STUDIES OF ION PERMEABILITY. III. DIFFUSION OF Br^- ION IN THE EXTRACELLULAR SPACE OF FROG MUSCLES

GILBERT N. LING

Department of Molecular Biology, Division of Neurology, Pennsylvania Hospital, Philadelphia, Pennsylvania 19107

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SUMMARY

Using the rapid flow U-tube well-counter assembly, we studied the Br^--ion efflux from frog sartorius muscle and thin connective tissues isolated from an area adjacent to the muscle in the same frog. It was shown that a very slow fraction from the muscle originates completely or nearly so from its connective tissue contents. Comparing the zero time intercepts of the connective tissue with those of the same type of connective tissues within the sartorius muscle, we obtained a value for the connective tissue content of the sartorius muscle of 8.7% which agrees with an earlier reported value (9.09%) based on an entirely different method. By 'subtracting the slow fraction due to the connective tissue, the remaining efflux curve of the sartorius muscle was shown to be further resolvable into 2 fractions. The slower one is due to Br^- from the cell; the 'faster from the extracellular space. The data permits another new method for estimating the extracellular space; the value of 8.2 ± 0.13%, still considered a ceiling value, agrees well with results from 3 other different methods recently introduced. The time constant of Br^--ion efflux from the muscle cells is 0.13 ± .012 min^{-1}. The rate of Br^--ion diffusion in the extracellular space indicates that in this space there is no unusual diffusion barrier and that the time course of Br^--ion diffusion is predictable from the thickness of the muscle, a tortuosity factor, and the normal diffusion coefficient of the ion in an 0.1 N NaCl solution.

In a preceding paper, it was shown that the Na^+-ion efflux from single frog muscle fibers (and multiple muscle fiber bundles) consists of two fractions: a fast fraction with an average half time of exchange ($t_{½}$) equal to 3.7 minutes and a slower fraction with $t_{½}$ equal to about 25 minutes.' It was concluded that the slow fraction, which has conventionally been regarded as representing the rate of intracellular-extracellular exchange, is actually rate-limited by desorption from sites located primarily in the cells. It is the fast fraction that is determined by the intracellular-extracellular exchange.

In order to study the fast fraction more closely, it would be highly convenient if, instead of relying solely on time-consuming single-muscle-fiber techniques, one could use whole sartorius muscles. However, an intact muscle, unlike a single muscle fiber, contains an extracellular space among the fibers. Diffusion of Na^+ ion in this space has not thus far been clearly separable from the fast exchanging fraction mentioned above. Indeed, these two fractions have conventionally been lumped together as the extracellular space (refs. 2-5; see also, ref. 6, chap. 11).

This paper will present an investigation of the Br^--ion efflux from intact frog sartorius muscles. With this ion it was possible to distinguish between the efflux from the cells and the efflux from the extracellular space. This separation in turn permitted an estimation of the effective diffusion coefficient of Br^- ion in the extracellular space. By comparing this diffusion coefficient with the diffusion coefficient of Br^- ion in a free aqueous solution, we can then determine what corrective factors, if any, need be introduced in order to describe diffusion in the extracellular space from the well-known theory of diffusion.

MATERIALS AND METHODS

Br-82 was obtained from Nuclear Science and Engineering Corp. of Pittsburgh, Pa., in the form of NaBr. The composition of the normal Ringer-phosphate solution used was: NaCl, 104.7 mM; KCl, 2.5 mM; $CaCl_2$, 1.0 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 6.6 mM; NaH_2PO_4, 2.0 mM; and Na_2HPO_4, 1.2 mM. In Br-Ringer solution, all NaCl was replaced by an equimolar amount of NaBr.

Sartorius muscles and loose connective tissue sheets were isolated from the legs of North American leopard frogs (Rana *pipiens pipiens*, Schreber) from Wisconsin and Vermont.' Both tissues from the same animal were incubated sterilely with shaking in Br-Ringer-GIB media[8] for various lengths of time in a constant-temperature room maintained at 25° ± 1°.

The U-tube y-well-counter technique for the study of ion efflux described earlier was used.[1,6] There was no discernible difference in the counting efficiency of Br-82 whether the isotope was in a muscle within a U-tube or uniformly dispersed in 2 ml of 0.1 M HCl in a lustered counting tube.

RESULTS

Efflux Curves of Muscle and Connective Tissues

Figure 1 shows the Br^--ion efflux curves from a sartorius muscle and from loose connective tissue dissected from the same frog and treated in a manner as similar as possible. The contours of the efflux curves bear a general resemblance to each other. Both consist

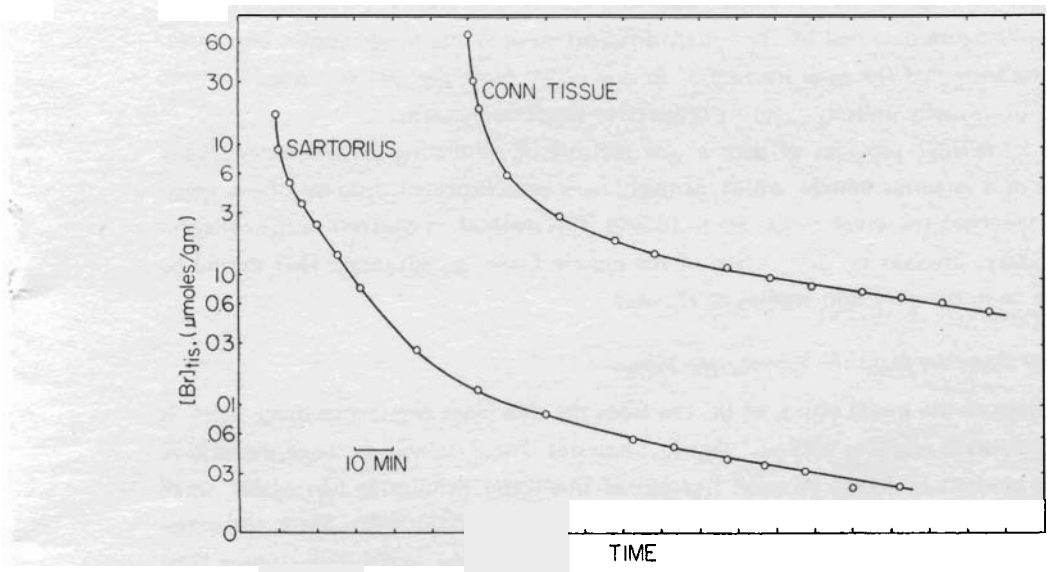

Figure 1. Time courses of Br^--ion efflux from sartorius muscle and from connective tissue sheets from the same animal. Experiment I of August 22, 1969. For additional details, see Tables 1 and 2.

of an initial rapidly exchanged fraction, a final slowly exchanged fraction, and a middle fraction exchanging at an intermediate rate.

Frog sartorius muscle contains considerable amounts of loose connective tissues, including small blood vessels, nerves, etc. Thin connective tissue sheets which run continuously into the fascia covering the outer surface of the sartorius muscle are composed of the same ingredients. By analyzing and comparing the collagen and elastin contents of the sartorius muscles with those of the loose connective tissue sheets, we found that, in terms of fresh weight ratio, there is an equivalent amount of connective tissue in the sartorius muscles equal to 9.09% (ref. 6, p. 210).

A survey of the Br^--ion efflux curves obtained revealed that by and large the slower fraction in one gram of sartorius muscle was roughly 10% of that in one gram of connective tissue. This suggests that virtually all of the slowly exchanging fraction of Br ion in the muscle might originate from the connective tissue in the muscle.

To test this assumption, we obtained the total amount of this slowly exchanging Br-ion in both tissues by extrapolating the slowest fraction in each tissue to zero time. A correlation coefficient of +0.94 was found from 13 pairs of the initial values of the slow fraction of the sartorius muscles and those of the connective tissues.

By dividing the amount of slowly exchanging Br^- ion from the muscle by the amount

from the connective tissue, we obtained the average (wet) connective tissue equivalent in each gram of fresh sartorius muscles equal to 0.087±0.0082 (S.E.). This value is close to the 0.0909 figure obtained by the totally different method mentioned above. This agreement confirms that the slow fraction of Br^--ion efflux from the sartorius muscle comes entirely or virtually entirely from its connective tissue component.

This knowledge provides us with a new method of estimating the connective tissue content of a sartorius muscle, which, as might have been expected, does vary from specimen to specimen (observed range: 5% to 13.8%). This method, in contrast to the collagen-elastin assay, involves no destruction of the muscle tissue—an advantage that should be valuable in future precision studies of effluxes.

The Fast Fraction from the Connective Tissue

The rate of the initial efflux of Br^- ion from the thin loose connective tissue sheets is very rapid, often reaching 90% exchange in 2 minutes. These connective tissue sheets have a water content of 85%.[7] A small fraction of this water belongs to fibroblasts, small blood vessel cells, and other cellular elements; the bulk is extracellular. Thus, the initial rapidly exchanged Br^- ion must be largely the Br^- ion in the extracellular phase. The initial readings usually provide enough data to predict, on the basis of the laws of diffusion, a rough estimate of the entire time course of Br^--ion diffusion from the extracellular space (see fraction labeled E_x in Fig. 2).

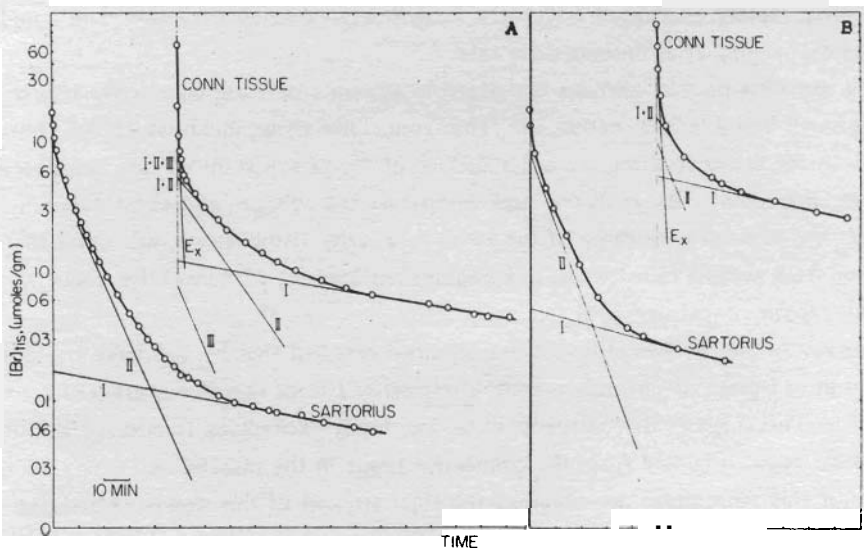

Figure 2. Analysis of the time course of Br^--ion efflux from sartorius muscles and from connective tissues from the same animals. In the set to the right referred to as A, the connective-tissue-efflux curve can be resolved into 3 fractions: I, II, and E_x. In the lefthand set, Set B, the curve is resolved into 4 fractions: I, II, III and E_x. (A), Expt. I, April 23, 1969; (B), Expt. III, April 26, 1969.

By "peeling oft" the slow fraction (Fraction I, discussed in the last section) from the connective-tissue-efflux curve, we obtained a second fraction with a $t_{1/2}$ of about 10 min. This fraction (Fraction 11) in addition to Fraction I and the fast initial fraction (Fraction E_x), accounted, in some cases, for the entire connective tissue Br^- efflux curve (Fig. 2B). In other cases, a small fourth fraction had to be included (Fig. **2A).** The collected data are given in Tables 1 and 2.

Table 1. Experimental Data on the Br^--Ion Efflux of Frog Connective Tissues at 25°C.

Date	Frog No.	Incub. Time (hrs)	Weight (mg)	$[Br]^{c.t}_{tis}$ (%).	$[Br]^{c.t}_{I}$ (%)
4/19/69	I	40	18.8	57.2	2.2
4/23/69	I	26	13.0	55.7	1.2
4/26/69	I	96	19.0	64.0	2.0
	III	96	18.4	80.5	5.3
4/29/69	I	3.2	26.0	63.0	1.2
	II	3.5	22.0	49.0	1.0
8/18/69	II	17.5	12.5	47.0	0.8
	III	17.5	12.0	46.0	1.4
	IV	17.5	7.8	53.0	1.3
8/22/69	I	72	27.0	73.0	2.4
	II	72	8.0	68.0	1.6
	III	72	25.0	7.0	1.9
	IV	72	9.0	50.0	1.8
AVERAGE±S.E.				59.9±3.02	1.85±0.31

$[Br]^{c.t.}_{tis}$ is the total Br^- ion concentration in the connective tissue; $[Br]^{c.t.}_{I}$ is the slowly exchanging Br^- ion concentration. In this and the following table, the Br^- ion contents are expressed as percentages of the B r ion concentration in the external medium (104.7 mM).

Non-connective Tissue Br^--Ion Efflux from the Sartorius Muscle

Our next task is to use the data thus obtained to make a suitable correction for the contribution of the Br^- ion from the connective tissues in the muscle. As mentioned above, the magnitude of Fraction I already provides us with a measure of the percentage of connective tissue in the muscle. We shall call this percentage σ. The formula used to obtain the concentration of non-connective tissue, labeled Br^- ion in μmoles per gram of whole fresh muscle, t minutes after washing began is:

$$[Br]^{t}_{n.c.t.} = [Br]^{t}_{m.t.} - \sigma\left\{[Br]^{t}_{c.t.(I)} + [Br]^{t}_{c.t.(II)} + [Br]^{t}_{c.t.(III)}\right\}, \quad (1)$$

where the symbols from left to right refer, respectively, to the concentration of non-connective tissue Br^- ion, the total muscle tissue Br^- ion, and the Br^- ion belonging to the three fractions of the connective tissues t minutes after washing began.

Table 2. Experimental Data on the Br^--Ion Efflux of Frog Sartorius Muscles.

Date	Frog No.	Incub. Time (hrs)	Weight (mg)	$[Br]^{e}_{tis}$ (%)	$[Br]^{e}_{I}$ (%)	$[Br]^{s}_{I} / [Br]^{e}_{c.t.}$	$[Br]_{in}$ (%)	$t_{½}$ min.	k $(min)^{-1}$	$[Br]^{e}_{e.c.s}$ (%)	$t_{0.1}$ Theor.	Obs. (min)
4/19/69	I	40	85	14.5	0.22	0.10	5.2	5.0	0.14	8.5	0.7-1.6	2.0
4/23/69	I	26	107	16.9	0.165	0.14	9.4	4.8	0.14	8.1	1.0-2.2	1.8
4/26/69	I	96	112	16.9	0.15	0.082	6.2	7.4	0.094	8.2	1.3-2.9	2.0
	III	96	113	17.3	0.5	0.077	10.8	3.1	0.22	7.6	1.3-2.9	1.9
4/29/69	I	3.2	101	15.9	0.09	0.079	6.5	7.0	0.10	8.0	1.0-2.2	2.0
	II	3.5	72	19.7	0.05	0.11	7.0	5.5	0.13	8.5	0.6-1.4	2.0
8/18/69	II	17.5	87	15.3	0.11	0.079	5.5	6.8	0.10	8.5	0.8-1.9	2.0
	III	17.5	80	18.2	0.14	0.044	5.0	4.0	0.17	9.0	0.7-1.6	1.5
	IV	17.5	115	18.0	0.10	0.075	5.2	7.3	0.095	8.5	1.3-2.9	1.5
8/22/69	I	72	136	17.2	0.19	0.050	6.8	8.2	0.11	8.9	1.6-3.7	1.0
	II	72	156	17.1	0.18	0.14	5.5	8.8	0.078	8.3	2.0-4.6	2.0
	III	72	162	13.0	0.15	0.075	4.0	5.3	0.13	7.2	2.4-5.5	2.0
	IV	72	119	12.3	0.08	0.095	4.6	3.2	0.22	7.8	1.3-2.9	1.5
Average± S.E.				16.3± 0.58	0.159± 0.03		6.28± 0.53	5.8± 0.47	0.13± 0.013	8.2± 0.13	(1.2-2.8)	1.8± 0.1

Muscle thicknesses (d) in cm were either directly measured or obtained from the muscle weight in grams (w), using an empirical relation: $d = 0.58w + 0.016$. $[Br]^{s}_{I}$ is the slowly exchanging fraction of Br^- ion concentration in the muscle cells, but expressed as a percentage of external Br^- ion concentration. To convert to millimolar concentrations per unit weight of fresh cells, the data have to be multiplied by $104.7 \times \frac{1}{(1-[Br]_{e.c.s.}-0.15\,[Br]^{c.t.}}$, where 0.15 is the percentage of dry weight in connective tissues.[1] $t_{½}$ is the half time of cell Br^--ion exchange and k is the rate constant of Br^--ion efflux.[20] $[Br]^{s}_{e.c.s.}$ is the percentage of extracellular space in fresh muscle tissue. $t_{0.1}$ is the time "for 90% exchange" of the exponential part of the Br^--ion efflux from the extracellular space of the muscle. "Theor." and "Obs." refer to theoretically calculated and observed values.

Figure 2 shows the results of this type of analysis. The corrected non-connective tissue curves can, in turn, be resolved into an exponential fraction with a $t_{1/2}$ of approximately 6 minutes and a much faster initial fraction. This is illustrated more clearly in Figure 3, which represents a magnified version of more complete data for the first 10 minutes of efflux in the three experiments shown in Figures 1 and 2.

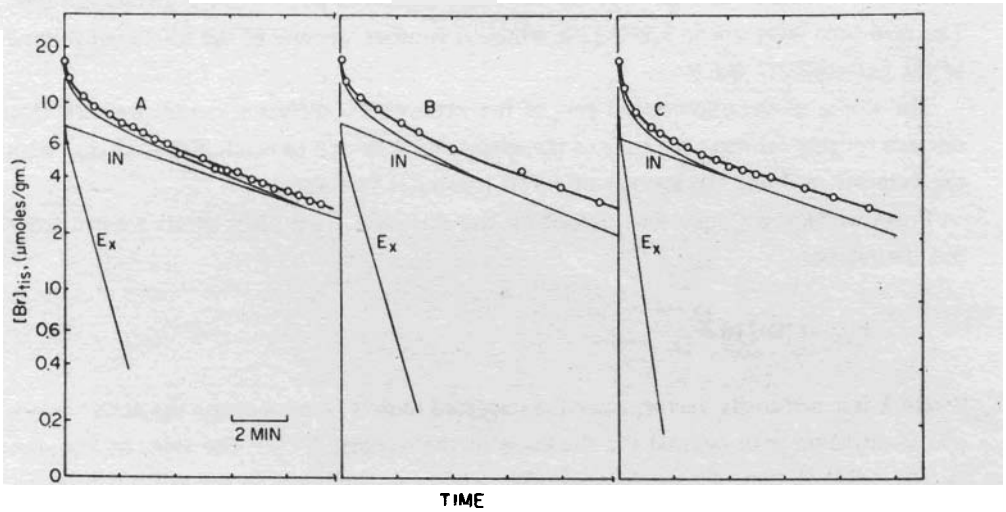

Figure 3. Analysis of the initial Br^- ion from the sartorius muscles. (A) Same experiment as in Figure 1. (B) and (C) are, respectively, the experiments marked (A) and (B) in Figure 2.

The Assignment of the Fractions from Muscle Cells

Since we have already shown the slowest fraction of the Br^--ion efflux trom the sartorius muscle to be due to connective tissues, the next question is: Is the remaining curve after the correction for the contribution of the connective tissue due to extracellular Br-ion? If the answer is affirmative, it would mean that no Br^- ion can enter the muscle cells. Yet Conway and Moore[9] long ago demonstrated that Br^- and the other halide ions do enter and leave these cells. Similar Br^--ion permeability of erythrocytes has also long been known.[10]

There is, then, no choice but to attribute the slower fraction to an intracellular origin and the faster fraction to an extracellular origin.

By extrapolating the slow fraction to zero time, we obtain an intracellular Br^--ion concentration of 6.28±0.53 as a fraction of the external Br^--ion concentration (104.7 mM); or, in absolute concentration, 6.5 μmoles per gram of whole; sartorius muscle. The average $t_{1/2}$ is 5.8±0.47 minutes; the Br^- ion-permeation-rate constant k, is (see Table 2):

$$\frac{1n2}{t_{½}} = \frac{0.693}{5.8} = 0.119\ min^{-1}$$

The Size of the Extracellular Space and the Rate of Br^--Ion Diffusion

The fast fraction, as expected, has the shape of a bulk-phase limited diffusion curve. The zero-time intercept is 7.9±0.32%, which is another measure of the fractional volume of the extracellular space.

The slopes of the exponential part of the extracellular diffusion curves are such that one can roughly estimate the time of the extracellular Br^- ion to reach 90% exchange with the external medium. An average of 1.7±0.1 minutes was obtained.

From well-known equations describing the diffusion from plain sheets we can write out the relation:[11]

$$t_{0.1} = 0.236 \frac{(\lambda d)^2}{D},$$

where λ is a tortuosity factor, since the staggered muscle fibers increase the actual length of the diffusion path beyond the thickness of the muscle, d.[3,7,11] The value of λ cannot exceed 1.57. In fact, it must be lower. We could not find data on the self-diffusion coefficient of Br^- ion, but a reasonably accurate estimate can be made in the following manner.

It is well known that the mobilities of Cl^-, Br^-, and I^- ion in water are approximately equal (65.2; 67.4; and 66.1). The self-diffusion coefficient of Cl^- ion in a 0.1 N salt solution at 25° is 1.95 x 10^{-5} cm^2/sec, and that of I^- ion is virtually the same, 1.94 x 10^{-5} cm^2/sec.[12] Thus, a value of 1.94 x 10^{-5} cm^2/sec for Br^- ion can be assumed with the necessary accuracy.

In Table 1 we have compared the experimentally estimated $t_{0.1}$ with two values calculated from Equation 1 with λ = 1.5 and 1.0, respectively. The average of the estimated $t_{0.1}$ values for λ equal to 1.0 is 1.2 minutes and that for λ equal to 1.5 is 2.8 minutes. The observed average for all 13 sets of experiments is 1.7±0.1 min, which falls between the two limiting theoretically calculated values.

DISCUSSION

Table 2 shows the variety of quantitative data on the nature of the frog muscle provided by the study of Br^--ion efflux using the U-tube y-counter assembly. The data also provide answers to some important questions that have been raised in the past.

(1) ***The Sarcoplasmic*** Reticulum

It has been suggested that the fast fraction of Na^+-ion efflux from single muscle fibers (ref. 1 and 6, chap. 11) originates from Na^+ ion diffusing from the tortuous sarcoplasmic reticulum.[12,13]

However, cytological evidence does not support the idea that the reticulum is directly open to the external media;[14] the distribution revealed by extracellular space probes like ferritin also shows clearly that only the T-tubules, comprising 0.2 to 0.4% of the total cell volume, are open to the outside.[15–17]

In the present study of Br^--ion efflux, we have shown that after correction for the contribution of the connective tissue only two fractions remain: one exponential fraction belonging to the cells and the other belonging to the extracellular space. If the fast Na^+-ion efflux is truly due to Na^+ ion in the sarcoplasmic reticulum space, the same space should have produced a similar third fraction for Br^--ion efflux. Since this is not observed, and was not observed in muscle briefly exposed to 0.1 N $K^{42}Cl$,[18] one cannot attribute the fast fraction of Na^+-ion efflux to this cause.

Nor does the intracellular freezing pattern lend support to the speculation that the sarcoplasmic reticulum represents tubules filled with a normal aqueous solution. If it did, ice crystals should grow both longitudinally and in other directions. In fact, they grow only lengthwise.[19]

(2) The Size of the Extracellular Space

Using poly-L-glutamate, we obtained a ceiling value of 8.9% for the extracellular space of frog muscles including the sartorius muscle.[7] The analysis of the sucrose and mannitol uptake of single frog muscle fibers offered a similar ceiling value.[7]

The present study offers still another estimate of the ceiling value of the extracellular space (8.2%). This estimate is based on two assumptions, as follows:

1. The Br^--ion efflux is surface-limited. If the Br^--ion efflux is bulk-phase limited, then the cell Br^- ion would be raised to 8.6% and the extracellular space reduced to 5.8%. At this moment there is not clear evidence favoring either alternative.

2. There is little or no rapidly exchanging Br^- ion adsorbed on the connective tissue or other exposed components of the muscle. For this reason, it will be safer to consider even the figure of 8.2% as a ceiling value.

It should be emphasized that the size of the extracellular space obviously depends on the blotting procedure used. The conclusion we reached was based on the use of the specific blotting procedure described earlier:[7] the muscle was placed between four sheets of wet Whatman No. 1 filter paper and the forefinger was then run gently but firmly over the muscle four different times.[7]

(3) The Rate of Br^--Ion Diffusion in the Extracellular Space

The time for the exponential part of the bulk-phase limited diffusion of Br^- ion to reach 90% exchange ($t_{0.1}$) could be regarded only as a rough estimation. In spite of this lack of high precision, the general agreement with the theoretically calculated value leaves little doubt that diffusion in the extracellular space is properly described by the classical diffusion equation with a diffusion coefficient of Br^- ion in a 0.1 N NaCl solution and with a tortuosity factor, λ, somewhere between 1.0 and 1.5.'' In subsequent papers we shall employ this knowledge in our attempt to further understand water and solute distribution and the permeability properties of the living cells.

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