

Determination of Extracellular Space in Amphibian Muscle

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ABSTRACT The volumes of distribution of inulin and dextran in the sartorius, stomach, and cardiac muscle of the frog agree rather closely. That these spaces represent the volume of extracellular water is supported by the observation that efflux of sucrose can be divided into a fast and a slow phase and that the fast-moving fraction corresponds closely with inulin space determined in the same muscle. These and other findings confirm that sugars and related substances penetrate slowly into part of the fiber water and that, therefore, their volume of distribution does not accurately represent the volume of extracellular water. The kinetics of efflux of sucrose is consistent with the assumption that the movement of sugars is determined by the resistance of the cell surface as well as by internal diffusion. In connective tissue, sucrose and inulin are excluded only from a small part of the total water.

The volume of extracellular space is most frequently measured by determining inulin or sucrose space. However, in extensive studies on the sartorius and stomach muscle of the frog it was found that the volume of distribution of sugars and related substances is larger than that of inulin and decreases with increasing molecular weight according to the series: erythritol > arabinose > sucrose > inulin (2, 3). Similar results have been obtained for other muscles (10, 12, 1). In the perfused rabbit's heart sucrose space was found to be considerably larger than extracellular space determined histologically after freeze-drying (9). It was concluded on the basis of these and other findings that the substances mentioned, except inulin, penetrate into the fibers slowly and that their uptake by the fibers varies because it is limited by steric factors (3, 4). However, to explain variations in uptake it has also been proposed that some solutes are sterically excluded from a large part of the extracellular water (11, 7).

A firm basis for determining extracellular space would be established if the same values were obtained using different types of solutes. Therefore, volumes of distribution of several substances with high molecular weight were deter-

mined and compared with values obtained with a method based on the separation of efflux of sucrose into a fast and a slow phase.

METHODS

Uptake of solutes by the sartorius, stomach, and cardiac muscle of the frog *Rana pipiens* was determined. The sartorii used generally weighed less than 100 mg. Cardiac muscle was prepared by cutting base and apex from the ventricle with a razor blade and removing the spongy tissue from the interior so that a ring of muscle with a thickness of 0.5–1 mm was obtained. Tissues were blotted between two pieces of filter paper, the sartorius for 10 sec, stomach muscle for 2 min, and cardiac muscle for 1 min. Also fascia from the back of the frog was used. It had a dry weight of about 17% after blotting for 30 sec and an average thickness of about 0.1 mm.

In one series of experiments tissues were equilibrated, usually overnight, at 0°C, in Ringer's solution containing 1% inulin and one of several labeled macromolecular substances, dextran, polyvinylpyrrolidone (PVP), or serum albumin, then extracted for 3 hr with three changes of solution. The combined extract was made up to 5 ml, of which 2 ml were used for determining the radioactive substance and 2 ml, for the chemical determination of inulin (3). After extraction the muscles contained no inulin, but some dextran and ¹²⁵I. The former was determined by extracting the muscles further overnight at 6°C and counting the extracts separately. The remaining ¹²⁵I was measured by bringing the muscles into a well counter. After extraction for 3 hr about 2% of the total dextran L, 4% of dextran H, and 8% of PVP and serum albumin remained in the sartorius. Total ¹²⁵I activity, which was counted in some experiments before extraction using the well counter, agreed well with the values obtained by the method described.

In experiments in which inulin space and efflux of sucrose were determined in the same muscle, the tissues were equilibrated in Ringer's solution containing 1% inulin and 1 mM sucrose-¹⁴C for 1 or 3 hr, then transferred through a series of tubes containing 3 ml Ringer's solution. For the determination of inulin, 2-ml aliquots from all tubes were pooled. For measuring efflux, 1 ml from each tube was transferred to a counting vial. Sucrose was further extracted overnight in counting vials containing a solution prepared by mixing equal volumes of methanol and 0.025 HCl. After adding scintillation fluid, this extract was counted separately, the muscle being left inside.

Ringer's solution contained in millimoles: NaCl, 115; KCl, 2; CaCl₂, 1; MgSO₂, 0.6; and Na phosphate, 2 (pH 7). Inulin was obtained from Nutritional Biochemical Corp. (Cleveland, Ohio); sucrose-¹⁴C, inulin-carboxyl-¹⁴C (referred to here as radioactive inulin), and dextran-carboxyl-¹⁴C with a molecular weight in the range of 15,000–17,000 and 60,000–90,000 (referred to here as dextran L and H respectively) were obtained from New England Nuclear Corp. (Boston, Mass.); ¹²⁵I-labeled iodinated polyvinylpyrrolidone (PVP), from Nuclear-Chicago Corporation (Des Plaines, Ill.); and ¹²⁵I-labeled human serum albumin, from E. R. Squibb (New York, N.Y.). To remove impurities of low molecular weight, the preparations of sucrose-¹⁴C were washed once with ethanol, those of radioactive inulin and dextran twice with methanol. The tracers were added to Ringer's solution to give an activity of about 0.2 μc

per ml. ^{14}C was counted with a liquid scintillation counter, ^{125}I with a gamma spectrometer and well counter.

Experiments were carried out at 24–26°C. Results are given as averages and standard deviations. Volumes of distribution of muscle (spaces) will be expressed as percentages of wet weight after equilibration.

RESULTS

As shown in Table I, inulin and dextran L and H have nearly the same volume of distribution, decreasing only slightly with increasing molecular size, but serum albumin and PVP gave results which were about 10 and 20% lower respectively than those for inulin.

TABLE I
COMPARISON OF INULIN SPACE WITH THE SPACES
OF OTHER MACROMOLECULAR SOLUTES

Muscle	Inulin	Dextran L	Difference	Inulin	Dextran H	Difference
Sartorius	17 ± 2.4	17 ± 3.0	0.4 ± 0.4 (24)	23 ± 3.4	22 ± 3.0	0.7 ± 0.8 (16)
Stomach	21 ± 1.8	28 ± 3.5	0.4 ± 0.5 (24)	30 ± 2.6	29 ± 3.1	1.0 ± 0.7 (18)
Heart	28 ± 4.2	20 ± 2.1	0.8 ± 1.2 (8)			
Muscle	Inulin	PVP	Difference	Inulin	Albumin	Difference
Sartorius	18 ± 3.1	14 ± 2.8	4.4 ± 2.4 (12)	16 ± 2.6	14 ± 3.2	2.1 ± 0.8 (10)
Stomach						
Heart						

Spaces of inulin and one of the other solutes mentioned were determined in the same muscle. Spaces are averages based on wet weight. Differences are average differences between the two spaces determined for each muscle. In parentheses: number of muscles analyzed.

As reported previously, sucrose gives higher values than the macromolecular substances mentioned, but values which agreed closely with those for inulin were obtained by utilizing the fact that efflux of sucrose can be divided into a fast phase, lasting 10–20 min, and a very slow phase (3). Small muscles, weighing less than 40 mg, were used because the first phase was the briefer the smaller the muscle, while the size of the muscle had no influence on the slow phase, thus making the distinction between the phases particularly clear. By extrapolation total sucrose then could be separated into two fractions as shown in Fig. 1.

In 16 experiments with the sartorius the difference between the space corresponding to the fast sucrose fraction and inulin space of the same muscle was 0.62 ± 0.40 . The agreement was equally good whether the muscles were equilibrated for 1 or 3 hr, although the slowly moving fraction increased with the duration of equilibration. Results obtained for stomach and cardiac muscle were similar, but were less satisfactory, because the slow phase of efflux was faster than in the sartorius, making extrapolation less certain.

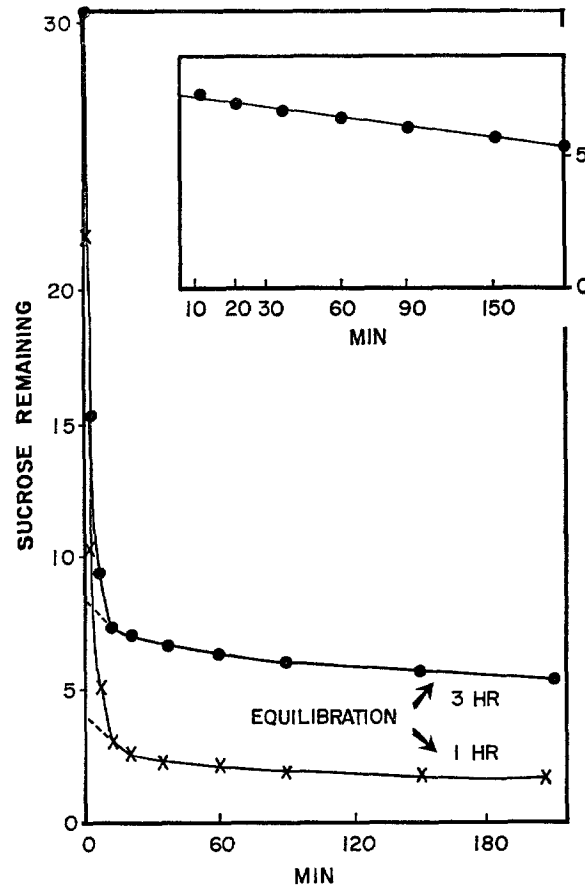


FIGURE 1. Efflux of sucrose from a pair of sartorii weighing 31.6 and 28.5 mg. They had been equilibrated for 1 and 3 hr respectively in Ringer's solution containing 1 mM sucrose- ^{14}C and 1% inulin. Ordinate: concentration of sucrose remaining expressed as spaces. The dotted line represents the extrapolation of the slow phase of efflux to time 0. This gave values for the slowly moving sucrose fraction of 3.5 and 8.0% respectively for the short and long period of equilibration. By subtracting these values from total sucrose spaces, 22.0 and 30.6%, the fast-moving fractions were obtained. They were 18.5 and 22.6%, while inulin spaces in the same muscles were 18.3 and 21.6%. Inset: data for one muscle plotted with abscissa representing square root of time.

The duration of the fast phase of efflux of sucrose in the small sartorii used was about 10 min. For comparison efflux of inulin was studied in separate experiments. Sartorii weighing 30, 50, and 80 mg, previously loaded with inulin, contained about 4, 6, and 10% respectively of the total uptake after washing for 30 min, as determined chemically or with radioactive inulin. On the basis of molecular weight it can be assumed that diffusion of sucrose is two to three times faster than that of inulin. Thus, the duration of the fast phase of efflux of sucrose in the muscle used agrees with the assumption that efflux is due to diffusion in inulin space.

Efflux of sucrose does not proceed exponentially at anytime, but in small muscles efflux curves are approximately straight lines after the first 10 or 20 min if the amount of sucrose remaining is plotted against the square root of time. This may have some theoretical significance because according to Harris (8) such a relationship is found if movement of the solute is determined both by internal diffusion and the resistance of a thin surface layer. If the straight line is extended according to the theoretical curves of Harris, an extrapolated value for the fast-moving fraction of sucrose is obtained which agrees with the estimate based on the procedure described above.

Connective tissue. To assess the role of the connective tissue of muscle also uptake of solutes by fascia from the back of the frog was studied. As seen from Table II, the solutes used penetrated into most of the tissue water, but spaces varied from 94 % for sucrose to 71 % for PVP. The differences probably are due to steric factors.

TABLE II
CONCENTRATION OF SOLUTES IN CONNECTIVE TISSUE

	Sucrose	Inulin	Albumin	PVP
Space, %.....	94±4 (14)	88±5 (14)	75±2 (8)	71±4 (8)

Tissues were equilibrated for 1 or several hr, extracted for 3 hr, and dried at 110°C for 1 hr. Values are average spaces based on tissue water. In parentheses: number of tissues analyzed.

DISCUSSION

The fact that the values for inulin and dextran L and H agree rather well, particularly also the close agreement between inulin space and the space corresponding to the rapidly moving sucrose fraction, support the assumption that inulin space is a good measure of the volume of extracellular water. That PVP and serum albumin give significantly lower values can be explained by assuming that these substances are excluded from a rather large part of the extracellular water. This assumption is supported by the finding that their volume of distribution in connective tissue is considerably lower than inulin space, that of PVP by 17 %. Assuming for the sartorius that the extracellular space of the muscle, which is roughly a fifth of the total volume, is quantitatively like the connective tissue used, the difference between inulin and PVP space would be expected to be about 3 %, while it was actually 4 %. Inulin space of connective tissue in turn is lower than sucrose space by about 6 %. Therefore, the volume of extracellular water may be larger than inulin space by about 1 %.

In the toad's sartorius, Tasker et al. (13) found values for inulin and serum albumin space similar to those reported here. Barr and Malvin (1), on the other hand, reported a large difference for the circular muscle of the dog's

intestine, an inulin space of about 40 % and serum albumin space of 18 %. Also in the frog's stomach immersed in arabinose solution inulin space is very high, while dextran space remains low (unpublished). Inulin space also is very high after prolonged washing in 2 mM CaCl_2 and recovery in Ringer's solution, while excitability and contractility are nearly normal (5). Thus it seems that slightly injured muscle fibers may be permeable to inulin but remain impermeable to larger molecules.

A completely different explanation of the results presented here is the assumption that sugars do not penetrate into the fibers and that their spaces closely represent the volume of extracellular water. According to this view, inulin space is smaller because it is sterically excluded from a large part of extracellular water (11, 7). That, contrary to this view, sugars penetrate into the fibers, although only into part of the fiber water, is supported by the following arguments.

1. According to Goodford and Leach (7) dextran space of the taenia coli of the guinea pig is about 25 %, sorbitol space 40 %. In the frog's stomach muscle inulin space is about 28 %, sucrose space 40 % (35–50 % depending on conditions), and erythritol space 60 % (3 and unpublished experiments with labeled erythritol). Thus, there seems to be a continuous spectrum of values ranging from that of dextran and inulin to that of glycerol, which penetrates into all the water (3). To assume that the volume of extracellular water is 40 % or more seems unacceptable on the basis of the histology of the muscles.
2. As shown above, sucrose and inulin are dissolved in most of the water of connective tissue. Sucrose space is slightly larger than inulin space, but the difference is far too small to account for the difference in the distribution of these substances in muscle.
3. The assumption that nonmetabolizable sugars penetrate into muscle fibers rests not only on their volume of distribution but also on other types of observations. Particularly significant is the fact that efflux has two distinct phases, and that the difference between sucrose and inulin space is due to a fraction which moves even more slowly than inulin and is equally slow in large and small muscles. Therefore, the slow phase of efflux of sugars certainly is not due to diffusion in extracellular space. Also influx has a slow phase, as shown particularly clearly by Barr and Malvin (1). The objection that the slow phase of influx and efflux is due to impurities of the tracer used or to metabolic breakdown of the sugar has been eliminated by experiments in which the substance washed out during the slow phase of efflux has been identified chemically and chromatographically (6). Thus the slow phase of efflux indicates that sucrose leaks slowly into the fibers.
4. The osmotic effects of nonelectrolytes differ and are inversely related to

their molecular size and their volume of distribution. For instance, less shrinking is produced by Ringer's solution with added erythritol than with added sucrose (4). The fact that stomach and cardiac muscle swell in isosmotic solutions of sugars and related substances is particularly remarkable. Osmotic equilibrium in these solutions could not be established without penetration of solute into the fibers, all the more because of a loss of electrolytes. At the same time, swelling is much smaller than that in water and, contrary to solutions of substances penetrating into all tissue water, such as glycerol, swelling is diminished by increasing the concentration of the solutes (2, 4). That connective tissue is not responsible for these volume changes is shown by the fact that isolated connective tissue, as mentioned above, does not swell in sugar solutions and that dextran space of muscle does not increase during swelling in such solutions (unpublished).

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