STUDIES ON INSULIN ACTION. II. THE EXTRACELLULAR SPACE OF FROG MUSCLE: DEMONSTRATION OF D-MANNITOL AND SUCROSE ENTRY INTO ISOLATED SINGLE MUSCLE FIBERS AND INTACT MUSCLES.

GILBERT N. LING, MARGARET C. NEVILLE, SANDRA WILL and PATRICIA SHANNON

Department of Molecular Biology, Division of Neurology, Pennsylvania Hospital, Philadelphia, Pennsylvania

(Received December 2,1968)

SUMMARY

The distribution of labeled D-mannitol, sucrose, and other widely used extracellular space probe materials was studied in four types of muscles from leopard frogs and bullfrogs as well as in connective tissues and isolated single and multiple muscle fiber preparations from bullfrog semitendinosus muscle. In leopard frog muscle the extracellular space complex (consisting of the extracellular space proper along with connective tissues, small blood vessels, nerves, the sarcolemma and the transverse tubular system), accommodates D-mannitol and sucrose to a total concentration of about 9% of that of the external solution. Both D-mannitol and sucrose enter into muscle cells; an equilibrium concentration in the cell water of from 12.5% to as high as 34% of that in the external solution was observed.

An accurate estimation of the extracellular space is essential to any quantitative study of intracellular solute distribution. For frog sartorius muscle an extracellular space of 13%, obtained from an estimation of short-time inulin distribution by Conway and his co-workers,[1] was widely accepted for some time. More recently there has been a trend toward the use of higher figures obtained from the distribution of sugars such as sucrose or labeled D-mannitol.[3-10] For frog sartorius muscle the figure obtained using these probe materials is 22.4%.[10] In 1967, Ling and Kromash reported results of studies using a new probe material, poly-L-glutamate (PLG).[11] They concluded that in frog sartorius, semitendinosus, tibialis anticus longus and iliofibularis muscles, the true extracellular space (i.e., space filled with Ringer solution or plasma) could not exceed 8%.

The true extracellular space is, however, not the only extracellular component of muscle tissue. Intact muscles also contain connective tissues, small blood vessels, nerve fibers, etc. These materials, which will be collectively referred to as the connective tissue

complex, may also accommodate the solute under study. Thus, to obtain the true intracellular solute concentration one must subtract from the total tissue content not only the solute in the extracellular space proper but also that in the connective tissue complex.

The studies reported in this paper were designed to answer two questions:

1. What is the origin of the discrepancy between the value of extracellular space obtained using probe materials such as D-mannitol and that obtained using poly-L-glutamate?
2. How does one obtain a reasonably accurate figure to correct for solute distribution in the nonmuscle cell components (henceforth collectively referred to as the extracellular space complex)?

MATERIALS AND METHODS

Materials

Sartorius, semitendinosus, tibialis anticus longus and iliofibularis muscles were isolated from leopard frogs (Ram *pipiens*, Schreber) and from bullfrogs (*Rana catesbiana*, Shaw). Single and multiple muscle fiber preparations were dissected from the white portions of the semitendinosus muscles of large bullfrogs and consisted primarily of "fast" fibers.[12] Connective tissues used were either tendons from the ends of the semitendinosus muscles or thin films of loose connective tissue from the ventral surface of the thigh and the dorsal surface of the leg of skinned frogs. The latter contained small nerve fibers and blood vessels.

All chemicals used were of c.p. grade; D-mannitol (Lot 6826) and D-sorbitol (N-2681) were obtained from Pfanstiehl Laboratories, Waukegan, Illinois. C-14-labeled D-mannitol (Lot 1055, 1-C-14) was obtained from Mallinkrodt Chemical Works, Orlando, Florida; H-3-labeled D-mannitol (1-H-3, Lot 292-47) from New England Nuclear Corp., Boston, Massachusetts. Both labeled D-mannitol preparations were certified chromatographically pure. Labeled D-sorbitol was obtained from Nuclear Research Chemicals, Orlando, Fla., (U-C-14, Lot 21). Labeled sucrose (U-C-14, Lot 48050) was obtained from Calbiochem, Los Angeles, California. C-14-labeled urea from Nichem, Bethesda, Maryland. I-131-labeled serum albumin was obtained from Abbott Laboratories; Cl-36 from Union Carbide.

Assay Methods

Radioactivity was assayed on a liquid 0-scintillation counter (Packard, Model 314 EX) using methods described previously.[13] Minor counting efficiency differences between samples were corrected on the basis of "silent" control experiments.[13] PLG was assayed according to the method described by Ling and Kromash."

Determination of Probe Material Distribution in Intact Muscles

The initial step in the procedure for the determination of probe material distribution was 18 hours of incubation at 0°C in a Ringer solution (for composition see ref. **14)** containing the probe material. This is far longer than is necessary for any of the probe materials used in this study to equilibrate in the extracellular space (Fig. **1).** The tissues were then blotted by placing the muscles between **4** layers of moist **Whatman** No. **1** filter

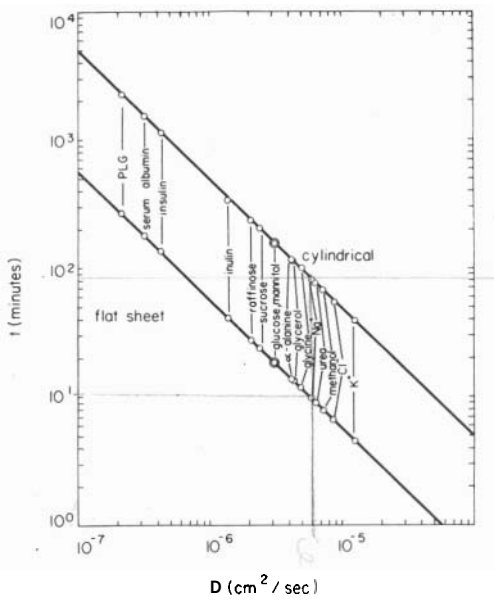

Figure 1. The Lengths of Time Necessary for Various Solutes to Reach 99% Equilibrium in the **Extra**cellular Space of Thin-sheet and Cylindrical Muscles at 0°C.
Calculated using equations for diffusion of solutes from thin-sheet and cylindrical muscles given in Ling and **Kromash,**[11] and a **λ-value** of 1.5 (ratio of diffusion path length over the geometric thickness or radius). Thin-sheet muscles considered to be 0.06 cm thick and cylindrical muscles, 0.2 cm in diameter. Diffusion coefficients were obtained from the following sources: **PLG,**[11] Bovine serum **albumin,**[27] insulin,[**] **inulin,**[28] **raffinose,**[29] **sucrose,**[29] **glucose,**[29] **D-mannitol,**[29] **α-alanine,**[29] **glycerol,**[30] Na^+ **ion,**[31] **urea,**[32] methanol,[**] Cl^- **ion,**[31] and K^+.[33] Diffusion coefficients at 0°C (D°) were calculated from values (D^t) obtained at a higher temperature, t°C, using the relation:

$$D^\circ = D^t \cdot \frac{273}{273 + t} \cdot \frac{\eta_0}{\eta_t}$$

where η_0 and η_t are the viscosity of water at 0°C and t°C respectively.[29] From this graph one may readily estimate the time necessary for a substance of given diffusion coefficient to reach 99% equilibrium in the extracellular space proper. For higher temperature, the diffusion coefficients may be corrected using the above equation. To obtain values for thicker or thinner tissue preparations, the times obtained from the graph are multiplied by a factor $(\frac{a'}{0.06})^2$ for flat tissues and $(\frac{r'}{0.2})^2$ for **cylindrical** tissues where a' or r' is the thickness or the radius of the **tissue** in question.

paper and running the index finger firmly over the muscle four times. Figure 2 shows that this amount of blotting leads to a constant I^{131}-labeled serum albumin space. After weighing, the muscles were, in most cases, placed in 2 ml 5.2% TCA for overnight extraction at 4°C. For determination of radioactivity, 0.5 ml aliquots of the extracts were placed in 5 ml of Bray's scintillation fluid.[15]

In a few cases the radioactivity was extracted in two steps. First the blotted muscles were shaken at 0°C in 2 ml of Ringer solution for 40 minutes. They were then blotted dry and extracted with 5.0% TCA as above. In this case the radioactivity in 0.5 ml aliquots of both the Ringer solution and the TCA extract was determined.

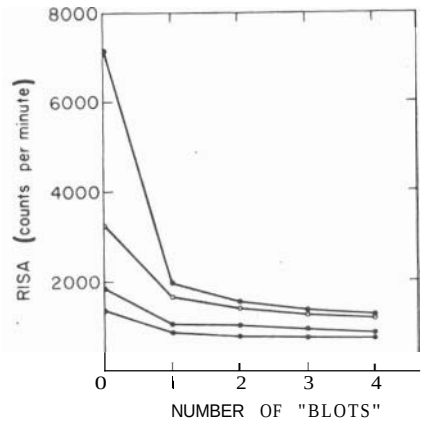

Figure 2. Efficiency of Blotting Procedure.
A sartorius muscle was incubated in Ringer phosphate containing I^{131}-labeled serum albumin. After removal from the incubation solution it was placed on 4 sheets of filter paper wetted with Ringer solution and covered with four more layers of wetted filter paper. The index finger was then drawn firmly across the muscle the number of times indicated on the abscissa. The muscle was then positioned on the end of a roll of moist filter paper and placed in a lusteroid counting tube. The tube and contents were counted for 0.5 minutes in the well scintillation counter (Packard) after which the muscle was replaced in the incubation solution preparatory to repeating the procedure. Each symbol represents a different muscle. RISA represents labeled albumin.

Determination of Probe Material Distribution in Single and Multiple Muscle Fiber Preparations

Single muscle fibers and multiple fiber preparations were isolated and kept overnight at 4°C in Ringer-phosphate solution. After this period of time, injured preparations could be easily recognized by their opaqueness and fragmented appearance and were discarded. All preparations were handled with No. 5 Dumont forceps. The humidity in the preparation room was maintained near 100%.

For incubation, good fibers were placed in 0.2 ml of Ringer solution containing about

10 μc/ml of C-14-labeled D-mannitol or sucrose. After incubation at 0°C for 18 hours or longer, the fibers were rinsed vigorously in a large volume of cold Ringer-phosphate solution at 0°C for 5 seconds (more than long enough to remove labeled material adhering to the surface of the fiber or in the sarcolemma, see discussion). After briefly touching a piece of wetted filter paper to drain off excess solution, the fiber was placed on a sheet of moistened Visking sausage casing on the stage of a dissecting microscope. The appearance of the fiber was checked and the terminal tendons and any other extraneous materials were removed (from this stage on intactness of the fiber is no longer essential). After weighing on a micro torsion balance (capacity 25 ml, Pacific Federal) the fiber was transferred to a scintillation vial containing 0.5 ml TCA. 5 ml Bray's scintillation fluid was added before counting. The small amount of muscle tissue in the vial does not significantly alter the counting efficiency.

RESULTS

The Distribution of D-mannitol, D-sorbitol, and Sucrose in Frog Muscles

Curve C of Fig. 3 shows the equilibrium distribution of labeled D-mannitol in leopard frog muscles. Curve B of Fig. 3 shows the amount of labeled D-mannitol recovered after 40 minutes of washing at 0°C. As shown by Fig. I, washing for this length of time is enough to extract 99% of the labeled D-mannitol from the extracellular space proper of even the thickest muscles used. Curve A shows the residual amount of labeled D-mannitol left in the muscle after 40 minutes of washing. The existence of this residual fraction suggests that this substance is not confined exclusively to the extracellular space proper.

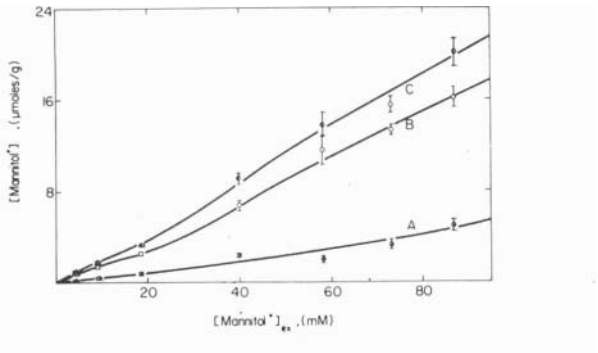

Figure 3. The Distribution of Labeled D-mannitol in Leopard Frog Muscles in Varying External D-mannitol Concentrations.
In this and following figures with similar symbols, each point represents a semitendinosus, a tibialis anticus longus, and an iliofibularis muscle. The distance between the two horizontal bars represents twice the standard error. In this and similar figures following, the ordinate represents the concentrations of the labeled material in the μmoles per gram of fresh tissue.

Figures **4** and 5 show the equilibrium distribution of D-sorbitol and sucrose, respectively, in **4** types of leopard frog muscles. Figure **6** shows the equilibrium distribution of Pmannitol and sucrose in bullfrog muscles.

The results shown in Figures **3,4,5,** and 6 are typical of all the results we have obtained for the distribution of D-mannitol, D-sorbitol, and sucrose in frog muscles. In every case the distribution curve shows a slight curvature indicating a concentration dependence of the distribution space for these substances. Thus, taking Fig. **3** as an example, at **20 mM** external concentration the D-mannitol space is 17.5%; at **60 mM** it is **23.4%.** The latter figure agrees with the D-mannitol space measured by Narahara et al. for frog sartorius muscles.[10]

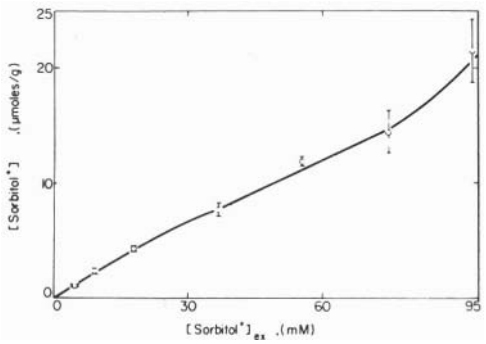

Figure 4. The Distribution of Labeled D-sorbitol in Leopard Frog Muscles at Varying External Concentrations.

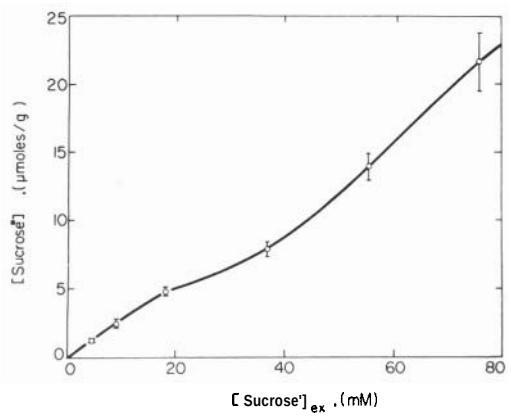

Figure 5. The Distribution of Labeled Sucrose in Leopard Frog Muscles at Varying External Concentrations.

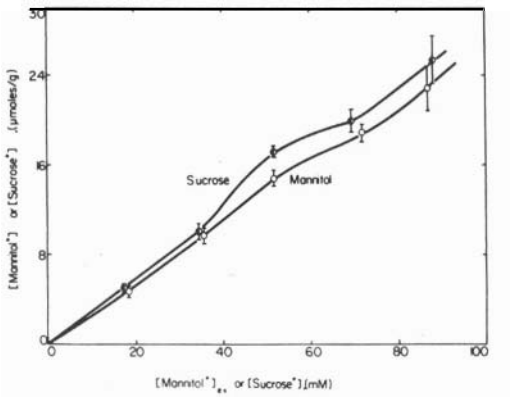

Figure 6. The Distribution of Labeled **D-mannitol** and Sucrose in Bullfrog Muscles.

The Distribution of Urea in the Extracellular Space of Leopard Frog Muscles

Mixed muscles were incubated overnight at 0°C in Ringer solution containing labeled urea. Muscles were then washed for 40 minutes in Ringer solution. As shown by Fig. 1, this is more than 4 times the time necessary to obtain 99% recovery of labeled urea from the extracellular space proper (9 minutes). The amount of labeled urea recovered in the washing solution is plotted as a function of the labeled urea concentration in the incubation medium in Fig. 7. The curve has the appearance of a hyperbola (as in a **Langmuir** adsorption isotherm) superimposed on a straight line. The dotted line, drawn through the origin, and parallel to the straight portion of the curve has a slope of 0.10 **(g/ml),** indicating a maximum extracellular space proper of **10%.**[11]

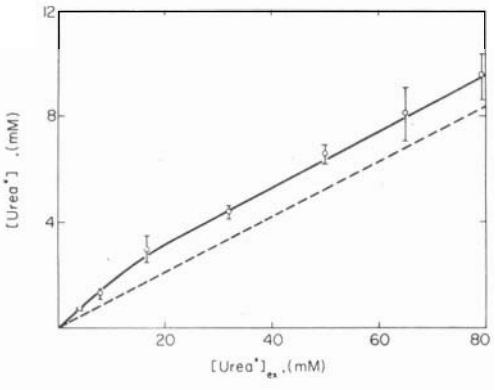

Figure 7. The Distribution of Labeled Urea in Leopard Frog Muscles at Varying External Concentrations.
Muscles incubated overnight at 0°C with labeled urea followed by washing for 40 minutes at 0°C in Ringer solution. Values on graph show recovery of the labeled urea from the washout solution.

The Equilibrium Distribution of D-mannitol, D-sorbitol, Sucrose, and Urea in Connective Tissues

Figures 8 and 9 show the distribution of D-mannitol, methanol, urea, sorbitol, and sucrose in thin films of connective tissue from leopard frogs as a function of the concentration of these substances in the bathing medium. All show a straight line relationship. The slopes of the lines are 0.78, 0.78, 0.84, 0.78, and 0.88 for D-mannitol, D-sorbitol, sucrose, methanol, and urea respectively. From eight measurements we found the dry weight of these loose connective tissues to be 15 ± 0.7% of the total weight. The water content is thus 85%. The above substances are, therefore, within a 10%variation, equally distributed in the connective tissue water and the external medium.

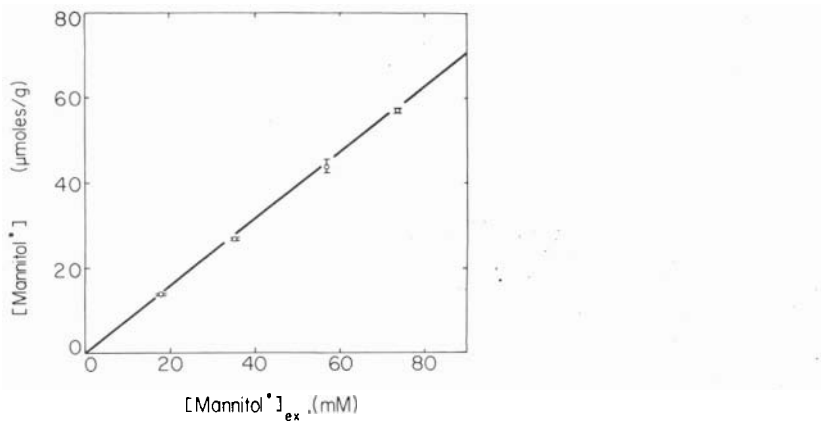

Figure 8. The Distribution of Labeled D-mannitol in Loose Connective Tissue in Leopard Frogs. The procedure used was the same described for studying D-mannitol distribution in muscles.

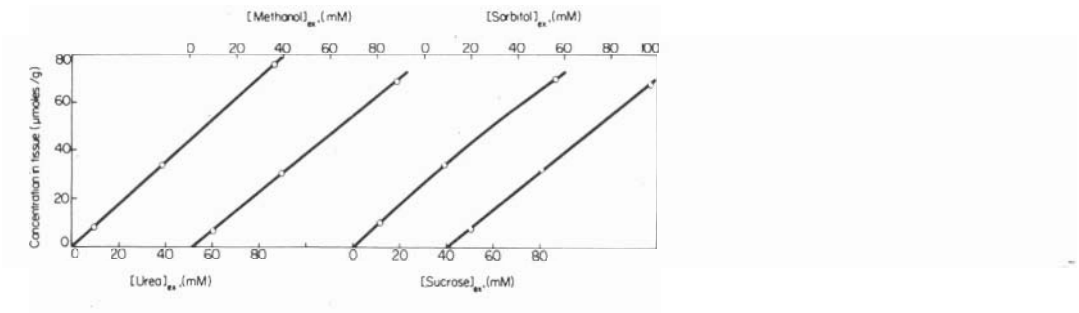

Figure 9. The Distribution of Labeled Sorbitol, Sucrose, Urea, and Methanol in Loose Connective Tissue of Leopard Frogs.

Table 1 shows that the connective tissue distribution of glucose or Cl is in the same range as the above probe materials. On the other hand, only about 30%of the connective tissue water is accessible to poly-L-glutamate, a substance we have previously used as a measure of the extracellular space proper.''

Solute	Tissue Weights (mg.)	equilibrium External Concentration (mM)	Equilibrium Tissue Concentration (μmoles/g.)	Equilibrium Distribution Ratios Between Tissue and External Solution (av. ± S.E.)
Glucose	46.0	81.9	62.6	0.77
	35.0	81.9	69.7	0.85
	66.8	22.4	16.7	0.74
	43.8	22.4	15.9	0.71
				0.76 ● 0.05
Chloride	48.0	105.6	82.2	0.78
	40.5	105.6	80.5	0.77
	61.0	101.5	67.5	0.67
	24.0	101.5	65.2	0.64
				0.70 ± 0.06
PLG	98.0	8.8*	2.78*	0.34
	90.0	8[illegible]	3.08*	0.35
	90.0	4[illegible]	1.08*	0.26
	75.6	4.1*	0[illegible]	0.24
				0.30 ± 0.05

* in mg./ml.

Table 1. Equilibrium Distribution of Glucose, Chloride and Poly-L-glutamate in Washed ''Connective Tissues.''
Connective tissues were washed for 6 hours in three changes of Ringer Phosphate with 24 mM glucose before incubation for 18 hours at 0°C in Ringer solution containing H^3-labeled glucose or Cl^{36} labeled chloride, or (for 20 hours) in Ringer solution containing PLG. Labeled glucose andchloride contents of the connective tissues were from boiling extracts. PLG contents were obtained by equilibrating blotted tissues at 0°C in 1.5 ml of Ringer-phosphate solution (washing-out solution, containing no PLG) for 24 hours and assaying the PLG content of the washing-out solution.

The Efflux of D-mannitol from Dense Connective Tissues

Small strips of tendon about 1 mm wide were incubated in Ringer solution containing 20 mM labeled D-mannitol for 18 hours at 4°C. The efflux of the labeled D-mannitol was followed by washing these strips for specified lengths of time in successive vials of Ringer solution at 0°C. The resulting efflux curve is shown in a logarithmic plot as a function of time in Fig. 10. The curve can be resolved into two fractions. By extrapolation one finds

that the slower fraction amounts to 8% of the total D-mannitol content of the tissue.* The rapidly exchanging fraction comprises about 92% of the total.

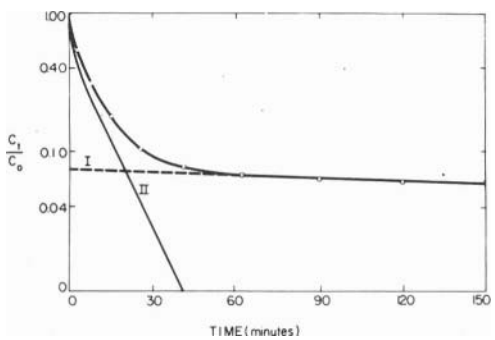

Figure 10. The Time Course of Efflux of Labeled Dmannitol from a Piece of Bullfrog Muscle Tendon at 0°C.

The efflux curve can be resolved into two exponential fractions (I and II). From the slope of the faster fraction and the equation for diffusion of solutes out of cylinders,[13] a diffusion coefficient for D-mannitol in the tendon water of 1.22 x 10^{-6} cm^2/sec was obtained. C_t represents the concentration of labeled mannitol in the tissue t minutes after washing begins; C_o represents the initial tissue mannitol concentration.

The Accumulation of Labeled Dmannitol and Sucrose in Washed Single and Multiple Muscle Fiber Preparations from Bullfrogs

Single muscle fibers were incubated with labeled D-mannitol overnight at 0°C followed by brief washing in nonlabeled Ringer solution and the labeled D-mannitol content determined as in the methods section. Figure 11 shows the results as a function of the external D-mannitol concentration. The rectilinear curve has a slope of 0.1 (g/ml). The water content of whole muscles is 80%.[13] Of this 8.9% belongs to the extracellular complex. Muscle cells comprise 90% of the total muscle weight. Hence the water content of single muscle fibers is $\frac{.80 - .089}{0.9}$ = 79%. Thus, the distribution ratio of D-mannitol between intracellular water and extracellular water is $\frac{0.1}{0.79}$ = 12.5%. Figure 12 shows that single and multiple muscle fiber preparations also accumulate labeled sucrose. In this case the curve is not rectilinear. The slope is 0.1 up to 40 mM, becoming as high as 0.27 at higher sucrose concentrations. The corresponding distribution coefficient of sucrose between intracellular and extracellular water thus varies between 12.5% and 34%.

* It is unlikely that this slow "tail" represents an impurity in the D-mannitol preparation for the following reason: Both a C-14-labeled preparation from Mallinkrodt and a tritium-labeled preparation from New England Nuclear Corp. were used. Both preparations were certified chromatographically pure and gave similar results. An impurity amounting to 8% in both products is highly unlikely (see also Narahara and Özand[10]).

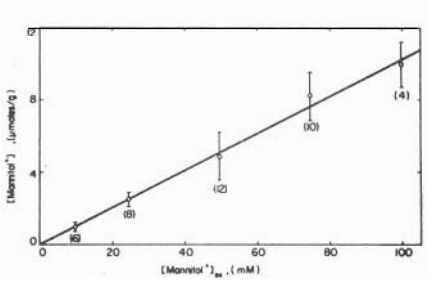

Figure 11. The distribution of Labeled D-mannitol in Washed Single and Multiple Muscle Fiber Preparations from Bullfrog Semitendinosus Muscle.
Of the total of 40 preparations, 22 were single muscle fiber preparations, the remainder were 2,3, or 4 fiber preparations. No significant differences were observed among them. Incubation in labeled D-mannitol varied from 18 to 24 hours at 4°C. Distance between the bars is twice the standard error. The number under each point represents the number of determinations at that concentration.

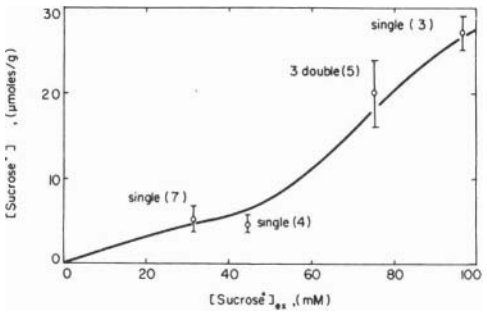

Figure 12. The Distribution of Labeled Sucrose in Single and Multiple Muscle Fiber Preparations from Bullfrog Semitendinosus.
Of the total preparations, 16 were single muscle fibers and the others are double muscle fiber preparations.

DISCUSSION

In order to determine the intracellular concentration of a solute, one must take into account not only the solute in the "extracellular space proper" but also that in other extracellular elements including:

1. The transverse tubules (T-system) of the sarcoplasmic reticulum.
2. The sarcolemma.
3. The connective tissue complex, including tendons, fascia, small nerves and small blood vessels with their occasionally trapped blood cells.

In a preceding paper, Ling and Kromash[11] conclude that the extracellular space proper of frog muscles cannot exceed 8% of the muscle weight. This conclusion was reached on the basis of the assumption that the concentration of poly-L-glutamate (PLG), a newly introduced extracellular space probe material, in the extracellular space proper is linearly related to the PLG concentration in the bathing medium.

The T-system is the only part of the sarcoplasmic reticulum directly open to the exterior[16] and occupies 0.2 to 0.4% of the entire muscle tissue volume.[17-19] These tubules are accessible to ferritin[19] which has a molecular weight of 750,000, more than ten times that of PLG (50,000-61,000) and thus should be freely accessible to PLG. This system has therefore been included in the extracellular space proper measured with poly-L-glutamate.

The sarcolemma is a complex structure considerably thicker than the plasma membrane (i.e., Robertson's unit membrane) with a total thickness of about 0.1 μ* (ref. 20). It consists of at least 4 layers, two of which contain collagen fibers; the rest is an amorphous ground material. The diameter of the average fiber in leopard frog muscles is about 60 μ. Thus, the sarcolemma constitutes about 0.3% of the volume of the leopard frog muscle cell, an amount below the experimental error in our results and thus insignificant.

The thin sheets of loose connective tissue usually found in varying degrees of intactness on the ventral surface of the skinned thighs and the dorsal surfaces of the skinned legs of leopard frogs can be seen to run continuously into the connective tissues in the muscles themselves. Further, the gross composition of these tissues closely resembles that of the connective tissue complex. Thus, we have chosen it as a model of the connective tissue complex.

The proportion of connective tissue complex in frog muscles has been investigated in two ways: 1. The total content of collagen and elastin in intact sartorius muscles was analyzed and compared to that of the connective tissue model. This led to an estimated weight of connective tissue complex equivalent to 9.1% of the fresh sartorius muscle weight.[14] This is likely to be an upper limit because the collagen in muscle connective tissue is probably higher than in the model. 2. Connective tissues contain a component that strongly adsorbs Na^+ ion. In efflux studies[2] on connective tissues containing labeled Na^+ ion, this fraction is very slowly released when the tissue is washed in a nonradioactive solution. A similar, very slowly exchanging fraction of labeled Na^+ ion is also present in intact sartorius muscles as well as in sartorius muscles allowed to degenerate for 24 hours after incomplete transection at 2 mm intervals with a razor blade. By comparing the levels of the labeled Na^+ ion in this slowly exchanging fraction in the connective tissue model with the same fraction in intact and cut muscles, we estimated the connective tissue complex to constitute about 5% of the fresh sartorius muscle weight.

***An EM plate kindly prepared for us by Dr. Sumner Zacks shows that the sarcolemma of bullfrog muscle fibers has a similar thickness.**

Both values of 9.1% and 5% were obtained from sartorius muscles. We estimate that the heavier tendons found in the cylindrical muscles (semitendinosus, tibialis anticus longus, and iliofibularis) are at least partially compensated by the dense fascia found on the external surface of the sartorius. Further, we have found no significant difference between the sugar distribution in the two types of muscles, suggesting that the extracellular space complex in all these tissues does not differ widely.

Since the extracellular space complex is the sum of the extracellular space proper and the connective tissue complex, the total extra-muscle-cell solute is the sum of the solute in these two components. From PLG distribution (see also the 40 min. urea distribution shown in Fig. 7), the extracellular space proper has a ceiling value of 8%. Comparing the PLG distribution curves with theoretical models, we find that a value of 5% is probably the best estimate.

We have shown that D-mannitol, D-sorbitol, sucrose, and D-glucose have the same concentration in the connective tissue water as in the external bathing solution. The connective tissue complex contains 85% water. Of this, 30% is accessible to PLG and has already been counted as part of the extracellular space proper. The remainder is 55%. Thus, if we use the 9.1% figure the connective tissue complex would account for 0.091% x 0.55 = 5% of muscle weight. If the 5% figure is used, it would account for .05 x 0.55 = 2.7% of the fresh muscle weight. The total extracellular space complex therefore occupies 5% + 5% = 10% or 5% + 2.7% = 7.7%, averaging 8.9% of the muscle tissue.

The total uptake of D-mannitol in whole leopard frog muscle lies between 17.5% and 23.4%, averaging 20.4% (Fig. 3). There is then 20.4% - 8.9% = 11.5% not accounted for by the extracellular space complex. For bullfrog muscles the average D-mannitol uptake is 26.5% (Fig. 6). Here 26.5% - 8.9% = 17.6% is not accounted for.

Similarly in leopard frog muscle, the total sucrose space averages 25% (Fig. 5); in bullfrog muscle, it averages 31% (Fig. 6). The sucrose space unaccounted for by the extracellular space complex is 16% and 22% respectively.

The Uptake of D-mannitol and Sucrose by Single and Multiple Muscle Fiber Preparations of Bullfrog Muscles

Figures 11 and 12 show that washed single and multiple muscle fiber preparations contain about 10% of the concentration in the external medium of D-mannitol and sucrose (at low external concentration). The only extracellular space component which these fibers possess is the sarcolemma. The question is – does this accumulation represent solute present in the sarcolemma?

The diffusion coefficient of the bulk of D-mannitol in the sarcolemma cannot be much slower than in the much denser connective tissue studied in this series (1.22×10^{-6} cm^2/sec from Fig. 9). Assuming that it is of the same order of magnitude one can calculate (see Ling and Kromash[11] for equation) that the time for 99% of the D-mannitol to be removed

from the 0.1 μ thick sarcolemma is 10^{-5} second. Thus, 5 seconds of rinsing is very much more than adequate to wash out 99% of the D-mannitol from the sarcolemma.* In fact, some of the intracellular D-mannitol is removed as well. We must therefore conclude that the sarcolemma is not the source of the D-mannitol and sucrose in the single muscle fibers and that these substances enter the cells. For bullfrog muscle cells the intracellular D-mannitol (in moles per gram of fresh cells) is at least 10% of the external D-mannitol concentration. This accounts for the bulk of the difference between the D-mannitol calculated to be in the extracellular space complex and the total tissue D-mannitol actually measured; a small remainder is at least partly accounted for by the loss during the 5 second washing.

Similarly sucrose also enters into the single fiber preparations in a quantity comparable to the deficit mentioned above. In view of the gathering evidence that macromolecules as big as DNA,[22] proteins,[23-26] and inulin[26] all enter into and exit from intact cells (for review and additional reference see Ryser[26]), that D-mannitol and sucrose should do the same is hardly surprising.

ACKNOWLEDGEMENT

This investigation was supported by National Science Foundation Research Grant (GB-2637 and GB-3921) and by the Pennsylvania Hospital General Research Support Grant (5-SO1-FR-05590; NIH award). The senior investigator was supported by Public Health Service Research Career Development Award K3-GM-19,032.

We thank Dr. Sumner Zacks for his invaluable help.

We also thank the John A. Hartford Foundation, Inc. for providing much of the basic equipment.

* It might be noted that the "tail" on the curve for the efflux of D-mannitol from connective tissue shown in Fig. 10 comprises too small a percentage of the total D-mannitol in these tissues to account for the D-mannitol remaining in the single muscle fibers after washing. Thus, 0.08 x 0.3% = 0.024%. This fraction could account for only 0.024% of the muscle fiber D-mannitol space.

REFERENCES

1. P. J. Boyle, E. J. Conway, F. Kane, and H. L. O'Reilly, *J. Physiol. (London), 99,* 401 (1941).
2. J. E. Desmedt, *J. Physiol. (London), 121,* 191 (1953).
3. D. Conway, M. G. Harrington, and M. Mullany, *J. Physiol. (London), 165,* 246 (1963).
4. H. W. Davenport, *J. Physiol. (London), 165,* 246 (1963).
5. C. Edwards and E. J. Harris, *J. Physiol. (London), 135,* 567 (1957).
6. E. J. Harris and H. J. Martins-Ferreina, *J. Exptl. Biol. 32,* 539 (1955).
7. J. A. Johnson, *Am.J. Physiol. 181,* 263 (1955).
8. L. J. Mullins and O. A. S. Frumento, *J. Gen. Physiol. 46,* 629 (1963).
9. P. Tasker, S. E. Simon, B. M. Johnstone, K. H. Shankley, and F. H. Shaw, *J. Gen. Physiol. 43,* 39 (1959).
10. H. T. Narahara and P. Özand, *J. Biol. Chem. 238,* 40 (1963).
11. G. N. Ling and M. H. Kromash, *J. Gen. Physiol. 50,* 677 (1967).
12. S. W. Kuffler and E. M. Vaughan-Williams, *J. Gen. Physiol. (London), 121,* 318 (1953).
13. G. N. Ling, M. C. Neville, P. Shannon, and S. Will, *Physiol. Chem. Physics 1,* 42 (1969).
14. G. N. Ling, *A Physical Theory of the Living State: The Association-Induction Hypothesis.* Blaisdell, New York, 1962.
15. G. Bray, *Analyt. Biochem. 1,* 279 (1960).
16. K. R. Porter and M. A. Bonneville, *An Introduction to the Fine Structure of Cells and Tissues.* Lea and Febiger, Philadelphia, 1964.
17. L. D. Peachy, *J. Cell Biol. 25,* 209 (1965).
18. D. K. Hill, *J. Physiol. 175,* 275 (1964).
19. H. E. Huxley, *Nature 202,* 1067 (1964).
20. S. L. Rosenthal, P. M. Edelman, and I. L. Schwartz, *Biochem. Biophys. Acta 109,* 512 (1965).
21. G. N. Ling, (in press).
22. O. T. Avery, C. M. McLeod, and M. McCarty, *J. Expt. Med. 79,* 137 (1944).
23. D. M. Dawson, *Biochem. Biophys. Acta 113,* 246 (1966).
24. A. D. McLaren, W. A. Jensen, and L. Jacobson, *Plant Physiol. 35,* 549 (1960).
25. K. Zierler, *Ann. N.Y. Acad. Sci. 75,* 223 (1958).
26. H. J. P. Ryser, *Science 159,* 390 (1958).
27. J. T. Edsall, in *The Proteins,* Vol. 1, Part B, H. Neurath and K. Bailey, eds. Academic Press, New York, 1953.
28. J. J. Bunin, W. W. Smith, and H. W. Smith, *J. Biol. Chem. 118,* 667 (1957).
29. C. D. Hodgman, R. C. Weast, and S. M. Selby, *Handbook of Chemistry and Physics, 43rd edition.* The Chemical Rubber Publishing Co., Cleveland, 1961-1962, p. 2229.
30. F. H. Garner and P. J. M. Marchant, *Trans. Inst. Chem. Engra. (London), 39,* 397 (1961).
31. R. Mills, *Rev. Pure and Applied Chem. 11,* 77 (1961).
32. L. J. Gosting and D. F. Akeley, *J. Amer. Chem. Soc. 74,* 2058 (1952).
33. A. M. Friedman and J. W. Kennedy, *J. Amer. Chem. Soc. 77,* 4499 (1955).