# The Extracellular Space of Voluntary Muscle Tissues

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ABSTRACT The volume occupied by the extracellular space has been investigated in six types of voluntary muscles: sartorius (frog), semitendinosus (frog), tibialis anticus longus (frog), iliofibularis (frog), rectus abdominis (frog), and diaphragm (rat). With the aid of four types of probe material, three of which are conventionally employed (inulin, sorbitol, sucrose) and one of which is newly introduced (poly-L-glutamate), and a different experimental method, we have demonstrated that the "true" extracellular space of frog sartorius, semitendinosus, tibialis anticus longus, and iliofibularis muscle and of rat diaphragm muscle is equal to, or probably less than, 8–9% (v/w) of the tissue. The frog rectus muscle shows a somewhat higher ceiling value of 14%.

Living tissue consists of cells and "extracellular space." A precise knowledge of the percentage of extracellular space is essential if one is to obtain meaningful values for the concentrations of solutes or water within the cell. In the case of solutes like $Na^+$ ion and glucose, which are usually found at lower concentrations within the cell than in the extracellular fluid, an erroneous value for the extracellular space may lead to the conclusion that these solutes are altogether absent in the cell, while in truth they are present in sizable concentrations (or vice versa). Yet published values for the extracellular space vary widely. Thus, for frog sartorius muscle the proportion of extracellular space given in the literature varies from 13% of the tissue (1–4) to as high as 35% (5–9). With this degree of uncertainty, meaningful interpretation of quantitative data on "glucose transport" or "sodium transport" in muscle tissues, as examples, is virtually impossible. Such problems are of fundamental importance in understanding the behavior of the living cell.

In this communication we present results of investigations of the extracellular space of several types of voluntary muscles of amphibian and mammalian origin. Using new techniques, we have found the extracellular spaces of these tissues to be surprisingly low and constant.

## MATERIALS AND METHODS

### *Materials*

We studied five types of muscles from leopard frogs (*Rana pipiens*, Schreber)—sartorius, semitendinosus, tibialis anticus longus, iliofibularis, and the rectus abdominis—and one type of muscle from albino rats (Wistar), the diaphragm muscle. The dissection and isolation of the first four muscles named above presented no special problem. The rectus abdominis was isolated with considerable adjacent tissue attached; without further dissection it was equilibrated with Ringer's solution containing the probe material under study (for example, inulin). Before the probe material was extracted, the cut peripheral tissue was trimmed away. In this process, injury to some muscle fibers was inevitable, but intactness of the cells at this stage was of little consequence. For the rat diaphragm muscles, the rib cage preparation would have been a better choice, but this would have entailed the use of large quantities of certain probe material that is costly (see below). We chose to employ, instead, "cut" diaphragm muscles. Before recovery of the probe material, the regions of the muscle near the cut ends were trimmed away.

All chemicals used were of cp grade. Inulin was obtained from Pfanstiehl Laboratories, Waukegan, Ill. (Lot 6198); poly-L-glutamate, from Mann Biochemicals, New York (Lot B2523, mol wt 50,000; Lot N2461, mol wt 61,000); safranin O, from Harleco, Philadelphia (Lot 30101); labeled inulin (carboxyl-$^{14}$C, mol wt 3000–4000, Lot 96-192-I-22) from New England Nuclear Corp., Boston; labeled D-sorbitol (ul-$^{14}$C), from Nuclear-Chicago, Des Plaines, Ill. Batch 21); labeled sucrose (ul-$^{14}$C), from Nichem, Farmingdale, N.Y.

### *Assay Methods*

Inulin was determined according to the method of Roe et al. (10). All $^{14}$C-labeled compounds were assayed with a Packard liquid $\beta$-scintillation counter (Model 314E) using Bray's scintillation fluid[1] (11). As a rule, 0.5 ml of Ringer's solution containing the labeled compound to be assayed was added to 10 ml of scintillation fluid. Assay of $^{14}$C-labeled inulin in this manner presented some difficulty since, in this scintillation fluid, inulin slowly settles out and the number of counts per minute decreases. This difficulty was overcome by sandwiching the unknown between standards and by counting samples as soon as possible after their preparation. The assay of poly-L-glutamate (PLG) is adapted from the procedure given by Bovarnide et al. (12): 0.5 ml of Ringer's solution containing a known or an unknown amount of PLG is added to 1 ml of a 0.06 M NaOH-citrate buffer (pH 5.98), 1 ml of 0.1 % (w/v) safranin solution, and 2.5 ml of 0.118 M NaCl solution in a centrifuge tube. The tubes are al-

[1] 4.0 g of PPO (2,5-diphenyloxazole) (Packard); 200 mg of POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene] (Packard); 60 g of naphthalene (recrystallized from alcohol, Matheson, Coleman, and Bell); 100 ml methanol (Merck); 20 ml ethylene glycol (Merck); and enough *p*-dioxane (purified, Fisher catalogue No. 53) to make up 1 liter. We are indebted to Dr. H. B. Horowitz and Dr. R. Fenichel for informing us of this particular brand of *p*-dioxane, which their investigations have shown to be perfectly satisfactory for the present purpose, but more economical.

lowed to stand for 30 min at room temperature, after which the precipitate is spun down at 900 g, in a horizontally swinging centrifuge for 10 min. The safranin remaining is determined colorimetrically at 540 mμ in a Klett colorimeter on 1:50 dilution of the clear supernatant. The amount of PLG in the sample is calculated from the decrease of safranin color. It is important to use a different standard curve for each batch of safranin solution prepared, a precaution pointed out by Bovarnide and his coworkers in their original paper.

## EXPERIMENTAL PROCEDURES AND METHODS OF DATA ANALYSIS

Muscles were incubated in Ringer's solutions containing varying concentrations of the probe material (the "soaking-in" solutions) long enough to ensure equilibrium distribution in the extracellular space (see below and Table I). Samples of the bathing solution removed at this time were assayed for the concentration of probe material remaining. Each muscle or group of muscles was then removed from the bathing solution, blotted dry, and introduced into a known volume of Ringer's solution containing no probe material (the "washing-out" solution). A standardized procedure for thorough blotting of adhering fluid is as follows. The tissue was placed between decks (five sheets on each side) of Whatman No. 1 filter paper wetted with Ringer's solution, and the index finger was run firmly back and forth four times over the paper above the tissue. We discovered considerable experimental error due to adhering fluids on the tissue surface if the blotting was less careful. After blotting, the tissue was incubated until the probe material in the extracellular space again reached equilibrium with the external solution. Unless otherwise stated, both soaking-in and washing-out procedures were carried out at 0°C.

The present procedure demands a knowledge of the time necessary for the probe molecule to reach equilibrium in the extracellular space. For this reason, we shall discuss this problem first.

### A. *Equilibrium in the Extracellular Space*

1. DIFFUSION COEFFICIENT OF THE PROBE MOLECULES The diffusion coefficients for chicory inulin at 37°C in water ($D_{37,w}$) was given by Bunim et al. (13) as 4.65 × $10^{-6}$ cm²/sec and of dahlia inulin as 4.10 × $10^{-6}$ cm²/sec. Using the lower figure and correcting for temperature,[2] one obtains a minimum value of 1.40 × $10^{-6}$ cm²/sec at 0°C.

There are no data available on the diffusion coefficient of PLG. We were able to estimate this value on the basis of an empirical correlation between the molecular weights and diffusion coefficients of some 69 macromolecular compounds as shown in Fig. 1 (taken from W. B. Dandliker as cited by Edsall, 15). This correlation permits, on the basis of the molecular weight of the samples used (i.e. 50,000 and 61,000), an evaluation of the PLG diffusion coefficients. These should be not lower than 4 × $10^{-7}$ cm²/sec at 20°C or 2.14 × $10^{-7}$ cm²/sec at 0°C.

[2] The temperature correction was made on the basis of the relation $D_{0,w} = [273/(273 + 37)] \cdot [\eta_0/\cdot\eta_{37}] \cdot D_{37,w}$, where $D_{0,w}$ and $D_{37,w}$ refer to the diffusion coefficient in water at 0°C and 37°C, respectively. $\eta_0$ and $\eta_{37}$ are the viscosities of water at these temperatures (see reference 14).

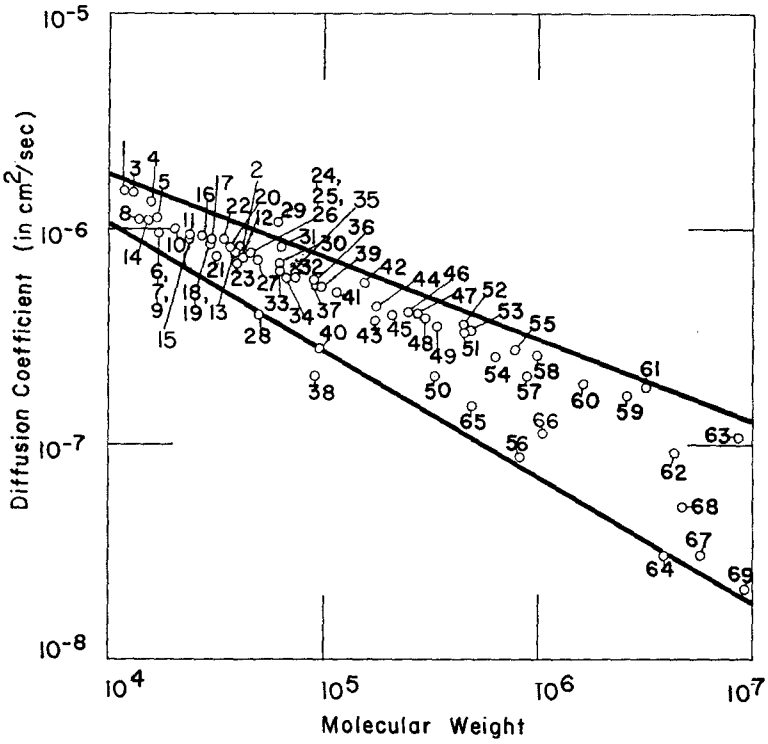


FIGURE 1. The relation between molecular weight and diffusion coefficients. The diffusion coefficients are for 20°C in water. Data are taken from the table of Dandliker as cited by Edsall (15). The numbers refer to the various proteins as follows:

*1*, insulin (monomer) (*k*)
*2*, insulin (trimer, or tetramer)
*3*, ribonuclease
*4*, cytochrome *c* (cow heart)
*5*, myoglobin
*6*, *Bacillus phlei* protein
*7*, erythrocruorin (*Lampetra*)
*8*, lysozyme
*9*, lactalbumin (cow)
*10*, ACTH (sheep)
*11*, α-chymotrypsin (monomer)
*12*, α-chymotrypsin (dimer)
*13*, chymotrypsinogen
*14*, trypsin
*15*, trypsinogen
*16*, scarlet fever toxin
*17*, carbonic anhydrase
*18*, crotoxin
*19*, oxytocin
*20*, human tuberculosis bacillus protein
*21*, prolactin
*22*, pepsin
*23*, peroxidase
*24*, β-lactoglobulin
*25*, concanavalin B
*26*, ovalbumin
*27*, growth hormone
*28*, zein
*29*, L-amino acid oxidase
*30*, pyrophosphatase
*31*, enolase
*32*, follicle-stimulating hormone
*33*, bovine serum albumin
*34*, hemoglobin (man)
*35*, diphtheria toxin
*36*, luteinizing hormone
*37*, diphtheria antitoxin
*38*, tropomyosin
*39*, concanavalin A
*40*, hexokinase
*41*, rabbit muscle glyceraldehyde phosphate dehydrogenase

2. TRUE DIFFUSION PATH LENGTH The muscles used in the present investigation fall into two categories: in the first belong the frog sartorius muscle, the frog rectus abdominis muscle, and the rat diaphragm muscle; all are thin sheets, 0.4–0.8 mm thick. In the second belong the rest of the frog muscles: tibialis anticus longus, semitendinosus, and iliofibularis. These three muscles have the form of double or single tapering cylinders, each with a diameter of 2 mm or less.

The bulk of the extracellular space is simple physical space filled with tissue fluid.[3] Thus, diffusion from either the thin sheet type of muscle or the cylindrical type can be predicted from the well-known solutions of classical diffusion problems (see below, also reference 16). There is, however, one departure from these classical problems. The extracellular space constitutes only part of the volume of the sheets or cylinders and has the form of irregular interstices. Thus, the actual diffusion path is longer than the geometric thickness of the sheet or the diameter of the cylinder. The ratio of the true diffusion-path length to the thickness or radius of the tissue has been represented by $\lambda$ (Harris and Burn, 17). If all the muscle fibers are arranged in perfectly aligned rows, the shortest diffusion path is equal to the depth of the tissue, but in this case there are "pockets" of space between neighboring channels which will slow diffusion. If, on the other hand, the fibers are arranged in a staggered fashion, the diffusion path will be longer than the over-all depth or diameter of the muscle. In the case of cylindrical fibers, the upper limit is the ratio of one-half the circumference, $\pi r$, divided by the diameter, $2r$, or $\pi/2 \sim 1.57$, which is the value employed by Harris and Burn. The actual value, however, must be somewhat lower, because the fiber arrangement that will permit no "short cuts" other than the circumference cannot be exactly circular in cross-section. For staggered squares, the $\lambda$-value is 1.5. It is still lower for closely packed hexagons (1.15), but again "pockets" exist in this system. Taking all into consideration, we estimate that a ratio of 1.5 is close to the truth; the value cannot exceed 1.57.

[3] The reader can most readily convince himself of this point by dissecting some single muscle fibers.

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*42*, $\beta$-amylase (sweet potato)
*43*, $\gamma$-globulin (man)
*44*, aldolase
*45*, fumarase
*46*, catalase
*47*, phycocyan
*48*, phycoerythrin
*49*, phosphorylase (rabbit muscle)
*50*, fibrinogen (cow)
*51*, hemocyanin (*Palinurus*)
*52*, apoferritin
*53*, urease
*54*, thyroglobulin (pig)
*55*, hemocyanin (*Homarus*)
*56*, myosin
*57*, toxin (*Clostridium botulinum*)
*58*, glutamic acid dehydrogenase
*59*, $\beta_1$-lipoprotein, serum (man)
*60*, erythrocruorin (*Planobris*)
*61*, erythrocruorin (*Lambricus*)
*62*, pyruvic oxidase
*63*, hemocyanin (*Helix pomatia*)
*64*, actomyosin
*65*, turnip yellow mosaic virus
*66*, tomato bushy stunt virus
*67*, tobacco mosaic virus
*68*, rabbit papilloma virus
*69*, polyhedral virus (silkworm)

3. TIME TO REACH EQUILIBRIUM IN THE EXTRACELLULAR SPACE The classic equation for diffusion of a solute into or out of a slab is (16, 18)

$$\frac{\bar{C} - C_f}{C_i - C_f} = \frac{8}{\pi^2} \sum_{\nu=0}^{\infty} \frac{1}{(2\nu + 1)^2} \exp\left[\left(-\frac{(2\nu + 1)\pi}{\lambda h}\right) Dt\right] \quad (1)$$

where $\bar{C}$ is the average concentration of solute in the slab at time $t$; $C_f$, the final, and $C_i$, the initial, concentrations within the slab; $D$, the diffusion constant of the solute; $h$, one-half the thickness of the slab; and $\nu$, an integer from zero to infinity.

TABLE I

TIME FOR PROBE MATERIAL TO REACH EQUILIBRIUM IN THE EXTRACELLULAR SPACE OF THIN-SHEET AND CYLINDRICAL TYPES OF MUSCLES AT 0°C

Data for the cylindrical type of muscle were calculated for a diameter of 2 mm; data for the thin-sheet type were calculated on the basis of a thickness of 0.6 mm. The sources of the diffusion coefficients are discussed in the text. The molecular weight of (dahlia) inulin was from Westfall and Landis (19). It should be pointed out that the $^{14}C$-labeled inulin which we used has a lower molecular weight of 3000–4000 (see Materials and Methods).

| Probe material | Inulin | Poly-L-glutamate |
|---|---|---|
| Molecular weight | 5,100 | 50,000–61,000 |
| Diffusion coefficient ($cm^2/sec$) | $1.40 \times 10^{-6}$ | $2.14 \times 10^{-7}$ |
| Time to reach 90% exchange in extracellular space | | |
| Thin muscles | 19 min | 2.0 hr |
| Cylindrical muscles | 1.6 hr | 10 hr |
| Time to reach 99% exchange in extracellular space | | |
| Thin muscles | 41 min | 4.4 hr |
| Cylindrical muscles | 3.3 hr | 21 hr |

For diffusion of a solute into or out of a cylinder of radius $r_0$, the equation is

$$\frac{\bar{C} - C_f}{C_i - C_f} = \sum_{1}^{\infty} \frac{4}{\xi_\nu^2} \exp\left[-\frac{\xi_\nu^2 Dt}{\lambda^2 r_0^2}\right] \quad (2)$$

Here the $\xi_\nu$ are the roots of the equation $J_0(x) = 0$, where $J_0(x)$ is the Bessel function of zero order ($\xi_\nu = 2.405, 5.520, 8.654, 11.792, \cdots$). Multiplying the half-thickness or radius of the tissue by the factor 1.5 and substituting this value ($\lambda h$ or $\lambda r_0$), along with the diffusion coefficient of the substance in question, into the above equations, we may obtain the times for the various probe materials to reach 90% and 99% of equilibrium in the extracellular space. The results for both thin-sheet and cylindrical types of muscles are shown in Table I.

## B. *The Equilibrium Method*

In the conventional methods for determining extracellular space (1, 2, 20, 21), it is assumed that the probe material (e.g. inulin) is ideal, that is, that it neither enters

the cell nor is adsorbed on extracellular components of the tissue. The tissue is soaked for a variable length of time in a solution containing an arbitrary concentration of probe material. It is then washed in a solution containing no probe material. The entire amount of probe material recovered in the washing-out solution is assumed to have come from the extracellular space.

Our equilibrium procedure described above is similar to those conventionally employed. The chief departure lies in the fact that we did not assume that an ideal probe molecule exists and can be found. Instead, we made the safer assumption that cell penetration and adsorption is always possible, and proceeded from these premises to isolate the "true" extracellular space component from the interfering fractions on the basis of its distinctive characteristics.

The theoretical equation for solute distribution in whole tissues is given in the Appendix as Equation C. This equation includes solute in the extracellular space, in the intracellular water, and adsorbed on two kinds of sites: one kind showing site-to-site interaction and another kind showing no site-to-site interaction (22–25). For illustration, let us examine a simpler model containing one type of adsorption site with no interaction and another type of adsorption site with interaction. In this case, Equation C for the concentration of the $i$th solute in the tissue, $[p_i]_{tis}$, is

$$[p_i]_{tis} = [\beta + \alpha(1 - \beta)q_i][p_i]_{ex} + \frac{[f]_L \tilde{K}_i^L [p_i]_{ex}}{1 + \tilde{K}_i^L [p_i]_{ex}} + \tilde{\tilde{K}}_i^K \{[p_i]_{ex}\}^{n_i^K} \qquad (3)$$

where $\alpha$ is the percentage of water in the tissue in liters per kilogram; $\beta$, the fraction (v/w) of the tissue occupied by the extracellular space; $q_i$, the mean equilibrium distribution coefficient of the $i$th solute between the intracellular water and extracellular water; $[p_i]_{ex}$, the equilibrium extracellular concentration of the $i$th solute; $[f]_L$, the concentration of (intracellular or extracellular) adsorption sites showing no site-to-site interaction and is in moles per kilogram of whole tissue; $\tilde{K}_i^L$, the adsorption constant on the $K$th type of interacting site; and $n_i^K$, the interaction parameter. Both $\tilde{\tilde{K}}_i^K$ and $n_i^K$ are pure numbers.

Fig. 2*a* shows a plot of Equation 3. The three broken curves, *A*, *B*, and *C*, in this figure represent, from left to right, the three components on the right-hand side of this equation. The dashed line, *D*, in Fig. 2*b* is a straight line drawn through the origin, parallel to the flat part of the curve nearest the origin.

If there is no adsorption, the *B* and *C* components vanish. The lines *D* and *A* then coincide and the slope of *D* yields the value of $\beta + \alpha(1 - \beta)q_i$. If $q_i$ is also equal to zero, then the slope of the straight line *D* is $\beta$, which is the proportion of extracellular space in the tissue. However, if *B*, *C*, and $q_i$ are not equal to zero, the slope of *D* would give us a ceiling value for $\beta + \alpha(1 - \beta)q_i$ and, hence, a ceiling value of $\beta$. In the present theoretical case, the slope of *D* is 0.834, whereas the sum of $\beta$ and $\alpha(1 - \beta)q_i$ is only 0.500.

The equilibrium method cannot differentiate between probe material in the extracellular space and that in some other compartments, but if more than one type of probe molecule is used, a better approximation of the extracellular space can be obtained from the material which yields a *minimum value* for $\beta + \alpha(1 - \beta)q_i$.

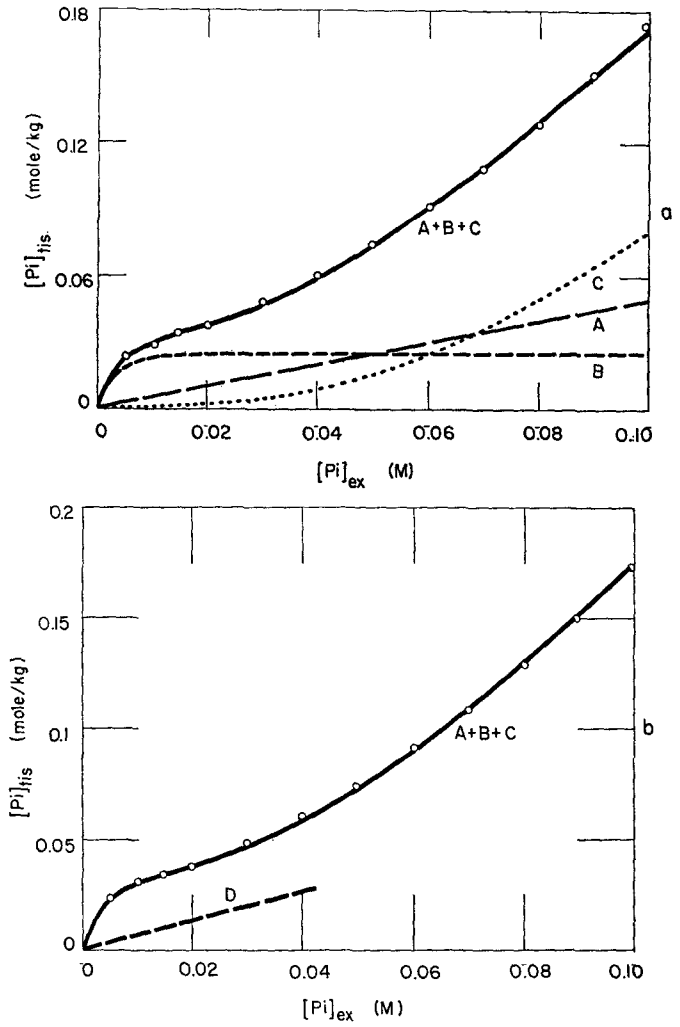


FIGURE 2. Theoretical equilibrium distribution of a probe material at varying external concentrations. The theoretical curve marked $A + B + C$ is calculated according to Equation 3. It is composed of the three component curves shown in *a* as *A*, *B*, and *C*. These curves refer to the first, second, and last terms of the right-hand side of Equation 3, respectively. The various parameters used are as follows: $\beta = 0.1$, $\alpha(1 - \beta) = 0.4$, $[f]_L = 2.5 \times 10^{-3}$ mole/kg, $\tilde{K}_i^L = 10^3$ (M)$^{-1}$, $\tilde{\tilde{K}}_i^K = 10$, and $n_i^K = 2$. The straight line marked *D* in *b* is drawn through the origin, parallel to the initial flat portion of the curve; it gives a ceiling value to line *A* in *a*.

## RESULTS

### *Equilibrium Inulin Distribution Studies*

In Table II the apparent "extracellular space" of frog sartorius, tibialis anticus longus, semitendinosus, and iliofibularis muscles was determined

TABLE II

APPARENT EXTRACELLULAR SPACE OF FROG MUSCLES, ASSAYED WITH A CONVENTIONAL INULIN METHOD

The inulin concentration was 0.2%. The soaking-in time was 18 hr and the washing-out time was 15 hr. Both processes were conducted at 4°C with occasional agitation.

| Muscle type | No. of muscles | Weight | Insuline space | Extracellular space | |
|---|---|---|---|---|---|
| | | *mg* | *ml* | % *v/w* | |
| Sartorius | 2 | 135.0 | 0.0195 | 14.40 | |
| Sartorius | 2 | 86.2 | 0.0077 | 8.90 | |
| Sartorius | 2 | 119.0 | 0.0109 | 9.16 | 10.3 |
| Sartorius | 2 | 160.0 | 0.0139 | 10.00 | |
| Sartorius | 2 | 128.0 | 0.0114 | 8.94 | |
| Iliofibularis | 2 | 104.0 | 0.0108 | 10.44 | 11.0 |
| Iliofibularis | 2 | 103.0 | 0.0120 | 11.64 | |
| Semitendinosus | 2 | 147.0 | 0.0107 | 7.25 | |
| Semitendinosus | 2 | 140.0 | 0.0124 | 8.45 | 8.8 |
| Semitendinosus | 2 | 158.0 | 0.0170 | 10.60 | |
| Average | | | | | 10.0 |

TABLE III

APPARENT EXTRACELLULAR SPACE OF FROG MUSCLES, ASSAYED WITH $^{14}C$-LABELED INULIN WITH PROLONGED EQUILIBRATION TIME

Isolated muscles were shaken in a Ringer's phosphate solution containing $^{14}C$-labeled inulin in tracer amounts for 60 hr at 0°C. The muscles were then blotted on wetted filter paper and incubated in 2 ml of nonradioactive Ringer's solution for 84 hr at 0°C. The specific activity was calculated from the radioactivity of the soaking-in solution.

| Muscle No. | Muscle type | No. of muscles | Muscle weight | Apparent extracellular space |
|---|---|---|---|---|
| | | | *mg* | % |
| 1 | Sartorius | 2 | 141 | 16.4 |
| 2 | Sartorius | 2 | 136 | 19.2 |
| 3 | Semitendinosus | 2 | 136 | 20.4 |
| 4 | Semitendinosus | 2 | 136 | 24.4 |
| 5 | Tibialis anticus longus | 2 | 156 | 18.3 |
| 6 | Tibialis anticus longus | 2 | 119 | 24.2 |
| 7 | Iliofibularis | 2 | 99 | 15.4 |

according to the conventional method, using 0.2% inulin in Ringer's phosphate solution with a soaking-in time of 18 hr, a washing-out time of 15 hr, and occasional agitation. As shown in Table I, the time for inulin to reach 99% equilibrium is only 3.3 hr.

Table III shows that further prolongation of the soaking-in time to 60 hr and of the washing-out time to 84 hr further increases the inulin space.[4]

B. *The Equilibrium Distribution of Labeled Sucrose and of Labeled Sorbitol in Comparison with Inulin*

Extensive unpublished studies on the time course of uptake of labeled sucrose, glucose, and many other related compounds by frog muscle tissue at 0°C showed that, as a rule, equilibrium is reached in less than 10 hr (usually in

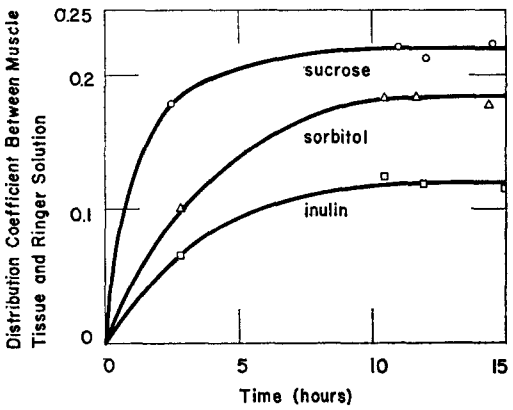


FIGURE 3. Sucrose space and sorbitol space in comparison with inulin space. Time course of uptake of $^{14}$C-labeled sucrose (1 mM), $^{14}$C-labeled sorbitol (1 mM), and $^{14}$C-labeled inulin (tracer amount, no carrier added) as assayed from the decrease of radioactivity in the bathing solution. Prior to incubation with the probe molecule, the muscles were washed for 3.5 hr at 25°C in three changes of Ringer's phosphate solution with continual shaking to remove preexisting insulin. The incubation solution (3.5 ml, 0°C) contained frog muscles (two each: sartorius, tibialis anticus longus, semitendinosis, and iliofibularis) weighing in toto between 0.6 and 1.3 g. The ordinate (in milliliters per gram) represents the concentration of labeled probe material in the tissue divided by the concentration of probe material in the bathing medium.

5–6 hr). From then on, a constant concentration is maintained for as long as we observed (24 hr). Fig. 3 shows the time course of uptake of labeled sucrose, labeled sorbitol, and labeled inulin by frog muscles. The space available to sucrose and to sorbitol exceeds that available to inulin.

[4] The question may be raised: "Why, even with the 60 hr–84 hr schedule, do the extracellular space values still fall short of the figure often quoted, i.e. 25–30%?" We believe that in some specific cases very high values reported could be attributed to broken fibers in the muscle preparation. Such broken fibers would accommodate large amounts of inulin. A more general explanation is the much higher inulin concentration usually employed (approximately 1%). The data shown in Figs. 4 and 5 demonstrate the trend toward abrupt increase of apparent "inulin" space at higher inulin concentration.

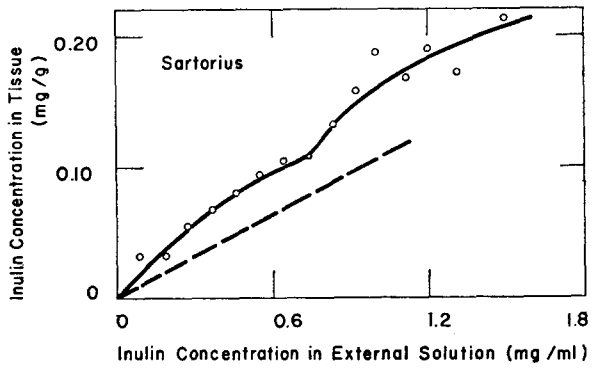


FIGURE 4. Equilibrium distribution of inulin in frog sartorius muscles. Sartorius muscles were shaken in 2 ml of Ringer's phosphate solution (glucose-free) containing varying concentrations of inulin at 0°C for 24 hr. After blotting, the muscles were washed for 24 hr, also at 0°C. The inulin assayed from the wash-out solution yields the inulin concentration in the muscles as given on the ordinate. The concentrations of inulin in the soaking-in solution at equilibrium are shown on the abscissa. Each point represents two or three sartorius muscles treated as a group. (13 September 1965.)

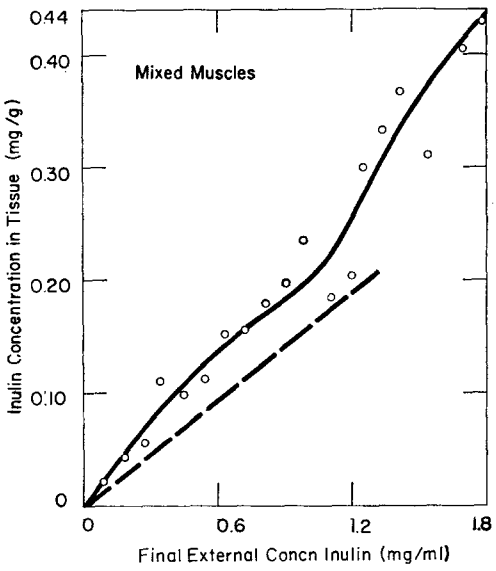


FIGURE 5. Equilibrium distribution of inulin in mixed frog muscles. Mixed muscles were treated as described in the legend to Fig. 4. Ordinate and abscissa as in Fig. 4. Each point represents one each of sartorius, semitendinosus, tibialis anticus longus, and iliofibularis muscles treated as a group. (13 September 1965.)

### C. *The Effect of External Inulin Concentration on Inulin Distribution in Tissue*

The equilibrium distribution of inulin after 24 hr of soaking-in and 24 hr of washing-out is illustrated in Fig. 4 for sartorius muscle alone and Fig. 5 for a group of "mixed muscles."[5] The secondary rise observed at higher external

[5] As used in this paper, a group of "mixed muscles" contains one each of the following: sartorius, tibialis anticus longus, semitendinosus, and iliofibularis.

inulin concentrations may or may not be pronounced. The curves are not straight lines as would be the case if inulin were an ideal probe material; instead, they resemble the theoretical curves shown in Fig. 2. The dashed lines were drawn through the origin parallel with the initial flat portion of the curves; the slopes are 0.107 for sartorius muscle and 0.15 for mixed muscles.

D. *Equilibrium Distribution of Poly-L-glutamate in Various Muscle Tissues*

Fig. 6 shows the recovery of PLG from sartorius muscles that had been equilibrated in solutions containing various concentrations of PLG as a function of the loss of PLG from the equilibrium solutions. The points can be fitted with a straight line with a slope of unity; this shows that PLG is not

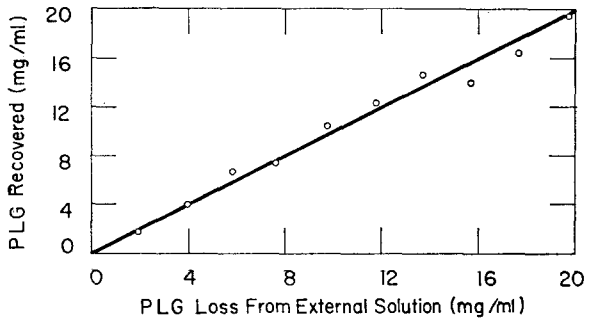


FIGURE 6. Comparison of loss of PLG from the soaking-in solution with that recovered in the washing-out solution. Two sartorius muscles were soaked for 21 hr at 0°C in 1 ml of Ringer's phosphate solution containing varying concentrations of PLG and were washed out in 1 ml of normal Ringer's phosphate solution for 24 hr. The abscissa represents the loss of PLG from the soaking-in solution; the ordinate, the PLG recovered from the washing-out solution. Each point represents two sartorius muscles treated as a group. The slope of the curve is unity. (23 August 1965.)

metabolically altered (or irreversibly taken up) by the tissue and, therefore, is a satisfactory probe material for determination of the extracellular space. The results also show that under the experimental conditions, neither concentration-dependent viscosity change nor concentration-dependent monomer -polymer transformation (if they exist) significantly affect our results by slowing down diffusion.

1. FROG SARTORIUS MUSCLE Fig. 7 shows the equilibrium distribution of PLG in sartorius muscles after 21 hr of soaking in and 24 hr of washing out. In general, the curve resembles the inulin distribution curves shown in Figs. 5 and 6, as well as the theoretical curve in Fig. 2. Note the secondary rise at high PLG concentrations. The straight line drawn through the origin and parallel to the initial flat portion of the curve has a slope of 0.083.

Fig. 8 shows another series in which the muscles were soaked in PLG solu-

tion for 42 hr and washed out for 24 hr. After such prolonged exposure to PLG, in some cases one or both muscles in each group went into partial contracture. Nevertheless, the total PLG distribution in these muscles was not materially different from the distribution in muscles that had remained

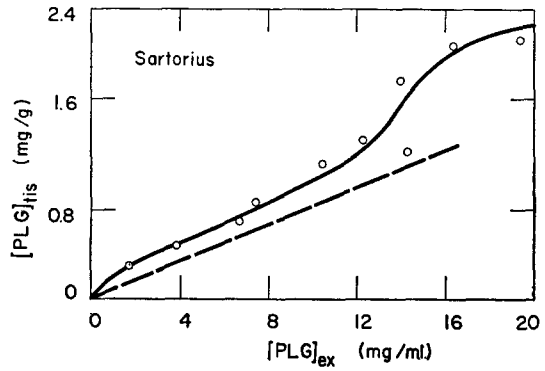


FIGURE 7. The equilibrium distribution of PLG in frog sartorius muscles at varying external PLG concentrations. Data were obtained from the experiment described in Fig. 6. The slope of the dashed line is 0.083.

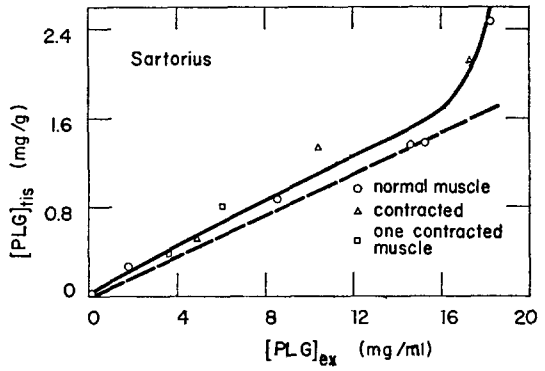


FIGURE 8. Equilibrium distribution of PLG in sartorius muscles at varying external concentrations of PLG. The soaking-in time was 42 hr and the washing-out time was 24 hr, both at 0°C. Each point represents two sartorius muscles treated as a group. Normal appearance of both muscles at the end of the soaking-in period, ○; one normal and one in contracture, □; both in contracture, △. The slope of the dotted line is 0.091. (21 September 1965.)

perfectly normal in appearance. The slope of the initial flat portion of the curve in this case is 0.091.

2. MIXED MUSCLES Fig. 9 shows PLG distribution in mixed muscles from two experiments; in one, the soaking-in time was 17 hr and the washing-out time 23 hr; in the other, the soaking-in time was 42 hr and the washing-

out time 24 hr. Within the limits of experimental accuracy, there is no difference between the results. This shows that it takes no more than 17 hr for PLG to reach equilibrium in these muscles, in agreement with our calculated equilibration time given in Table I. It also shows that, unlike inulin, PLG is not steadily taken up by muscles after the estimated time for PLG to reach equilibrium in the extracellular space. The initial flat portion of the curve has a slope of 0.093.

3. FROG RECTUS MUSCLE The data shown in Fig. 10 were obtained after a soaking-in period of 19.8 hr and a washing-out time of 24 hr (both at 4–5°C). The slope of the dashed line is 0.136.

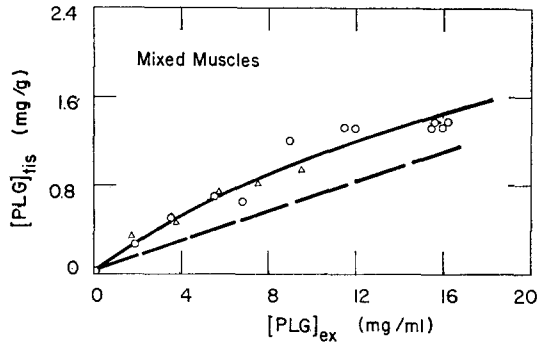


FIGURE 9. Equilibrium distribution of PLG in mixed muscles at varying concentrations of PLG. The soaking-in time for one set of experiments (○, 21 September 1965) was 42 hr, and the washing-out time, 24 hr. The soaking-in time for the other set of experiments ( △, 16 August 1965) was 17 hr, and the washing-out time, 23 hr. The slope of the dashed line is 0.093.

4. RAT DIAPHRAGM MUSCLE The data shown in Fig. 11 were obtained after 18.5 hr of soaking in and 24 hr of washing out (both at 4–5°C). The slope of the dashed line is 0.08.

## DISCUSSION AND CONCLUSION

In this series of experiments, we have studied the extracellular space of muscle tissues using four types of probe material; of these, three (inulin, sorbitol, sucrose) are conventionally used, and one (PLG) is newly introduced. None satisfies the criteria of an ideal probe molecule; all either absorb onto and/or enter other compartments. The highly negatively charged PLG shows the minimum of these undesirable traits as a probe molecule.

The calculated time necessary for inulin to reach equilibrium in the extracellular space is less than 4 hr at 0°C (Table I). The data in Tables II and III show that muscle continued to take up more inulin even after 18 hr. This slow rise of inulin concentration is too large to be attributed to a slower

phase of diffusion into the sarcoplasmic reticulum. Only the T-system tubules are confluent with the extracellular space; they have a total volume only 0.3–0.5% of the total muscle volume (26–28). A more likely explanation is that inulin becomes absorbed or enters into compartments other than the extracellular space. One can draw the same conclusion by comparing the equilibrium distribution curves for inulin (Figs. 5 and 6) with the theoretically

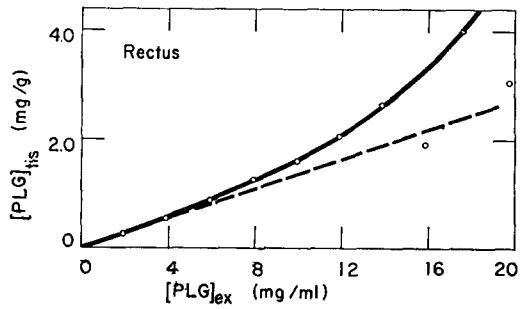


FIGURE 10. Equilibrium distribution of PLG in frog rectus abdominus muscle. The soaking-in time was 19 hr 45 min, and the washing-out time, 24 hr, both at 4°C. The slope of the dashed line is 0.136. (25 October 1965.)

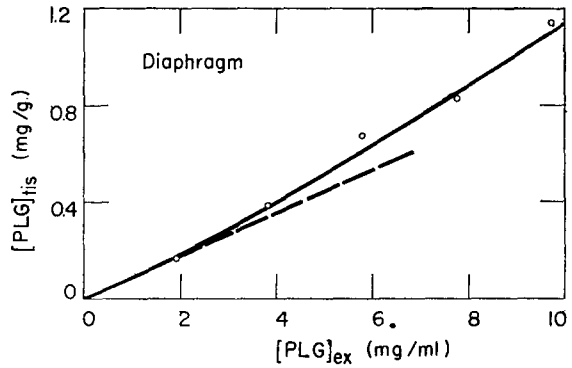


FIGURE 11. Equilibrium distribution of PLG in rat diaphragm muscle. The soaking-in time was 18 hr 25 min; the washing-out time, 24 hr, both at 4–5°C. The slope of the dashed line is 0.08. (27 October 1965.)

calculated curve of Fig. 2. Comparison of the "space" available to sucrose and sorbitol with that available to inulin (Fig. 3; see also Figs. 4 and 5) shows that sucrose and sorbitol deviate further from ideality as probe materials than does inulin. The shape of the equilibrium distribution curve of PLG illustrates that it also deviates from the behavior of an ideal probe material.

This departure from the properties of an ideal probe material notwithstanding, results from PLG and other studies have yielded a consistent

picture, which is summarized in Table IV. The frog sartorius, alone or with tibialis anticus longus, iliofibularis, and semitendinosus, and the rat diaphragm muscle have extracellular spaces no higher than 8–9%. The frog rectus abdominis shows a somewhat higher ceiling value of 13–14%.

TABLE IV

SUMMARY OF EXPERIMENTALLY DETERMINED VALUES OF EXTRACELLULAR SPACE IN FROG AND RAT MUSCLES

Because the values obtained are ceiling values, the sign $\leq$ is used.

| Muscle type | Animal source | Inulin equilibrium | PLG equilibrium |
|---|---|---|---|
| | | % | % |
| Sartorius | Frog | $\leq 10.7$ | $\leq \begin{cases} 8.3 \\ 9.1 \end{cases}$ |
| Mixed muscles | Frog | $\leq 15.0$ | $\leq \begin{cases} 8.2 \\ 9.3 \end{cases}$ |
| Rectus | Frog | — | $\leq 13.5$ |
| Diaphragm | Rat | — | $\leq 8.0$ |

## APPENDIX

The total concentration of the $i$th solute in the tissue is

$$[p_i]_{tis} = (1 - \beta)\,[p_i]_{cell} + \beta[p_i]_{ex} \tag{A}$$

where $[p_i]_{tis}$ is the concentration of the $i$th solute in this tissue in moles per kilogram of fresh tissue; $[p_i]_{cell}$, the concentration of the $i$th solute in the cell in moles per kilogram of fresh cells; $[p_i]_{ex}$, the concentration of the $i$th solute in the external solution in equilibrium with the cells; and $\beta$, the percentage (v/w) of extracellular space in the tissue.

Elsewhere we have shown (24, 25) that for many solutes studied, distribution in muscle cells follows a general equation which, for the present purpose, may be simplified into the following form:

$$[p_i]_{cell} = \alpha q_i [p_i]_{ex} + \frac{1}{(1-\beta)} \sum_{L=1}^{N} \frac{[f]_L^{cell} \tilde{K}_i^{L(cell)} [p_i]_{ex}}{1 + \tilde{K}_i^{L(cell)} [p_i]_{ex}} + \frac{1}{(1-\beta)} \sum_{K=1}^{M} \tilde{K}_i^{K(cell)} ([p_i]_{ex})^{n_i^{K(cell)}} \tag{B}$$

The three terms on the right-hand side of Equation A are, from left to right: $i$th solute in the intracellular water, $i$th solute adsorbed on noninteracting sites (Langmuir adsorption isotherm), and $i$th solute adsorbed on interacting sites (cooperative adsorption isotherm). $\alpha$ is the percentage of water in the cell in liters per kilogram of

fresh cells. $q_i$ is the mean equilibrium distribution coefficient of the $i$th solute between the intracellular and extracellular water. $\tilde{K}_i^L$ is the association constant of the $i$th solute on the $L$th type of site among a total of $N$ noninteracting sites. The concentration of the $L$th type of site is $[f]_L$ in moles per kilogram of fresh muscle tissue. $\tilde{\tilde{K}}_i^K$ is the adsorption constant of the $i$th solute on the $K$th type of interacting site among a total of $M$ types of sites of this kind. $n_i^K$, an exponent, is a quantitative measure of the magnitude of site-to-site interaction; its explicit relation to the nearest neighbor interaction energy has been given elsewhere (22, 23, 25, 29).

The extracellular phase of the tissue consists of extracellular space filled with bathing medium and also certain adsorption sites on the connective tissue elements, etc. Combining the extracellular and intracellular $i$th solute, and considering that there are altogether $S$ types of noninteracting sites and $T$ type of interacting sites in the whole tissue, we have

$$[p_i]_{\text{tis}} = \{\alpha(1-\beta)q_i + \beta\}[p_i]_{\text{ex}} + \sum_{L=1}^{S} \frac{[f]_L \tilde{K}_i^L [p_i]_{\text{ex}}}{1 + \tilde{K}_i^L [p_i]_{\text{ex}}} + \sum_{K=1}^{T} \tilde{\tilde{K}}_i^K ([p_i]_{\text{ex}})^{n_i^K} \quad \text{(C)}$$

where $[f]_L$, $\tilde{K}_i^L$, and $\tilde{\tilde{K}}_i^K$ refer to both intracellular and extracellular sites.

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