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Uptake of Sorbitol by Chick Embryo Heart Cells at Various Stages of Development

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Summary. Sorbitol enters chick embryo heart cells from five days of development on. The rate of sorbitol entry becomes slower as development proceeds and the data suggest this is principally due to an increase in the apparent K_m of transport, the V_{max} remaining relatively constant. The uptake of sorbitol displays saturation kinetics and is believed on this ground to be carrier-mediated. Sorbitol does not appear to be actively transported since it is not concentrated against a gradient and its uptake is not inhibited by iodoacetate or 2,4-dinitrophenol. Sorbitol does not appear to be taken up via the glucose transport system since uptake is not stimulated by insulin or inhibited by glucose or phloretin.

Guidotti and Foa (1961) found that sorbitol readily enters the cells of the hearts of 5-day chick embryos. This is noteworthy because most cell membranes are effectively impermeable to sorbitol. In fact, sorbitol has been frequently used as a marker for the extracellular space of the heart (Morgan, Randle & Regen, 1959). The aim of my work has been to further characterize the mode of sorbitol entry as simple diffusion or carrier-mediated transport and to determine the kinetic properties of the transport process.

Guidotti and Foa (1961) found that uptake of sorbitol by 7-day chick embryo hearts is significantly slower than by 5-day hearts and that by 10 days sorbitol distributes in a space not much larger than the extracellular space. They postulate that by 10 days of development the heart cell membranes have become impermeable to sorbitol. I also find that as embryonic development proceeds, sorbitol enters the intracellular space less and less rapidly, but in my experiments sorbitol enters the heart cells at all stages of embryonic life.

In agreement with the results of Guidotti and Foa, I find no effect of various metabolic inhibitors on sorbitol uptake and that insulin is also

without effect. Glucose at concentrations in excess of the K_m for glucose transport does not inhibit sorbitol uptake; neither does 0.2 mM phloretin.

Estimates of the kinetic parameters of sorbitol transport were obtained. The decrease in the rate of sorbitol transport with developmental age occurs chiefly by an increase in the K_m of transport, the V_{max} remaining relatively constant.

Materials and Methods

Sorbitol Transport Experiments

Embryo hearts were dissected out, allowed to beat for 2–3 min in Hanks (Hanks, 1948) solution containing 1 unit/ml heparin, then transferred to ice cold Hanks. Hearts from 5- 6- and 10-day embryos were used whole, those from 15- and 20-day embryos were cut lengthwise into halves and quarters, respectively. 12 to 15 hearts (or heart pieces) were placed in 2 ml of Hanks solution in a 25-ml Erlenmeyer flask and put on an incubator-shaker (37 °C) for a 5-min warm-up period. The incubator-shaker was continally flushed with 95% O_2, 5% CO_2. Then 50 µl of 0.1 M ^{14}C-sorbitol (50 µCi/ml) and a variable amount of unlabeled sorbitol were added and the experiment begun. At 5, 10, 20, 30, and sometimes 60 min three hearts or heart pieces were removed and small medium samples taken in triplicate. The tissue samples were blotted, rinsed with Hanks solution, blotted, weighed, put in a test tube with 1.5 ml water and boiled for 30 min. Medium samples were also put in 1.5 ml water and boiled for 30 min. 0.75 ml of tissue extracts and medium samples was mixed with 5 ml Triton-Toluene scintillation fluid and counted in a Beckman LS-230 liquid scintillation counter. The channels ratio method was used for quench corrections, but there were negligible differences in counting efficiency from one sample to the next. Tissue sample data were expressed as *CPM*/mg wet tissue and the three values for each time point averaged. The sorbitol space (V_{sbtl}) was then calculated by

$$V_{sbtl} = \frac{CPM/\text{mg wet tissue}}{CPM/\mu\text{l medium}}. \tag{1}$$

Kinetics of Sorbitol Transport

I have treated the kinetics of sorbitol uptake in terms of a simplified kinetic model. When ln V_{sbtl} is plotted versus time, curves of the type shown in Fig. 1 are obtained. Note that after 10 min the curves are linear in time. These simple kinetics are consistent with the idea that by 10 min the sorbitol concentration in the interstitial fluid (C_{isf}) has become constant, so that thereafter the entry of sorbitol into the cells shows first-order kinetics. Furthermore, since the rate at which sorbitol can leave the medium and enter the extracellular space is much larger than the rate at which it can leave the extracellular space to enter the cells, I made the assumption that from 10 min on the concentration of sorbitol in the interstitial space is equal to its concentration in the medium (C_m). Assuming first-order kinetics for sorbitol entry into the cells from 10 min on,

$$\frac{dC_{cell}}{dt} = \frac{k}{V_w^{cell}}(C_m - C_{cell}) = k'(C_m - C_{cell}) \tag{2}$$

where k is the apparent first-order rate constant for sorbitol entry into cells and V_w^{cell} is cell water (ml/g wet tissue). The solution to Eq. (2) is

$$C_{cell} = C_m(1 - e^{-k't}) \tag{3}$$

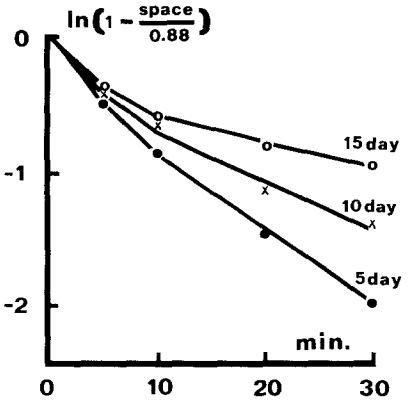

Fig. 1. A plot of the natural logarithm of (1 − space/0.88) versus time for the uptake of 16 mM sorbitol by chick embryo hearts at 5–6, 10, and 15 days of embryonic life. The slope of the linear portion of each curve is taken to be equal to the apparent first-order rate constant for entry of sorbitol into the cells

so that

$$\ln\left(1-\frac{C_{\text{cell}}}{C_m}\right)=-k't. \tag{4}$$

Assuming that

$$C_{\text{cell}}=\frac{Q_{\text{cell}}}{V_w^{\text{cell}}} \tag{5}$$

where Q_{cell} is the quantity of sorbitol in the cells and V_w^{cell} is the volume of water in the cells and realizing that Eq. (1) implies that

$$Q_{\text{cell}}=V_{\text{sbtl}}^{\text{cell}}\,C_m \tag{6}$$

we can transform Eq. (4) into

$$\ln\left(1-\frac{V_{\text{sbtl}}^{\text{cell}}}{V_w^{\text{cell}}}\right)=-k't. \tag{7}$$

If we express

$$V_{\text{sbtl}}^{\text{cell}}=V_{\text{sbtl}}-V_{\text{isf}} \tag{8}$$

and

$$V_w^{\text{cell}}=V_w-V_{\text{isf}} \tag{9}$$

where V_{isf} is the extracellular space (as measured from sucrose distribution) and V_w is the total tissue water, we obtain

$$\ln\left(1-\frac{V_{\text{sbtl}}}{V_w}\right)=\ln\left(1-\frac{V_{\text{isf}}}{V_w}\right)-k't. \tag{10}$$

Thus a plot of $\left(1-\frac{V_{\text{sbtl}}}{V_w}\right)$ versus t should have (for t greater than 10 min) a slope of $-k'$.

For each experimental time point we averaged the three values of V_{sbtl} obtained. The average values were then used to construct a graph of $\ln\left(1-\frac{V_{\text{sbtl}}}{V_w}\right)$ versus time. A straight line was drawn by means of linear regression methods through the points for $t=10$, 20, and 30 min and its slope taken to be equal to $-k'$.

Eq. (2) implies that the maximum (i.e. initial) velocity of entry occurs when $C_{cell}=0$. The theoretical initial velocity of sorbitol entry is thus calculated from

$$\left(\frac{dC_{cell}}{dt}\right)_i = k' C_m.$$

So then

$$v_i = V_w^{cells}\left(\frac{dQ_{cell}}{dt}\right)_i = k' V_w^{cells} C_m = k'(V_w - V_{isf}) C_m$$

where v_i is the initial rate at which sorbitol would enter the cells in a unit mass of tissue if we could load up the extracellular space before any sorbitol entered the cells and then allow the cellular uptake process to begin.

I constructed double-reciprocal plots by plotting $1/v_i$ versus $1/C_m$ and computed the values of the apparent K_m and V_{max} for sorbitol transport from the regression parameters of the line.

Chemical Form of Accumulated Radioactivity

Tissue extracts obtained by boiling tissue samples in 1.5 ml of water were taken to dryness and redissolved in a small volume of water. An aliquot of this solution was spotted on Whatman 3 mm paper or on an Eastman cellulose thin-layer plate and developed with butanol/pyridine/water (6:4:3). The paper or thin-layer chromatograms were dried and cut into strips and each strip put in a scintillation vial and counted in toluene-based scintillation fluid. After an appropriate background was subtracted from each count, the fraction of the total counts in each lane that co-migrated with an authentic sorbitol standard was calculated.

Determinations of Total Tissue Water

Tissue samples were taken at the beginning of a mock experiment and after 5, 10, 20, 30 and 60 min of incubation under the conditions of my experiments. After being rinsed with Hanks buffer and blotted, the tissue samples were placed in tared aluminum pans and weighed on a Mettler H54 balance. The pans were then placed in an oven at 100 °C and the heart tissue dried to constant weight, allowed to cool in a desiccator, and reweighed. From the wet and dry weights the amount of tissue water per unit mass wet tissue was computed. The average tissue water was 0.88 ml/g wet tissue. This value was not significantly altered by incubation under the conditions of our sugar uptake experiments.

Determination of the Extracellular Space

Initial experiments demonstrated that inulin and sucrose have the same volume of distribution within the tissue. Sucrose distributes in its space considerably faster than inulin. Sucrose was taken to be the marker for the extracellular space and experiments of the sort described for measuring sorbitol uptake were performed with ^{14}C-sucrose in the medium. Sucrose space at the various time points was calculated from an expression like Eq. (1). Chromatography of tissue extracts demonstrated that the radioactivity accumulated by the tissue was essentially all in the form of sucrose. The presence of unlabeled sorbitol at the concentrations used in the sorbitol uptake experiments did not have a significant effect on the sucrose space. It was found that a plot of ln $V_{sucrose}$ versus 1/time is linear. The straight line was extrapolated to the ordinate (time $=\infty$) and the antilog of the intercept taken to represent the ultimate sucrose space which is taken to be equal to the volume of the extracellular space (V_{isf}).

Critical Oxygen Tension of the Tissue Pieces

Tissue hypoxia is known to increase the rate of transport of glucose in heart (Morgan *et al.*, 1959) and skeletal muscle (Ozand, Narahara & Cori, 1962). It was thus important to be

sure that the oxygen tension in the medium was sufficiently high to provide adequate oxygenation for the tissue. Tissue samples were incubated in a fashion identical to that used in the sugar uptake experiments for about 15 min. Then both tissue and medium were rapidly transferred to a 2 ml O_2-electrode chamber (Gilson Medical Electronics, water-jacketed at 37 °C) and the P_{O_2} monitored by means of a Clark O_2 electrode (Instrumentation Laboratories). The electrode current was recorded on a recording nanoammeter (Heath). The P_{O_2} fell in a linear fashion with time for a variable period, indicating a constant rate of O_2 consumption. Eventually the slope of the curve began to diminish. The P_{O_2} at which this occurred is called the critical P_{O_2} and was taken to indicate the lowest oxygen tension in the medium that will support the maximum rate of oxygen consumption. With hearts from embryos of all ages, incubation conditions were able to maintain an oxygen tension in the medium at least 50% above the critical P_{O_2}.

Results and Discussion

The Extracellular Space

A typical plot of ln $V_{sucrose}$ versus 1/time is shown in Fig. 2. Such plots always showed a high degree of linearity. The line was extrapolated to the ordinate and the antilog of the y-intercept taken to represent the ultimate sucrose space. The rate at which sucrose distributes is almost independent of the age of the hearts used, the approximate half-time for this process being 5 min.

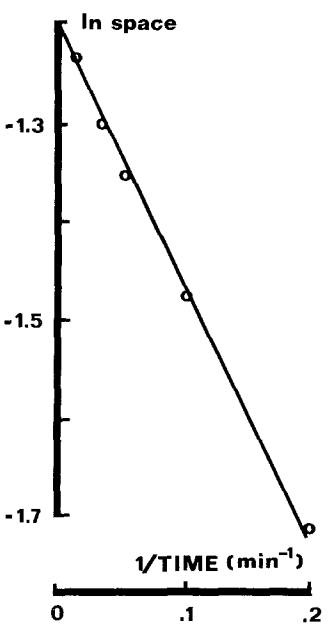

Fig. 2. A plot of the natural logarithm of the sucrose space (ml/gm wet tissue) versus the reciprocal of time for experiments in which the uptake of 8 mM sucrose by 15-day hearts was measured. Each point represents the mean of three determinations in a typical experiment

Table 1. Sucrose space of chick embryo hearts as a function of developmental age[a]

Age (days)	Sucrose space ±SD at 60 min (ml/gm wet)	True sucrose space ±SD (ml/gm wet)
5–6	0.390±0.041	0.374±0.023
10	0.318±0.036	0.330±0.010
15	0.298±0.029	0.305±0.005
20	0.361±0.035	0.359±0.011

[a] True sucrose space was determined by the extrapolation procedure described in the text. At least 15 determinations were made for each age.

Table 1 shows values for the ultimate sucrose space in hearts from embryos of various ages. I will assume this to be equal to the extracellular fluid volume (V_{isf}). These values are close to inulin spaces of chicken embryo heart by Guidotti, Loretti, Gaja and Foa (1966) and for the sorbitol space of rat heart by Morgan *et al.* (1959). Kipnis and Cori (1957) found that in the rat diaphragm prepared by cutting the muscle from its insertions, sucrose distributed in a larger space and with a longer time course than in the "intact" rat diaphragm in which none of the muscle fibers are cut. The fact that the sucrose spaces in 15- and 20-day heart pieces are similar to those in intact 5- 6- and 10-day whole hearts suggests that damage done by slicing the 15- and 20-day hearts lengthwise is not extensive.

Lack of Sorbitol Metabolism

Data from an experiment in which tissue extracts were analyzed by paper chromatography are shown in Table 2. Even after 60 min of incubation almost all the radioactivity in the tissue co-chromatographs with an unlabeled sorbitol standard. This is consistent with the findings of

Table 2. Identity of radioactive material taken up by chick embryo heart[a]

Time (min)	% of total counts that co-migrated with a sorbitol standard
10	99.5
20	96.5
30	96.8
60	96.5

[a] Results of a typical experiment on uptake of 16 mM sorbitol by 10-day hearts. Tissue extracts were concentrated and chromatographed on Whatman 3 mm paper with butanol/pyridine/water (6/4/3, descending).

Guidotti and Foa (1961) that sorbitol is not metabolized by chick embryo heart and that the levels of sorbitol dehydrogenase are extremely low. These results also suggest I am observing the uptake of sorbitol rather than of a radioactive contaminant.

Sorbitol Transport

As shown in Fig. 3 the rate at which embryonic heart cells take up sorbitol decreases from 5 to 15 days of embryonic life. Results from 15-day and 20-day hearts are similar. The data suggest that sorbitol distributes in a smaller fraction of the total tissue water as development proceeds, but at all ages it distributes in a volume that exceeds the extracellular space.

Fig. 4*A* shows the effect of the metabolic poisons 2,4-dinitrophenol and iodoacetate on sorbitol transport. Glucose is the only exogenous substrate available to the cells, so the lack of an effect by iodoacetate (a powerful inhibitor of glycolysis) argues against metabolic energy being required for sorbitol transport. Iodoacetate at 0.1 mM produced a 40% reduction in the rate of glucose uptake by 20-day hearts.

2,4-dinitrophenol, which is an agent that uncouples oxidative phosphorylation from electron transport, is also without effect on sorbitol transport. Dinitrophenol is known to stimulate certain glucose facilitated transport systems including the one in chick embryo heart (Guidotti & Foa, 1961). In our hands, 0.25 mM DNP causes a transient (20 min) stimulation of glucose uptake (average about 30% stimulation). The lack of an effect of dinitrophenol is consistent with the idea that sorbitol transport does not occur via the glucose carrier.

Glucose does not compete with sorbitol for entry into the heart cells. Fig. 4*B* shows that the uptake of sorbitol is not affected by a fourfold

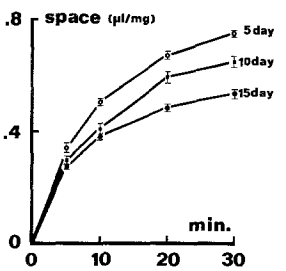

Fig. 3. Uptake of 16 mM sorbitol by chick embryo hearts of various stages of development. The numbers of experiments done were 7 with 5–6-day hearts, 8 with 10-day hearts, and 12 with 15-day hearts. The bars represent standard errors of the mean. (Standard errors were usually about 5% of the mean value; the largest standard error in all the experiments was about 10%.)

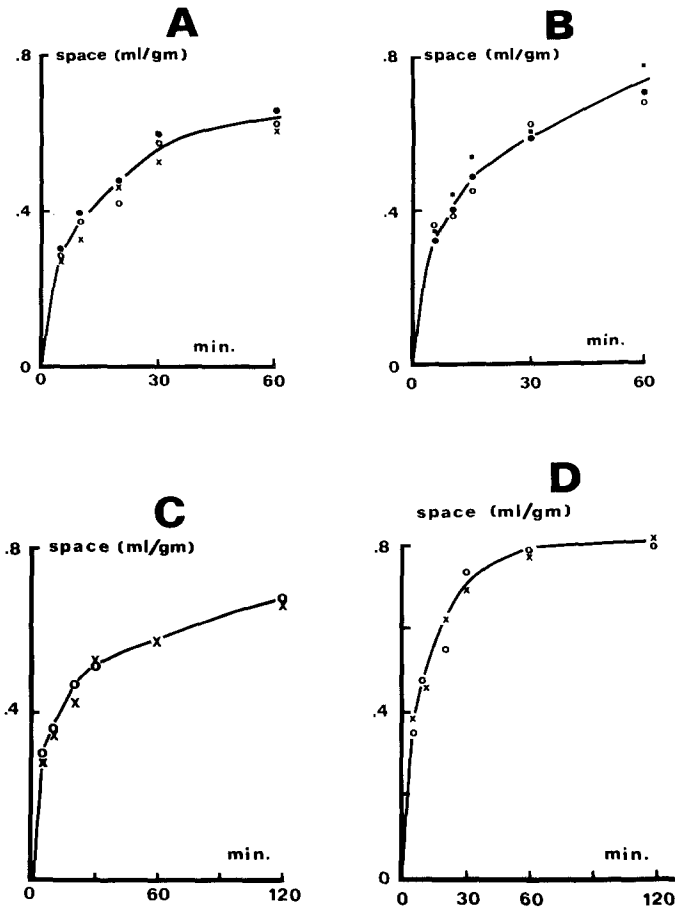

Fig. 4. (*A*) Effect of metabolic inhibitors on the uptake of 8 mM sorbitol by hearts from 15-day embryos. Each point represents the mean of three heart pieces in one experiment. ● = no inhibitor present, ○ = 0.1 mM iodoacetate, and x = 0.025 mM 2,4-dinitrophenol. Hearts were preincubated for 30 min in the presence of inhibitors before sorbitol was added. (*B*) Effect of glucose on uptake of 8 mM sorbitol by 20-day embryo heart. Each point represents the mean of three heart pieces in one experiment. ● = 8 mM glucose, ■ = 16 mM glucose, and ○ = 32 mM glucose. 10-min preincubation before addition of sorbitol. (*C*) Effect of 0.2 mM phloretin on the uptake of 8 mM sorbitol by hearts from 15-day chick embryos. Each point represents the mean of three heart pieces in one experiment. ○ = control and x = phloretin. 20-min preincubation before addition of sorbitol. (*D*) Effect of insulin on uptake of 8 mM sorbitol by hearts from 20-day chick embryos. Each point represents the mean of three heart pieces in a typical experiment. ○ = insulin-free and x = 1 unit of insulin/ml. 30-min preincubation with insulin

increase in the concentration of glucose in the medium. This suggests that sorbitol uptake does not occur via the glucose transport system. As shown in Fig. 4 *C* the glucose transport inhibitor phloretin (0.2 mM) is without effect on sorbitol uptake.

Guidotti, Kanameishi and Foa (1961) and Guidotti and Foa (1961) showed that beginning about 7 days of embryonic life glucose transport is increased by insulin. We find that insulin stimulates 2-deoxyglucose uptake by 6- and 15-day chick embryo hearts at levels from 0.0005 to 0.5 units/ml *(unpublished results)*. I find that sorbitol transport does not respond to insulin at any stage of development (Fig. 4*D*) even when doses of insulin as high as 1 unit/ml (far in excess of physiological levels) are used. This finding also suggests that sorbitol uptake occurs by some other route than the glucose transport system.

Kinetics of Sorbitol Transport

Sorbitol uptake experiments were performed with sorbitol concentrations in the medium of 4, 8, 16, 32 and 64 mM. The data were analyzed as described in Materials and Methods. Fig. 5 shows a double-reciprocal plot of the data. The slopes and intercepts of the lines were calculated by linear regression and the K_m and V_{max} for the cellular phase of sorbitol uptake computed as described above. The kinetics of transport in 15-day hearts were not significantly different from the results for 20-day hearts. It is noteworthy that the decrease in sorbitol transport with development is principally the result of a progressive increase in the K_m of transport, the V_{max} being relatively constant (Table 3).

Conclusions

I have shown that sorbitol enters chick embryo heart cells at all stages of development. The lack of any effect by glucose, insulin, or phloretin suggests that sorbitol is not taken up via the glucose transport system.

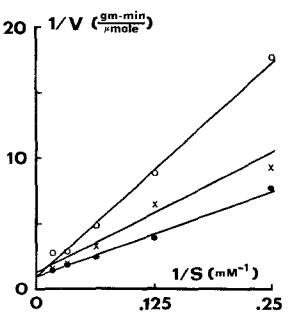

Fig. 5. Plot of the reciprocal of the theoretical sorbitol uptake rate versus the reciprocal of the medium sorbitol concentration for ● = 5–6-day hearts, x = 10-day hearts, and ○ = 20-day hearts

Table 3. The apparent values of kinetic constants of sorbitol uptake by chick embryo hearts as estimated from linear regression analysis of the data shown in Fig. 5[a]

Age (days)	$K_m \pm$SD (mM)	*t*-test	$V_{max} \pm$SD [μmoles/(gm min)]	*t*-test
5–6	31.6±5.9	S	1.16±0.21	NS
10	47.0±6.9	S	1.29±0.17	NS
20	64.8±8.3		0.98±0.12	

[a] The Student's *t*-test was used to test the significance of differences between means: S denotes significance at the 5% level; NS denotes lack of significance at the 5% level of the difference between successive means.

The lack of effect of dinitrophenol and iodoacetate and the fact that I have never observed accumulation of sorbitol above the level in the medium argue against this being an active transport mechanism.

It seems unlikely that sorbitol enters the heart cells by simple diffusion. I know of no plasma membrane that has been shown to have a high simple permeability to 6-carbon sugars. The only evidence we have to support the idea that sorbitol uptake is carrier-mediated is the fact that transport displays saturation kinetics with K_m values from 30 to 65 mM depending on the stage of development. It should be pointed out that definitive evidence of carrier-mediated transport should include evidence of competition by other substrates for the carrier and preferably counter-flow induced by a competing substrate.

A number of interesting questions remain to be resolved. It is puzzling that the embryonic heart should have a system for transporting sorbitol, which it cannot utilize. In this regard it will be interesting to see what other substances can enter the cell via the sorbitol carrier. It is not known whether sorbitol permeates the heart cells of adult chickens. If adult heart cells are impermeable to sorbitol it will be important to characterize the process by which the sorbitol transport system is lost.

Crofford, Jeanrenaud and Renold (1965) found that sorbitol permeates the cells of rat adipose tissue. In adipocytes, however, it seems likely that sorbitol transport occurs by the glucose carrier since uptake is inhibited by phloretin and 3-0-methyl glucose and stimulated by insulin. Systems for uptake of sorbitol by a phosphoenolpyruvate-dependent phosphotransferase system occur in bacteria (Roseman, 1969). It appears that sorbitol transport in embryonic chick heart, since it does not occur via the glucose carrier and sorbitol is not phosphorylated, is in some respects unique.

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Note Added in Proof: Experiments we have done with George Zavoico show that Langendorff-perfused adult chicken hearts admit sorbitol to the intracellular space under conditions that sucrose is confined to the extracellular space. Sorbitol distributes in approximately 90 % of the total tissue water in 30 min of perfusion.

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