STUDIES ON INSULIN ACTION. III. LINEAR DISTRIBTUION OF D-GLUCOSE, D-RIBOSE AND METHANOL IN FROG MUSCLE CELLS AT 0°C IN THE ABSENCE OF INSULIN.

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SUMMARY

In the effective absence of insulin, D-glucose, D-ribose and methanol accumulate inside frog muscle cells at 0°C. The steady level distribution coefficients (q), between the cell water and external solution are independent of concentration and are 0.25, 0.25 and 0.8 for D-glucose, D-ribose, and methanol, respectively. Preincubation of the muscle with insulin does not change the intracellular concentration of D-ribose or methanol, but adds another fraction of intracellular D-glucose. This additional fraction follows a Langmuir adsorption isotherm.

In the preceding paper of this series[1] we showed that D-mannitol and sucrose enter intact muscle fibers, reaching a steady concentration in the muscle tissue 20-30% of that of the external solution. In the absence of insulin, the distribution of glucose has been found to fall within this same range.[2–6] This finding has indicated to some that, in the absence of insulin, cells are effectively impermeable to glucose. With the finding that D-mannitol and sucrose, often considered to be distributed only extracellularly, do enter muscle cells,[1] this conclusion no longer seems warranted. For this reason we have re-examined the problem of the distribution of glucose in muscles effectively freed of insulin. We have used for this study a method introduced in a preceding paper,[7] which allows the study of glucose distribution in the effective absence of phosphorylation.

MATERIALS

All chemicals used were of c.p. grade. Insulin was obtained from the Sigma Chemical Co. (Lot no. 26B-0700, PJ4609). I^{131}-labeled insulin was from Abbott (Lot no. IN360-10). Radioactively labeled glucose was obtained from Calbiochem (U-C-14), International Chemical and Nuclear Corp. (glucose-1-C-14, Lot no. 1203; glucose-1-H-3, Lot no. 10211),

and Schwartz, Orangeburg, N.Y. (U-C-14, Lot no. 6301). Frogs (Ranapipiens, Schreber) were maintained as previously described.[7] Mixed muscles (sartorius, semitendinosus, iliofibularis, and tibialis anticus longus) were used throughout.

METHODS

The methods used here have been described in detail in a preceding paper.[7] To effectively remove endogenous insulin, muslces were gently shaken for 6 hours at 25°C in three changes of Ringer solution containing neither insulin nor glucose (about six muscles to 250 ml of solution). This procedure as well as insulin pretreatment at 25°C were followed by overnight incubation at 0°C with varying concentrations of labeled glucose to obtain a steady level of glucose uptake in the effective absence of phosphorylation.

The efflux of I^{131}-labeled insulin was studied as follows: Muscles were incubated with I^{131}-labeled insulin (50 μc/ml) and 0.1 u/ml nonlabeled insulin for 6 hours at 25°C. The accumulated insulin was washed out at 25°C in successive 5 ml volumes of glucose-free Ringer solution. At the end of the washout period, the insulin remaining in the muscle was extracted by boiling 20 minutes in 2 ml of 0.1 N HCl. 0.5 ml aliquots of both washout solution and extract were added to 5.0 ml Bray's scintillation fluid[8] and counted on a Packard Tri-Carb β-scintillation counter. The amount of radioactivity remaining in the muscle at a given time was calculated by adding the radioactivity in the succeeding washout tubes to that remaining in the muscle at the end of the experiment.

Calculation of *Intracellular* Glucose

The total uptake of glucose by muscle tissue was corrected for glucose in the extracellular space complex (extracellular space proper, plus the nonmuscle cell components such as connective tissue, small blood vessels, etc.) according to the following formula:

$$[\text{glucose}]_{in} = \frac{[\text{glucose}]_{tis} - 0.089\,[\text{glucose}]_{ex}}{0.915} \qquad (1)$$

where $[\text{glucose}]_{in}$, $[\text{glucose}]_{tis}$, and $[\text{glucose}]_{ex}$ are the intracellular, tissue, and external concentrations respectively. The basis for this value of the extracellular space is given in the preceding paper.[1]

RESULTS

Effect of Washing at 25°C on the Subsequent Uptake of Labeled Glucose at 0°C.

Curve B of Figure 1 shows the steady level uptake (0°C) of labeled glucose by frog muscles, following washing for varying lengths of time at 25°C in Ringer phosphate containing no insulin and no glucose. As the figure shows, the level of uptake after some 6

hours of washing is maintained for at least 8 hours. The results are substantially the same if the muscles are preincubated at 25°C with insulin (0.1 u/ml) and glucose (24 mM) (Curve A) before washing begins (as indicated by arrow).

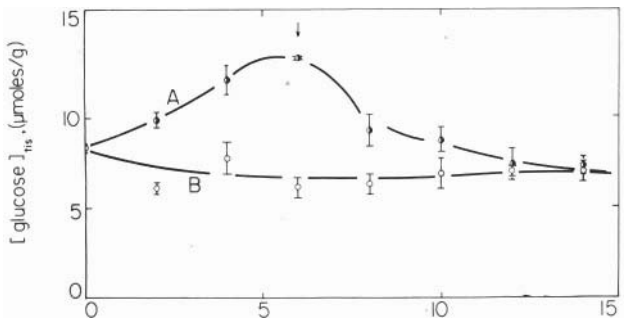

Figure 1. Effect of Duration of Washing at 25°C on the Subsequent Level of Glucose Uptake at 0°C. Mixed muscles were washed for the time shown on the abscissa in a large volume of Ringer phosphate solution containing no insulin and no glucose and then incubated overnight at 0°C in Ringer solution containing 24 mM glucose and labeled glucose. Values of labeled glucose taken up are the average of 4 determinations; the distance between the bars represents two standard errors. Lower curve, freshly isolated muscles; upper curve, muscles preincubated with 0.1 u/ml insulin and 24 mM glucose for 6 hours before washing begins (arrow).

The top curve of Figure 2 shows that when insulin is added to the preincubation medium following 7 hours of washing in the absence of insulin there is a prompt increase in the subsequent uptake of glucose at 0°C. D-ribose distribution in the same tissues (bottom curve), however, shows no response to the insulin-glucose treatment (see below). The response of glucose uptake shows that the muscles have not lost their ability to respond to insulin during the prolonged washing period and adds support to our earlier conclusion from K^+-ion assay and resting potential measurement that this procedure causes no injury to the muscles. It might be noted that the response time of these washed muscles to insulin is considerably shorter than that of freshly isolated muscles (see ref. 7, Fig. 7).

Time Course of Loss of I^{131}-labeled Insulin from Muscle Tissues

Muscles were preincubated in the presence of labeled insulin for 6 hours and then washed in successive portions of Ringer solution containing no insulin. The efflux curve so obtained is shown in Figure 3. After 6 hours, more than 95% of the labeled insulin is lost. As shown above, this amount of time is long enough to effectively remove the insulin effect on subsequent labeled glucose uptake. Some years ago, Stadie studied the time

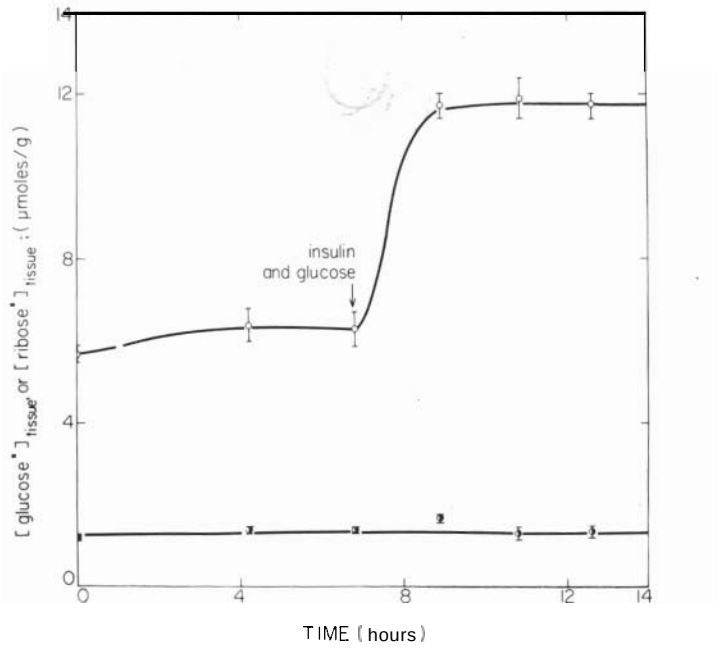

Figure 2. Effect of Addition of Insulin on the Uptake of Glucose and D-ribose by Previously Washed Muscles.
Muscles were washed as in Fig. 1. After 7 hours, insulin (0.1 u/ml) and glucose (24 mM) were added to the preincubation medium. Muscles were incubated overnight at 0°C with 24 mM glucose and H^3-labeled glucose and 5 mM ribose and C^{14}-labeled ribose. Top curve (glucose); bottom curve (ribose).

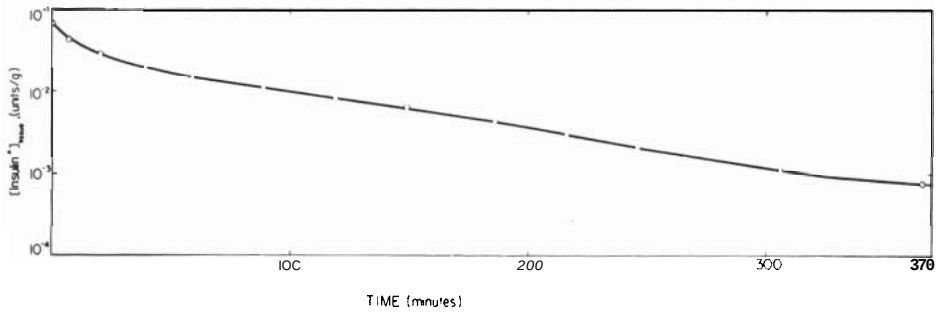

Figure 3. Efflux of I^{131}-labeled Insulin from Frog Sartorius Muscle.
Sartorius muscle was incubated with I^{131}-labeled insulin for 6 hours at 25°C and then washed in successive vials of Ringer phosphate solution containing no insulin, also at 25°C.

course of removal from rat diaphragm muscle of labeled insulin[g] introduced during a brief previous exposure (e.g., 1 minute). Since the survival time of these tissues was much shorter, the muscle was washed for a total of only 1 hour. It is not surprising that the slow release of labeled insulin reported here was not reported then.

The Quantitative Relation between Intracellular and Extracellular Labeled Glucose Concentrations

Figure 4 shows the steady level of labeled glucose accumulated at 0°C by frog muscles previously washed for 6 hours in Ringer solution containing neither insulin nor glucose (lower curve) and by muscles preincubated for 6 hours with 24 mM glucose and 0.1 u/ml of insulin (upper curves). The middle curves show the uptake of labeled glucose by insulin-treated muscles in the presence of 10 and 30 mM of nonlabeled glucose. The data are plotted in terms of μmoles of labeled glucose taken up per gram of fresh cells, using a value of 8.9% to correct for glucose in the extracellular space complex (see Methods). In the absence of insulin, the intracellular glucose concentrations are linearly related to the external concentration.

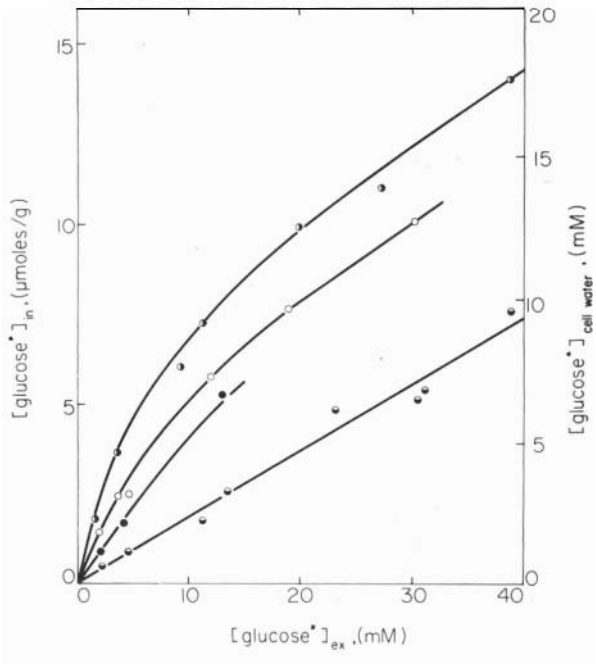

Figure 4. Steady Level of Glucose Uptake at 0°C in Insulin-treated (upper curve) and Washed Muscles (lower curve).

Mixed frog muscles were preincubated in Ringer solution containing no glucose and no insulin (lower curve) or 24 mM glucose and 0.1 u/ml insulin for 6 hours at 25°C. They were then incubated overnight at 0°C in Ringer solution containing varying concentrations of glucose and labeled glucose.

Figure 5 shows the effects of 0, 10, and 30 mM of nonlabeled glucose on the steady level of glucose uptake by washed muscles. As can be seen, the curve is again rectilinear; the addition of competing nonlabeled glucose has no effect. The slope of the curve, q, is 0.28 (right-hand ordinate), assuming all the intracellular glucose to be in the cell water. Table 1 summarizes the eleven sets of this type of experiment performed since 1964. The value of q varies between 0.19 to 0.33; the mean ± S.E. are 0.25 ± 0.01.

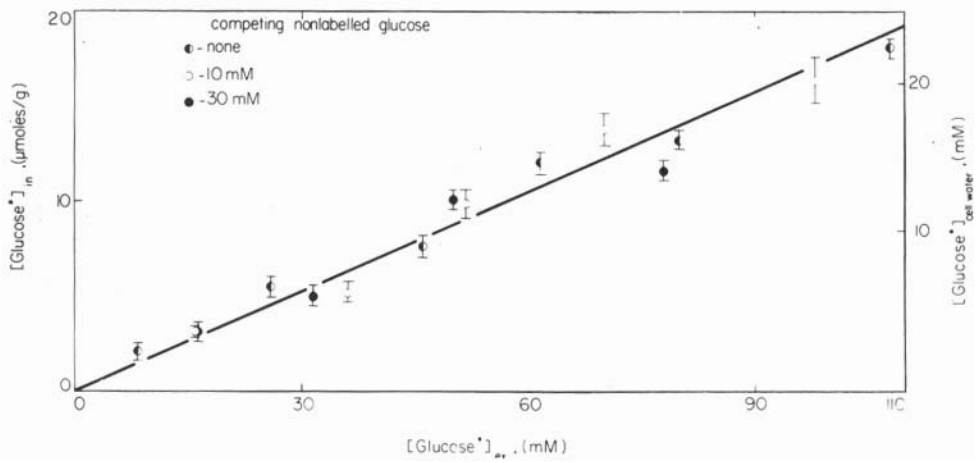

Figure 5. The Effect of Competing Nonlabeled Glucose on the Uptake of Labeled Glucose by Washed Frog Muscles.
Mixed muscles were washed for 6 hours in Ringer solution containing no glucose and no insulin at 25°C followed by incubation at 0°C with varying concentrations of glucose and labeled glucose.

The Steady Level of D-ribose and Methanol Accumulated at 0°C in Muscles Preincubated at 25°C in Ringer Solution Containing Insulin or No Insulin

Figure 6 shows that the intracellular D-ribose distribution is not changed by pretreatment with glucose and insulin. All points fall on a single straight line with a slope of 0.24 (right-hand ordinate), in terms of millimoles of D-ribose per liter of cell water. Two additional series of experiments were performed. One, without insulin, consists of a total of 26 determinations; the mean and standard errors are 0.26 ± 0.01. The other series, with 0.1 u/ml of insulin, consists of 22 determinations; the mean and standard errors are 0.26 ± 0.02.

Similarly, the distribution of methanol is rectilinear (Fig. 7); preincubation with or without insulin has no effect on the steady level of methanol taken up in subsequent incubation at 0°C. The slope of the straight line going through all the points is 0.91 (right-hand ordinate), i.e., q = 0.91. Another series of 4 individual determinations yields a mean

q value of 0.77 ± 0.01 (S.E.). A third series (4 determinations) gives a q value of 0.92 ± 0.01.

Table I. The Distribution Coefficient of D-glucose Between Intracellular and Extracellular Water at 0°C in Frog Muscles Effectively Freed of Insulin.

The second and third columns refer to the number of changes of preincubation solution (glucose-free Ringer phosphate solution containing no insulin) and the total duration of preincubation (25°C). The fourth column gives the range of D-glucose conditions in the incubation solution studies (0°C). The fifth column gives the total number of individual determinations in that experiment. The sixth column gives the mean ± S.E. of the equilibrium distribution coefficient of D-glucose between cell water and extracellular water. A value of q is calculated from each point obtained by dividing the intracellular D-glucose concentration by the final equilibrium external D-glucose concentration. In all cases the relation between intracellular and extracellular D-glucose concentrations is rectilinear as illustrated in Fig. 5.

	Changes	Duration (Hours)	Concentration Range mM	n	q
12-4, 1965	2	3	2-20	3	0.25 ± 0.01
4-5, 1965	2	4	2-40	8	0.19 ± 0.01
9-14, 1965	3	6	25-30	4	0.24 ± 0.014
12-9, 1965	3	6.5	25-45	8	0.24 ± 0.01
12-28, 1965	3	6	25-45	8	0.25 ± 0.02
1-6, 1966	3	6	25-80	10	0.25 ± 0.015
4-11, 1966	3	6	8-80	4	0.25 ± 0.02
4-14, 1966	3	6	8-80	4	0.33 ± 0.01
11-28, 1966	3	6	20-100	24	0.21 ± 0.02
7-1, 1967	4	6	5-100	44	0.25 ± 0.13
3-28, 1968	4	8	10-110	63	0.28 ± 0.8
Average					0.25 ± 0.01

The Equilibrium Distribution of Labeled D-glucose, D-ribose, and Methanol in Ion Exchange Resin

D-glucose, D-ribose, and methanol enter ion exchange resin beads rapidly, reaching equilibrium within 20 minutes at 0°C. Table 2 shows the equilibrium distribution of these substances in the water of the exchange resin at 0°C. The concentration of methanol in the resin water exceeds that of the external solution (slope roughly 1.3). The q values for D-glucose and D-ribose, on the other hand, are close to each other and well below unity. Similar distribution ratios, published earlier, for methanol and D-glucose (and D-xylose) in two other types of sulfonate ion exchange resins show a similar pattern and are also included in Table 3.

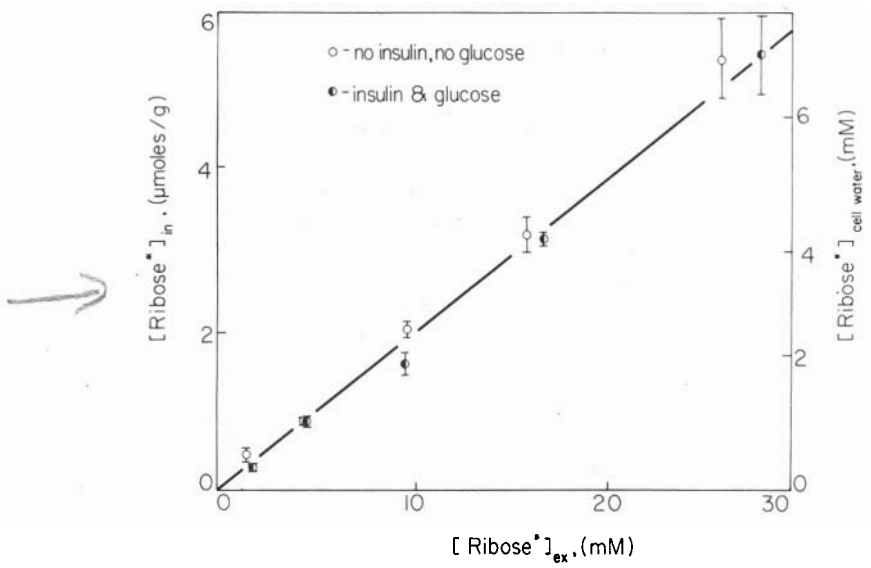

Figure 6. Steady Level of D-ribose Uptake in Washed Muscles and Muscles Preincubated with Glucose and Insulin.
Six hours of preincubation at 25°C with no insulin and no glucose or 24 mM glucose and 0.1 u/ml insulin were followed by overnight incubation at 0°C with varying concentrations of D-ribose and labeled D-ribose.

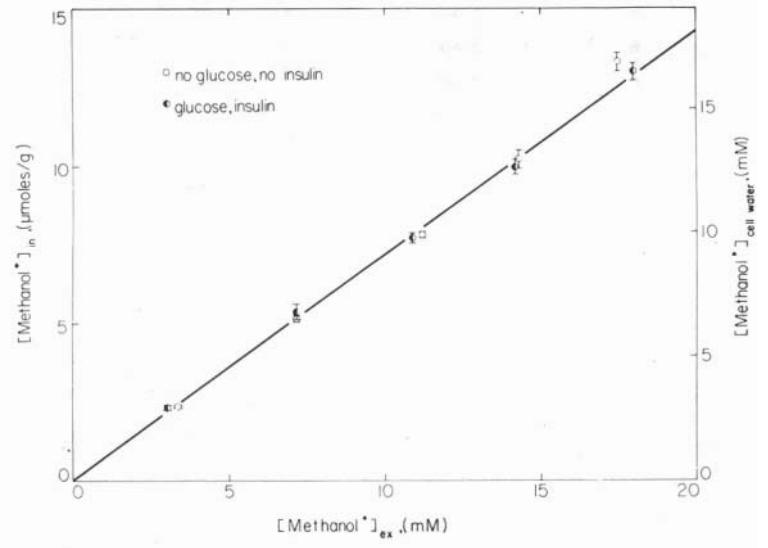

Figure 7. Steady Level of Methanol Uptake in Washed Muscles and Muscles Preincubated with Glucose and Insulin.
Six hours of preincubation at 25°C with no insulin or no glucose or 24 mM glucose and 0.1 u/ml insulin were followed by overnight incubation at 0°C with varying concentrations of methanol and labeled methanol.

Table 2. The Equilibrium Distribution of Methanol, D-ribose, and D-glucose Between the Water in a Sulfonate Ion Exchange Resin and an External Buffered Solution at 0°C.

The resin beads were equilibrated for 20.5 hours at 0°C with shaking in solutions containing different concentrations of labeled methanol, D-ribose, or D-glucose. After blotting dry on wet filter paper, quadruplicate samples were introduced into known value of distilled water in which the beads were shaken for another 24 hours at 0°C. Aliquots of this solution were assayed for radioactivity. Water content of the resin beads was obtained from the difference between wet weight and weight after oven-drying.

	Equilibrium Concentration in External Solution	Equilibrium Concentration in Resin Water (mM)	Distribution Ratio	Dowex 50(H^+) (Wheaton and Bauman[28])	Rexyn 50(H^+) (Ling[23])
Methanol	14.7	19.3 ± 1.3	1.31	0.61	0.94
	43.6	56.8 ± 1.8	1.30		
D-ribose	12.4	6.61 ± 0.12	0.53		(0.23)
	60.4	27.1 ± 3.7	0.45		
D-glucose	13.8	5.54 ± 0.34	0.40	0.22	0.23
	72.5	32.8 ± 0.84	0.45		

Table 3. The Total Concentrations of Glucose Adsorption Sites and the Glucose Adsorption Constant. Experimental procedures and methods of data calculation same as described for Fig. 8.

	[f] (mmoles/kg)		Kgl (M)	
12-9, 1965	7.7	6.6×10^{-3}	8.7×10^{-3}	8.7×10^{-3}
12-12, 1965	5.5	8.1×10^{-3}	9.1×10^{-3}	10.7×10^{-3}
3-7, 1967	15.6	6.7×10^{-3}		
10-10, 1967	12.5	7.5×10^{-3}		

DISCUSSION

I. D-glucose, D-ribose, and Methanol Distribution in Insulin-Deficient Muscles.

In the preceding paper of this series,' we showed that the total D-mannitol space in frog muscles is about 23%, in agreement with the value of Narahara and Özand.[10] Using single muscle fiber techniques, we have determined that, of this 23%, about **10%** to 15% is within the muscle cells. The remaining **8.9%** is dividied between the extracellular space proper and the connective tissue complex. We have found an average figure for the entire extracellular space complex of 8.9%, which applies equally to the distribution of D-glucose, D-mannitol, Sucrose, and urea.

Since D-mannitol and sucrose, hitherto considered impermeant, have been shown to enter muscle cells, it is not altogether surprising that D-glucose also enters muscle effectively freed of insulin by washing for 6 hours or more in Ringer solution containing no insulin. This finding is in general agreement with the radioautographic evidence that D-xylose penetrates into muscle cells in the absence of insulin (ref. 1 I; see also 3,4,12,13).

The most striking feature of the D-glucose distribution in these washed muscles is that the intracellular concentration is ***rectilinearly*** related to the extracellular concentration. This rectilinearity is also seen in the distribution of D-ribose and of methanol. The slopes are low for D-glucose **(0.25)** and D-ribose **(0.25)** and much higher but still below unity for methanol **(0.3).** The question arises, how can this asymetrical distribution be maintained? Let us consider three possible interpretation:

A. THE PUMP MODEL

Several more-or-less similar models have been proposed on the basis of a pump as a mechanism for the transport of solutes across cell membrane (see, for example, 14,15,16). The essential features of most of these models include the following:

1. A finite number of carriers are found in the continuous lipoid phase of the cell membrane.
2. The motion of the carrier with or without its passenger in the membrane is achieved by thermal agitation and is thus random.
3. The carriers make contact with the solutions at the inner and outer surfaces of the membrane where they can associate with a sugar molecule. The complex formed may dissociate immediately, or it may by thermal motion reach the other surface where it may again dissociate. If the dissociation constants at both surfacesare equal, there is no active transport, only what is referred to as "facilitated diffusion." If a concentration difference between the intra- and extracellular phases is achieved by the pump, the two dissociation constants must be different, energy must somehow be injected into the carrier system to achieve this difference.

4. The cell membrane may offer two-way leakage paths in addition to carriers.

In general, the existence of carriers and/or pumps is postulated when transport of a solute shows the following characteristics: ***saturability*** (the rate of transport or the steady level increases to a certain level as the concentration of solute increases and then levels off), and ***competition*** between similar species. In the absence of an effective insulin concentration, there is no evidence of saturability in the concentration range studied; nor does nonlabeled glucose compete for and thus decrease the steady level of labeled glucose (Fig. 5). Thus, under these conditions, there is no evidence for the existence of a carrier.

This conclusion can perhaps be made clearer through a more detailed analysis. From the essential features listed above, the most comprehensive pump model may be described by the following set of equations describing the rate of outward flux (F_{ow}) and the rate of inward flux (F_{iw}) of a sugar (or other solute) S:

$$F_{ow} = \frac{k_{ow}\,[C]\,[S]_{in}}{K_s^{ow} + [S]_{in}} + P_s\,[S]_{in} \tag{2}$$

$$F_{iw} = \frac{k_{iw}\,[C]\,[S]_{ex}}{K_s^{iw}\,[S]_{ex}} + P_s\,[S]_{ex} \tag{3}$$

where k_{ow} and k_{iw} are rate constants for the outward and inward movement of the carrier-sugar complex; [C] is the concentration of carriers or pumps in the cell membrane; $[S]_{in}$ and $[S]_{ex}$ are the concentrations of the sugar in the cell water and in the external medium respectively; and K_s^{ow} and K_s^{iw} are the dissociation constants of the sugar-carrier complex at the inner and outer surfaces of the cell membrane, respectively. P_s is a permeability constant for the "leakage."

Under steady-state conditions, there is no net movement. F_{ow} and F_{iw} are equal, thus

$$\frac{k_{ow}\,[C]\,[S]_{in}}{K_s^{ow} + [S]_{in}} + P_s\,[S]_{in} = \frac{k_{iw}\,[C]\,[S]_{ex}}{K_s^{iw} + [S]_{ex}} + P_s\,[S]_{ex} \tag{4}$$

The condition for $[S]_{in}$ to be rectilinearly related to $[S]_{ex}$ is that $K_s^{OW} >> [S]_{ex}$. In the case of D-glucose, since the rectilinearity of the experimental results (Fig. 5) persists to an internal D-glucose of 100 mM, K_s^{ow} and K_s^{iw} must be at least as large as 0.2 M and 1 M, respectively. Otherwise, both saturation and competition would be observed. Dissociation constants of 0.2 M and 1 M correspond respectively to free energies of binding of −1.0 Kcal/mole and 0 Kcal/mole, respectively. It is doubtful that this small amount of energy could permit the formation of a highly stereospecific complex between the sugar and its carrier or pump. Without high specificity, it would be difficult to explain the difference

in the distribution ratio between methanol (0.8, Fig. 7) and, say, D-ribose (0.25, Fig. 6) or D-glucose (0.25, Fig. 5), since all form H-bonds and hence would be capable of combining with the carrier.

B. VARIABLE ACCESSIBLE COMPARTMENTS

A rectilinear distribution with a slope less than unity is compatible with the idea that there are compartments in the cell which are not accessible to the sugars studied. As pointed out in an earlier communication, the difficulty of this theory lies in the demand for an absolutely ***impermeable*** barrier. Here we are dealing with ***steady*** level distribution and not with the rates of influx and efflux; there is no evidence that an absolutely impervious material exists in the living cell.[7] For this reason it is difficult to ascribe the distribution coefficient of less than unity to the membrane structure of subcellular particles. However, the basic concept of relative inaccessibility can be quite tenable if one broadens the concept to include exclusion in a statistical rather than an absolute sense and if one does not ascribe this exclusion property to a particular part of the living protoplasm. This will be made clear in the next section.

C. STATISTICAL MODEL FOR EXCLUSION FROM THE CELL

There is now strong experimental evidence both from the pattern of intracellular ice-formation[17,18] and from nuclear magnetic resonance studies[19] that the bulk of the intracellular water is in a different state from the water in, say, a dilute salt solution. Further, we have succeeded in demonstrating partial exclusion of other solutes (e.g., Na^+, K^+, Rb^+) from the water of an isolated cytoplasmic protein-water system, actomyosin gel.[20] These facts have been interpreted in terms of the association-induction hypothesis[21-24] as indicating that the water in the protein aqueous system found in living cells, as well as isolated actomyosin gel, is polarized into multilayers. Such water has different solvent properties from normal water.

Distribution of solutes in this polarized water can be described by Henry's law:

$$[\text{Ribose}]_{in} = \alpha q_{rib} [\text{Ribose}]_{ex} \tag{5}$$

$$[\text{glucose}]_{in} = \alpha q_{glu} [\text{glucose}]_{ex} \tag{6}$$

$$[\text{methanol}]_{in} = \alpha q_{meth} [\text{methanol}]_{,,} \tag{7}$$

where a is the percentage of water in the cell; q_{rib}, q_{glu}, and q_{meth} are the equilibricm distribution coefficients of D-ribose, D-glucose, and methanol, respectively. Now

$$q_{rib} = \frac{(p.f.)^{in}_{rib}}{(p.f.)^{ex}_{rib}} \exp(-\Delta H^{\circ}_{rib}/RT); \quad (8)$$

$$q_{glu} = \frac{(p.f.)^{in}_{glu}}{(p.f.)^{ex}_{glu}} \exp(-\Delta H^{\circ}_{glu}/RT); \quad (9)$$

$$q_{meth} = \frac{(p.f.)^{in}_{meth}}{(p.f.)^{ex}_{meth}} \exp(-\Delta H^{\circ}_{meth}/RT); \quad (10)$$

where $(p.f.)^{in}_{rib}$, $(p.f.)^{in}_{glu}$, $(p.f.)^{in}_{meth}$, $(p.f.)^{ex}_{rib}$, $(p.f.)^{ex}_{glu}$, and $(p.f.)^{ex}_{meth}$ are the partition functions or the number of ways in which energy can be partitioned among the quantum mechanically permissible states available to D-ribose, D-glucose, and methanol in the cell water and in the external solution, respectively. ΔH°_{rib}, ΔH°_{glu}, and ΔH°_{meth} are the standard enthalpy differences for D-ribose, D-glucose, and methanol in the external solution and these substances in the cell water. The differences between the partition functions in the internal and external solutions means differences in entropy. Thus, a low q value may reflect an unfavorable entropy; i.e., a more limited number of energy states. The senior author has, on theoretical grounds, suggested that this low entropy arises from the rotational restriction of the solute in polarized water.[24,25] We might note, in support of this interpretation, that the exclusion of ions from the water of the major cytoplasmic protein-water system (actomyosin gel) mentioned above has been shown to be entropic in nature.[20]

It now remains to point out that the larger the solute molecule, the greater its number of rotational states of motion. Thus, a greater proportion of the total entropy of larger molecules is made up by the rotational entropy than is the case for smaller molecules. The restriction of rotational motion suffered by the molecule on entering the polarized water of the cell will reduce the entropy of larger molecules more than the entropy of smaller molecules. Thus, larger molecules (such as sugars; e.g., D-ribose, D-glucose) will tend to be excluded from the polarized water system of the living cell to a greater degree than will smaller molecules (such as methanol). We have previously pointed out that the water in ion exchange resins also very likely exists in polarized multilayers.[22] For this reason, the distribution ratios of methanol, D-ribose, and D-glucose in the water of ion exchange resins are qualitatively similar to the ratios for the distribution of these molecules in living cells.

II. D-glucose, D-ribose, and Methanol Distribution in Insulin-Treated Muscles.

Figures 4, 5, and 6 have shown that insulin does not change the steady-level distribution of D-ribose and methanol in frog muscles, but profoundly alters the pattern of distribution of D-glucose. Let us again discuss the possible mechanisms of insulin action according to the three models presented above.

A. PUMP OR PERMEASE MODEL

Randle and smith[3] suggested that insulin changes the chemical structure and number of sites in the carrier. Fisher and Zachariah[26] suggested that insulin combines with a membrane carrier molecule, thereby changing its rate of glucose transport.

Much of their theoretical reasoning was based on the notion that, without insulin, glucose is kept out of the cell, and there is no pumping to maintain this state. Our present data show that, without insulin, glucose not only enters the cells but actually exists at a concentration farther away from the concentration in the external medium than it does in the presence of insulin. **Thus, in terms of the membrane pump model, the noninsulinized muscle is the one more in need of the outward pump.**

However, in Figure 4 as well as in the more extensive data presented in an earlier paper of this series,[7] we have shown that in an insulin-treated muscle at an external glucose concentration below 20 mM or so the intracellular glucose concentration exceeds the glucose concentration in the external solution; above 20 mM, it remains below the external glucose concentration. If one does not postulate'wasteful pumping in both directions at all concentrations, one must suggest that at high external glucose concentration insulin slows down an outward pump, while at low external glucose concentration insulin switches off the outward pump and switches on an inward pump. To bring about the smooth transition seen in Figure 4, from the low glucose to high glucose concentration range, a device of considerable complexity must be postulated. One fears that this model is becoming too complex to provide quidance in further understanding.

B. VARIABLY ACCESSIBLE COMPARTMENT THEORY

It has been suggested that barriers insurmountable to D-glucose may open up in response to insulin action. Again, the basic difficulty is finding an absolutely impermeable barrier in the first place. The difference between the ether-water distribution coefficient of D-ribose and D-glucose is very small[27] and in the reverse direction of that needed to explain an insulinized membrane barrier permeable to D-glucose but not to D-ribose. But, again, if the general concept of accessibility is broadened to include the opening up of adsorption sites, then the concept could merge with that offered in the next model.

C. THE ASSOCIATION-INDUCTION HYPOTHESIS

We have suggested above that the polarized state of water accounts for the low concentration of methanol, D-ribose, and D-glucose concentration in the cell. If insulin causes a rise of intracellular D-glucose concentration, it might produce this rise by altering the physical state of water. This is not the case, because such a change would cause similar changes in the distribution of D-ribose for example. The data of Figures 2 and 6 show that D-ribose distribution is not altered by insulin treatment.

In preceding papers[1,7] of this series we have suggested that the action of insulin is to create stereospecific D-glucose adsorption sites. If the total number of D-glucose adsorbing sites opened up by insulin is equal to [f], and if each site can adsorb only one glucose molecule, the total concentration of adsorbed labeled glucose, $[\text{glucose*}]_{ad}$ would be

$$[\text{glucose*}]_{ad} = \frac{[f]\ [\text{glucose*}]_{ex}}{K_{gl} + [\text{glucose*}]_{ex} + [\text{glucose}]_{ex}} \tag{11}$$

where [f] is the total number of glucose-adsorbing sites. $[\text{glucose*}]_{ex}$ and $[\text{glucose}]_{ex}$ are the external concentration at equilibrium of labeled and nonlabeled glucose, respectively, and K_{gl} is the adsorption constant for glucose. The total intracellular labeled glucose concentration $[\text{glucose*}]_{in}$ is the sum of the labeled glucose concentration in the cell water (Eq. 6) and the adsorbed labeled glucose:

$$[\text{glucose*}]_{in} = \alpha q_{glu}\,[\text{glucose*}]_{ex} + \frac{[f]\ [\text{glucose*}]_{ex}}{K_{gl} + [\text{glucose*}]_{ex} + [\text{glucose}]_{ex}} \tag{12}$$

Since the physical state of the intracellular water is not significantly changed by the action of insulin, q_{glu} in insulin-treated muscle should be similar to q_{glu} in insulin-poor muscle. Subtracting the labeled glucose concentration in washed muscles from the labeled glucose concentration in insulin-treated muscle should yield the concentration of adsorbed labeled glucose, described by Equation 11.

Equation 11 can be written in reciprocal form:

$$\frac{1}{[\text{glucose*}]_{ad}} = \frac{K_{gl}}{[f]}\left(1 + \frac{K_{gl}}{[\text{glucose}]_{ex}}\right)\frac{1}{[\text{glucose*}]_{ex}} + \frac{1}{[f]} \tag{13}$$

If one plots $\frac{1}{[\text{glucose*}]_{ad}}$ against $\frac{1}{[\text{glucose*}]_{ex}}$ at a fixed concentration of nonlabeled

competing glucose, one should obtain a straight line. Varying the nonlabeled external glucose concentration produces a family of straight lines converging at the same locus on the ordinate. This locus is the reciprocal of the total number of glucose adsorbing sites.

To obtain the curves shown in Figure 8, the intracellular-labeled glucose in washed muscles (lower line, Fig. 4) has been subtracted from the intracellular labeled glucose in insulin-treated muscles (upper lines, Fig. 4) and plotted reciprocally against the reciprocal of the external labeled glucose concentration. As can be seen, the result is a family of straight lines converging at the ordinate as predicted. In Table 3 we have tabulated the results of 4 sets of experiments. The total number of D-glucose adsorption sites varies between 5.5 and 15.6 μmoles per kilogram of fresh muscle cells, and the adsorption constant varies between 6.6 x 10^{-3} and 10.7 x 10^{-3} M.

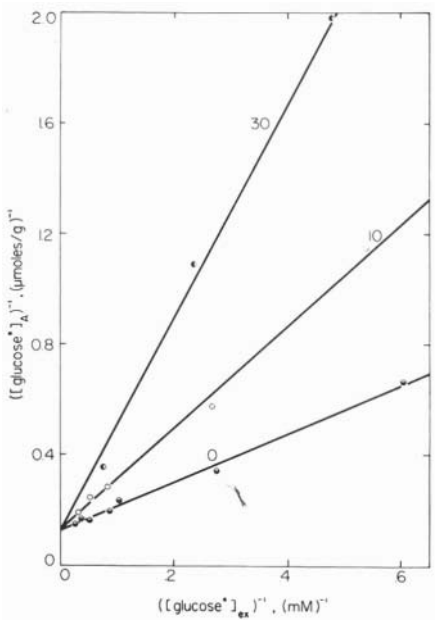

Figure 8. Reciprocal Plot of Data in Fig. 4.
The amount of glucose taken up by washed muscles (lower curve, Fig. 4) was subtracted from the glucose taken up by insulin-treated muscles (upper curves, Fig. 4). The reciprocal of the difference is plotted against the reciprocal of the external glucose concentration. The data conform to Eq. 13.

From the data presented in this report it becomes evident that the accumulation of glucose in frog muscles is consistent neither with a membrane-pump model nor with an inaccessible compartment interpretation. It is, however, consistent with the postulates of the association-induction hypothesis that in the absence of an effective concentration of

insulin, the unfavorable entropy of glucose in the cell water results in an intracellular concentration only a fraction of the extracellular concentration. Insulin increases glucose uptake by creating a set of specific protoplasmic glucose adsorbing sites.

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