles were loaded with xylose in the presence of insulin, they were transferred to insulin-free washing solution. As the hormone is washed off the muscles, the xylose moves out of the cell against its concentration gradient. In some cases, the internal xylose concentration reverses fully to the noninsulinized level. The movement is more dramatic at lower concentra-

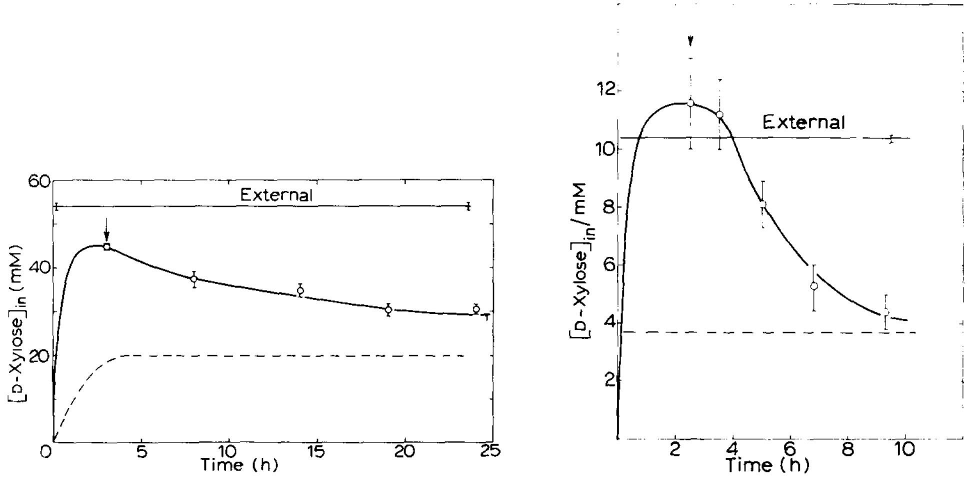

Fig. 8. Reversal of insulin effect in mouse diaphragm. Muscles were "loaded" with D-xylose by incubation in insulin-containing solution. At the arrow, they were transferred to a large volume (about a liter for 10 muscles) of insulin-free solution with the same xylose concentration as the loading solution. The internal concentration of xylose was followed during the "reversal" period. Dotted lines represent the levels of internal xylose which are observed for noninsulinized muscles. Each point represents the mean and standard error of 4–6 muscles. a. External xylose concn 54 mM. Temp. was 20 °C. Tissues water contents (g tissue water/g wet wt) as follows. Insulinized (3-h point): 0.796±0.002 (6): final (24-h point): 0.794±0.002 (5). b. External xylose concn 10.6 mM. Temp. was 37 °C. Insulinized water content: 0.766±0.003 (4); final water content: 0.770±0.006 (4).

tions and higher temperatures, but it is unmistakable and reproducible in all cases. Tissue water contents did not change during the reversal, as the legends to Fig. 8 show.

DISCUSSION

With the exception of the insulin reversal experiment, the effects reported here have been recognized qualitatively for many years. However, in past work, in-vitro incubation techniques were not well enough developed to enable very long-term steady levels of solutes to be determined quantitatively in mammalian muscle. These experiments have been done in order to test two opposing theories of the cell interior, and so the discussion will be structured accordingly.

From the standpoint of the compartmentation theory, the data of Table II make it clear that at least four compartments must be involved. Solutes fall into four classes: r values cluster about 0.08 (large sugars), 0.36 (many monosaccharides), 0.80 (D-ribose, insulinized sugars at 20 °C), and 1.00 (polyhydric alcohols, deoxy ribose, insulinized sugars at 34 °C).

It seems most likely that the low r value of sucrose and raffinose is due to entry into the sarcoplasmic reticulum. Though there is some controversy on this issue [29, 35] the weight of evidence indicates that the sarcoplasmic reticulum can be permeated from the extracellular space by small solutes [36–40]. Further, it has been pointed out [20] that the sucrose space of single frog muscle fibers is equal to the sarcoplasmic reticulum volume determined by electron microscopy. It is natural, then, to assign these large sugars to the sarcoplasmic reticulum, totally excluded from the rest of the cell. This assignment gives a sarcoplasmic reticulum volume of about 8% of the mouse diaphragm muscle fiber.

The real problem for the compartmentation theory is to explain the r value of 0.36 for the simple sugars. Assuming that glucose analogues enter the cytoplasm, the theory requires that 64% of the cell volume be non-cytoplasmic. Furthermore, in order to explain the r value of 0.80, it must be assumed that this inaccessible compartment is subdivided into at least two parts. Electron microscopy has not been carried out on the mouse diaphragm, but the possibility has been raised, albeit reluctantly, that the mitochondria (each of which is considered to be a two-compartment system [41–44]) could account for 64% of the cell volume in this red muscle [20]. The frequent observation of r values near unity indicates that there is no appreciable non-solvent water in this tissue.

The D-ribose anomaly has been discussed extensively in terms of the compartmentation theory [20]. The anomalous behavior has been explained by the fact that, of all the pentoses, only ribose is found in the furanose form in aqueous solution [45, 46]. This explanation predicts that if insulin has any effect on D-ribose, it should only be on the rate of entry; the steady level should not be affected by the hormone. Indeed, this is exactly the behavior observed. In analogy to the effect discussed here, it should be mentioned that D-ribose has an anomalously high permeability to isolated chloroplasts [47].

One serious problem with the compartmental explanation is that frog muscle, which has a much cleaner interior than mammalian red muscle, also has been reported to exclude sugars [8, 29] and amino acids [48, 49]. It would shed much light on the problem if a comparative study was performed on a variety of tissues to see if the r value for D-xylose correlates with, say, the non-mitochondrial volume fraction.

The data here raise some fundamental problems for the ordered water theory [20]. At first glance, the data of Table II seem to support the theory by demonstrating a range of r values for different hydrophilic solutes, but closer inspection reveals some problems. The r values do not show the true spectrum determined by solute size and hydrophilicity anticipated by the theory. As might be expected by this explanation [13], small solutes such as methanol are not excluded from the cell water at all. As the solutes become larger and more hydrophilic, however, the r values remain at unity instead of gradually decreasing. When the solutes become large enough (i.e. the glucose analogues), the r value suddenly jumps to 0.36.

Insulin-sensitive sugar binding is also not indicated by the data of Fig. 3. The insulin-dependent xylose concentration in the muscle does not saturate with external concentration, at least up to 50 mM; as is shown elsewhere [20]. This kind of data requires that the concentration of cytoplasmic xylose-binding sites available be at least in the order of 130 μmoles/g wet wt. It is difficult to imagine what cytoplasmic protein could provide so many adsorption sites.

Whereas the results just discussed are more easily handled by the compartmentation theory, the insulin-reversal experiments (Fig. 8), which yield qualitatively new observations, are more simply explained on the basis of the association–induction hypothesis. If insulin-dependent xylose is adsorbed to cellular binding sites, as insulin is washed away from the muscle, the sites should disappear (functionally, at least), and the xylose should move out of the cell against an apparent gradient.

The compartmentation theory, in its usual form, has difficulty explaining these data. It has often been implied that sugars are kept out of the intracellular compartments because they are impermeant. The data before us disprove this explanation. In order to save the compartment theory, an outward sugar pump (or coupled transport mechanism) would have to be placed in the compartmental membranes to provide the exclusion mechanism. Insulin must be postulated to inhibit this pump. Such a picture is not impossible, but it is certainly unexpected, since sugar transport in muscle has been thought to be passive [10].

The conclusions of this paper are thus not clear. Some of the data seem to support a compartmental explanation of sugar exclusion and insulin action in muscle. For this theory to be valid, however, nonmetabolized glucose analogues must be postulated to be actively transported in some manner. On the other hand, the association–induction hypothesis can explain the insulin-reversal experiments easily, but not the pattern of nonelectrolyte distribution. It has been pointed out that the association–induction hypothesis cannot tolerate the proposition that the insulin-sensitive sugar is not adsorbed in the cytoplasm [20]. In order to force a crucial test of the two theories, then, the next paper in this study [50] presents direct evidence bearing on the state of insulin-sensitive D-xylose in the cytoplasm. The possibility of sugar exclusion by ordered water will also be considered further.

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