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MEASUREMENT OF THE PERMEABILITY COEFFICIENT OF $^{22}Na^+$ THROUGH A SYNTHETIC PHOSPHOLIPID–PROTEIN MEMBRANE

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SUMMARY

The diffusion of $^{22}Na^+$ through a composite system consisting of a layer of phospholipid sol and a layer of bovine plasma albumin have been made using a modified form of the continuous monitoring method. When 0.001 M $CaCl_2$ and cholesterol is added to each layer a membrane is formed at the interface and affords a resistance to diffusional flow. Equations have been developed using Laplace transform theory from which the permeability coefficient of this membrane can be calculated.

INTRODUCTION

Conductimetric work by SAUNDERS[1,2] has shown that when an ultrasonicated phospholipid sol containing Ca^{2+} and Na^+ and cholesterol is layered on an albumin solution also containing these substances, a membrane is formed at the phospholipid–protein interface, and that there is a resistance to diffusion of the Na^+ at this interface.

In this paper we describe the measurement of the permeability coefficient of $^{22}Na^+$ across a synthetic phospholipid–protein membrane and we have used a modified form of the continuous monitoring apparatus[3,4] which we have described previously[5,6]. The composite system consisting of protein solution and phospholipid sol is contained in a length of precision bore capillary tubing which is placed in the well of a NaI crystal of a scintillation counter. Surrounding the crystal is a large reservoir of inactive NaCl solution of the same strength as that contained in the capillary, and it is stirred at such a speed that the concentration of $^{22}Na^+$ at the tip of the capillary is zero during the diffusion run. The method resembles, but is not equivalent to a self-diffusion technique. The equations which describe the activity of the tracer in the capillary are developed in the first part of this paper and is followed by a description of the experimental techniques and a discussion of the results.

THEORY

A finite system with a membrane at the interface

A diagrammatic representation of the system is given below

$x = 0$ (open end)
$x = l_1$ (membrane)
$x = l = l_1 + l_2$

Application of Fick's law to each layer gives

$$\left(\frac{\delta(c_1(x,t))}{\delta t}\right)_x = D_1\left(\frac{\delta^2(c_1(x,t))}{\delta x^2}\right)_t \tag{1}$$

$$\left(\frac{\delta(c_2(x,t))}{\delta t}\right)_x = D_2\left(\frac{\delta^2(c_2(x,t))}{\delta x^2}\right)_t \tag{2}$$

where D_1 and $c_1(x,t)$, D_2 and $c_2(x,t)$ are the diffusion coefficient and activity of $^{22}Na^+$ in layers I and II, respectively.

Eqns. 1 and 2 were solved to comply with the initial condition

$$c_1(x,t) = c_2(x,t) = c(x,0) \quad l > x > 0,\ t = 0 \tag{3a}$$

and the following boundary conditions

$$c_1(x,t) = 0 \text{ at } x = 0,\ t > 0 \tag{3b}$$

$$D_1\left(\frac{\delta c_1(x,t)}{\delta x}\right)_t = D_2\left(\frac{\delta c_2(x,t)}{\delta x}\right)_t \text{ at } x = l,\ t > 0 \tag{3c}$$

$$D_1\left(\frac{\delta c_1(x,t)}{\delta x}\right)_t = H(c_2(x,t) - c_1(x,t)) \text{ at } x = l_1,\ t > 0 \tag{3d}$$

where H is the permeability coefficient

$$\left(\frac{\delta c_2(x,t)}{\delta x}\right)_t = 0 \text{ at } x = l,\ t > 0 \tag{3e}$$

Using the notation of CARSLAW AND JAEGER[7], the Laplace transforms of Eqns. 1 and 2 are:

$$\frac{d^2\bar{c}_1}{dx^2} - q_1^2 c_1 + \frac{c(x,0)}{D_1} = 0 \tag{4}$$

$$\frac{d^2\bar{c}_2}{dx^2} - q_2^2 c^2 + \frac{c(x,0)}{D_2} = 0 \tag{5}$$

where $c_1 = c_1(x,t)/\rho$, $c_2 = c_2(x,t)/\rho$, $q_1 = (\rho/D_1)^{\frac{1}{2}}$ and $q_2 = (\rho/D_2)^{\frac{1}{2}}$

The transformed boundary conditions become:

$$\bar{c}_1 = 0,\ x = 0 \tag{6b}$$

$$D_1\frac{d\bar{c}_1}{dx} = D_2\frac{d\bar{c}_2}{dx} \text{ at } x = l_1 \tag{6c}$$

$$D_1\frac{d\bar{c}_1}{dx} = H(\bar{c}_2 - \bar{c}_1) \text{ at } x = l_1 \tag{6d}$$

$$\frac{d\bar{c}_2}{dx} = 0 \text{ at } x = l \tag{6e}$$

Solutions of the differential Eqns. 4 and 5 are:

$$\bar{c}_1 = A_1 \cosh q_1 x + B_1 \sinh q_1 x + \frac{c(x,0)}{\rho} \tag{7}$$

$$\bar{c}_2 = A_2 \cosh q_2 x + B_2 \sinh q_2 x + \frac{c(x,0)}{\rho} \tag{8}$$

The coefficients A and B in Eqns. 7 and 8 were evaluated using the boundary conditions and substitution of their values in Eqns. 7 and 8 yielded Eqns. 9 and 10

$$\bar{c}_1 = \frac{c(x,0)}{\rho} - \frac{c(x,0)}{\rho} \cdot \frac{[D_1 q_1 \sinh q_2 l_2 \cosh q_1(l_1 - x) + Hk \cosh q_2 l_2 \cosh q_1(l_1 - x) + H \sinh q_2 l_2 \sinh q_1(l_1 - x)]}{[D_1 q_1 \cosh q_1 l_1 \sinh q_2 l_2 + Hk \cosh q_1 l_1 \cosh q_2 l_2 + H \sinh q_1 l_1 \sinh q_2 l_2]} \tag{9}$$

$$\bar{c}_2 = \frac{c(x,0)}{\rho} - \frac{c(x,0)}{\rho} \cdot \frac{kH \cosh q_2(l_1 + l_2 - x)}{[D_1 q_1 \cosh q_1 l_1 \sinh q_2 l_2 + Hk \cosh q_1 l_1 \cosh q_2 l_2 + H \sinh q_1 l_1 \sinh q_2 l_2]} \tag{10}$$

where $k = (D_1/D_2)^{\frac{1}{2}}$. The transform of $c(x,0)/\rho$ is $c(x,0)$. Application of the inversion theorem for Laplace transformation to the second term on the right hand side of Eqns. 9 and 10 yielded

$$c_1 = c(x,0) - \frac{c(x,0)}{2\pi i} \int_{\gamma - i\infty}^{\gamma + i\infty} \frac{e^{\lambda t}}{\lambda} \cdot \frac{[D_1 \mu_1 \sinh \mu_2 l_2 \cosh \mu_1(l_1 - x) + Hk \cosh \mu_2 l_2 \cosh \mu_1(l_1 - x) - H \sinh \mu_2 l_2 \sinh \mu_1(l_1 - x)]}{[D_1 \mu_1 \cosh \mu_1 l_1 \sinh \mu_2 l_2 + Hk \cosh \mu_1 l_1 \cosh \mu_2 l_2 - H \sinh \mu_1 l_1 \sinh \mu_2 l_2]} \, d\lambda \tag{11}$$

In the above equation λ is written in place of ρ as c_1 is regarded as a function of a complex variable. Thus $\rho \to \lambda$, $q \to \mu$, therefore $\mu_1 = (\lambda/D_1)^{\frac{1}{2}}$, $\mu_2 = (\lambda/D_2)^{\frac{1}{2}}$, and $\beta = i\mu$, since the integrand in Eqn. 11 is equal to $2\pi i$ times the sum of the residues of its integrand at poles $\lambda = 0$, and $\lambda_n = -D_1 \beta_n^2$, where $\pm \beta_n$ ($n = 1, 2, 3 \ldots$) are the roots of the denominator of the integrand.

Making the appropriate substitutions for λ and μ, Eqn. 11 becomes

$$c_1 = c(x,0) - \frac{c(x,0)}{2\pi i} \int_{\gamma - i\infty}^{\gamma + i\infty} \frac{e^{+\lambda t}}{\lambda} \cdot \frac{[D_1 \beta_n \sin \beta_n k l_2 \cos \beta_n(l_1 - x) - Hk \cos \beta_n k l_2 \cos \beta_n(l_1 - x) + H \sin \beta_n k l_2 \sin \beta_n(l_1 - x)] \cdot d\lambda}{[D_1 \beta_n \cos \beta_n l_1 \sin k\beta_n l_2 - Hk \cos \beta_n l_1 \cos \beta_n k l_2 + H \sin \beta_n l_1 \sin \beta_n k l_2]} \tag{12}$$

The residue at the pole $\lambda = 0$, is $c(x,0)$, and the residues when $[D_1 \beta_n \cos \beta_n l_1 \sin k\beta_n l_2 - Hk \cos \beta_n l_1 \cos \beta_n k l_2 + H \sin \beta_n l_1 \sin \beta_n k l_2] = 0$ are

$$c(x,0) \sum_{n=1}^{\infty} \lim_{\lambda \to \lambda_n} \frac{(\lambda - \lambda_n)}{[D_1 \beta_n \cos \beta_n l_1 \sin \beta_n l_2 - Hk \cos \beta_n l_1 \cos \beta_n k l_2 + H \sin \beta_n l_1 \sin \beta_n k l_2]} \cdot \lim_{\lambda \to \lambda_n} \frac{e^{\lambda t}[D_1 \beta_n \sin \beta_n k l_2 \cos \beta_n(l_1 - x) - Hk \cos \beta_n k l_2 \cos \beta_n(l_1 - x) + H \sin \beta_n k l_2 \sin \beta_n(l_1 - x)]}{\lambda} \tag{13}$$

Applying L'Hospitals rule gives

$$2c(x,0)\sum_{n=1}^{\infty}\frac{e^{+\lambda t}}{\lambda}\cdot$$
$$\cdot\frac{[D_1\beta_n\sin\beta_nkl_2\cos\beta_n(l_1-x)-Hk\cos\beta_nkl_2\cos\beta_n(l_1-x)+H\sin\beta_nkl_2\sin\beta_n(l_1-x)]}{[\beta_nD_1kl_2\cos\beta_nl_1\cos\beta_nkl_2+(Hk^2l_2+Hl_1+D_1)\cos\beta_nl_1\sin\beta_nkl_2+ +Hk(l_1+l_2)\sin\beta_nl_1\cos\beta_nkl_2-\beta_nD_1l_1\sin\beta_nl_1\sin\beta_nkl_2]} \quad (14)$$

Summing the residues, Eqn. 12 therefore becomes

$$c_1(x,t)=2c(x,0)\sum_{n=1}^{\infty}\frac{e^{-D_1\beta_n^2t}}{\beta}\cdot$$
$$\cdot\frac{[D_1\beta_n\sin\beta_nkl_2\cos\beta_n(l_1-x)-Hk\cos\beta_nkl_2\cos\beta_n(l_1-x)+H\sin\beta_nkl_2\sin\beta_n(l_1-x)]}{[\beta_nD_1kl_2\cos\beta_nl_1\cos\beta_nkl_2+(Hk^2l_2+Hl_1+D_1)\cos\beta_nl_1\sin\beta_nkl_2+ +Hk(l_1+l_2)\sin\beta_nl_1\cos\beta_nkl_2-\beta_nD_1l_1\sin\beta_nl_1\sin\beta_nkl_2]} \quad (15)$$

Integration of Eqn. 15 with respect to x, from 0 to l will give the total activity in layer I at time t.

$$c_1=\int_0^{l_1}c_1(x,t)\mathrm{d}x \quad (16)$$

$$c_1=\frac{2c_0l_1}{(l_1+l_2)}\sum_{n=1}^{\infty}e^{-\beta_n^2D_1t}\cdot$$
$$\cdot\frac{[Hk\cos\beta_nkl_2\sin\beta_nl_1+H\sin\beta_nkl_2\cos\beta_nl_1-D_1\beta_n\sin\beta_nkl_2-H\sin\beta_nkl_2]}{[\beta_nD_1kl_2\cos\beta_nl\cos\beta_nkl_2+(Hk^2l_2+Hl_1+D_1)\cos\beta_nl_1\sin\beta_nkl_2+ +Hk(l_1+l_2)\sin\beta_nl_1\cos\beta_nkl_2-\beta_nD_1l_1\sin\beta_nl_1\sin\beta_nkl_2]} \quad (17)$$

where c_0 is the total activity (*i.e.* from 0 to l) in the capillary at $t=0$.

Eqn. 10 was inverted in a similar manner, and integrated with respect to x over the length l_1 to l_2 to give the total concentration c_2,

$$c_2=\frac{2c_0l_2}{(l_1+l_2)}\sum_{n-1}^{\infty}e^{-\beta_n^2D_1t}\cdot$$
$$\cdot\frac{H\sin\beta_nkl_2}{[\beta_nD_1kl_2\cos\beta_nl_1\cos\beta_nkl_2+(Hk^2l_2+Hl_1+D_1)\cos\beta_nl_1\sin\beta_nkl_2+ +Hk(l_1+l_2)\sin\beta_nl_1\cos\beta_nkl_2-\beta_nD_1l_1\sin\beta_nl_1\sin\beta_nkl_2]} \quad (18)$$

The total activity in the tube at time t is given by the expression

$$c=c_1+c_2 \quad (19)$$

Finite system without resistance to diffusion at the interface

The diagram shown in the 1st paragraph of this section is a diagrammatic representation of the system except now that at l_1 there is no membrane. The initial

condition is as Eqn. 3a, and the boundary conditions 3b, 3c, 3e apply. Boundary condition 3d is replaced by

$$c_1(x,t) = c_2(x,t) \text{ at } x = l, > 0 \tag{3f}$$

Proceeding as previously, the constants A and B for Eqns. 6 and 7 were found using the initial and boundary conditions described above. Thus,

$$\bar{c}_1 = \frac{c(x,0)}{\rho} - \frac{c(x,0)}{\rho} \cdot \left[\frac{\cosh q_2 l_2 \cosh q_1(l_1 - x) + \sigma \sinh q_2 l_2 \sinh q_1(l_1 - x)}{\cosh q_1 l_1 \cosh q_2 l_2 + \sigma \sinh q_1 l_1 \sinh q_2 l_2}\right] \tag{20}$$

and

$$c_2 = \frac{c(x,0)}{\rho} - \frac{c(x,0)}{\rho} \cdot \left[\frac{\cosh q_2(l_1 + l_2 - x)}{\cosh q_1 l_1 \cosh q_2 l_2 + \sigma \sinh q_1 l_1 \sinh q_2 l_2}\right] \tag{21}$$

where $\sigma = l/k$.

Inversion of Eqns. 20 and 21 yielded

$$c_1(x,t) = 2c(x,0) \sum_{n=1}^{\infty} \frac{e^{-D_1 \beta_n^2 t}}{\beta_n} \cdot \frac{[\cos \beta_n k l_2 \cos \beta_n(l_1 - x) - \sin \beta_n k l_2 \sin \beta_n(l_1 - x)]}{[(l_1 + l_2) \sin \beta_n l_1 \cos \beta_n k l_2 + (k l_2 + l_1) \cos \beta_n l_1 \sin \beta_n k l_2]} \tag{22}$$

$$c_2(x,t) = 2c(x,0) \sum_{n=1}^{\infty} \frac{e^{-D_1 \beta_n^2 t}}{\beta_n} \cdot \frac{\cos \beta_n k(l_1 + l_2 - x)}{[(l_1 + l_2) \sin \beta_n l_1 \cos \beta_n k l_2 + (k l_2 + l_1) \cos \beta_n l_1 \sin \beta_n k l_2]} \tag{23}$$

Integration of Eqns. 22 and 23 with respect to x gave expressions for the total concentration in each of the layers I and II and is given by Eqns. 24 and 25, respectively.

$$c_1 = \frac{2c_0 l_1}{(l_1 + l_2)} \sum_{n=1}^{\infty} \frac{e^{-\beta_n^2 D_1 t}}{\beta_n^2} \cdot \frac{[\cos \beta_n k l_2 \sin \beta_n l_1 + \sigma \sin \beta_n k l_2 \cos \beta_n l_1 - \sigma \sin {}_n k l_2]}{[(l_1 + l_2) \sin \beta_n l_1 \cos \beta_n k l_2 + (k l_2 + \sigma l_1) \cos \beta_n l_1 \sin \beta_n k l_2]} \tag{24}$$

and

$$c_2 = \frac{2c_0 l_2}{(l_1 + l_2)} \sum_{n=2}^{\infty} \frac{e^{-\beta_n^2 D_1 t}}{\beta_n^2} \cdot \frac{\sin \beta_n k l_2}{[(l_1 + l_2) \sin \beta_n l_1 \cos \beta_n k l_2 + (k l_2 + l_1) \cos \beta_n l_1 \sin \beta_n k l_2]} \tag{25}$$

The total concentration in the capillary at time t is thus given by Eqns. 24, 25 and 19.

When $D_1 = D_2$, the total concentration at time t is given by the equation

$$c = \frac{8c_0}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \cdot e^{\frac{-(2n+1)^2 D_1 \pi^2 t}{4l^2}} \tag{26}$$

which is valid for the finite system consisting of one layer only from 0 to l.

EXPERIMENTAL

The self-diffusion apparatus used in these experiments was a modification of the continuous monitoring apparatus and has been described previously; only a brief description will be given here. The reservoir of 5.5 l capacity which held the inactive liquid had a bottom which was shaped to fit around and into the well of a 2 inches NaI crystal of the scintillation counter. The reservoir was surrounded by a constant temperature water bath which was maintained at 25 $\pm$ 0.01°. When the reservoir was stirred at 72 rev./min (motor speed electronically controlled) the self-diffusion coefficient of Na^+ in aqueous 0.1 M NaCl solution was found to be (1.279 $\pm$ 0.006) $\cdot 10^{-5}$ cm$^2 \cdot$sec^{-1} compared with (1.2779 $\pm$ 0.0023) $\cdot 10^{-5}$ cm$^2 \cdot$sec^{-1} obtained by MILLS AND GODBOLE[3].

A 2-cm length of precision bore capillary tubing of 0.08 cm internal diameter was sealed at one end with a small globule of glass and the other end was carefully rounded to promote streamlined flow over the tip of the capillary tube. Before use its internal length was measured with a specially adapted depth micrometer, then cleaned with chromic acid and siliconed. 10% (w/w) borine plasma albumin and 10% (w/w) ultrasonicated lecethin sols were prepared as described in a previous publication, and each contained 0.1 M NaCl (labelled), 0.001 M $CaCl_2$, and cholesterol, and carefully adjusted for equal radioactivity. First the capillary tube was approximately half-filled with bovine plasma albumin, and then lecethin sol was carefully layered onto the albumin and filled the remainder of the tube. It was placed in the well of the scintillation counter, and the reservoir was filled with 0.1 M NaCl (not active) containing 0.001 M $CaCl_2$ until the level of the solution was just below the tip of the capillary. The system was allowed to come to temperature equilibrium. It was at this stage that the intial activity c_0 was determined, from the average of three, 10^6 counts. Inactive solution was added slowly to the reservoir until the capillary tip was immersed to a depth of 4 mm, and the time was taken to be zero at this point. The system was allowed to become finite and then the activities present in the capillary were determined at various times t. At each count, 10^6 counts were recorded, and the time t was taken to be the mid-point of this count.

RESULTS AND DISCUSSION

The permeability coefficients listed in Column 4 of Table I were calculated as follows. The times t, in these experiments were greater than 150000 sec, and as the root β_n was greater than 1 when $n = 2$, the exponential term was small and the second and subsequent terms of the summation of Eqns. 17 and 18 made a negligible contribution, and therefore the term when $n = 1$ was the only one that was considered in the calculation presented here.

TABLE I

DIFFUSION OF $^{22}Na^+$ IN A PHOSPHOLIPID–ALBUMIN COMPOSITE SYSTEM AT 25°

Expt. 1: $10^5 D_1 = 0.9396$ cm^2·sec^{-1}, $10^5 D_2 = 1.1180$ cm^2·sec^{-1}, $l_1 = 0.6600$ cm, $l_2 = 1.4865$ cm, $c_0 = 572.9$ sec^{-1}. Expt. 2: $10^5 D_1 = 0.7010$ cm^2·sec^{-1}, $10^5 D_2 = 1.1160$ cm^2·sec^{-1}, $l_1 = 0.8500$ cm, $l_2 = 1.2965$ cm, $c_0 = 700.8$ sec^{-1}.

Expt. No.	*Time (sec)*	*Experimental count (counts/sec)*	*Finite system with no resistance (counts/sec)*	$H \times 10^4$ *(cm/sec)*
1	150 093	310.7	268.5	0.8297
	162 470	297.4	251.1	0.6474
	175 549	284.5	234.0	0.5266
	236 921	231.5	168.0	0.3149
	252 742	219.3	154.3	0.2934
	265 101	211.6	144.3	0.2717
2	165 040	380.2	290.7	0.3150
	175 808	370.4	277.8	0.2625
	188 383	358.8	263.5	0.2268
	204 149	344.4	246.5	0.1993
	252 104	300.2	201.4	0.1688
	265 197	293.8	190.6	0.1492
	271 438	290.3	185.6	0.1431

To evaluate the root β_n when $n = 1$, the denominator of the integrand of Eqn. 12 was rearranged to give

$$k \cot \beta l_2 - \tan \beta l_1 - \frac{D_1}{H} = 0 \qquad (27)$$

A value of H was chosen *e.g.* 0.1, and β was found by iteration using the Newton–Raphson method, and using H and β, c_1 and c_2 were calculated using Eqns. 17 and 18 and hence the total theoretical activity. This was then compared with the experimental activity, and if this was larger than the theoretical value, a new value of H was chosen, *e.g.* 0.01, and the whole process was repeated until the theoretical and experimental activities differed by less than 0.1 %. A table of theoretical counts with their cor-

TABLE II

Root	*Theoretical count (counts/sec)*	*H (cm/sec)*	*Experimental count (counts/sec)*
0.71975	296.282	0.000068	
0.71921	296.614	0.000067	
0.71867	296.956	0.000066	
0.71810	297.307	0.000065	297.4
0.71752	297.669	0.000064	
0.71693	298.041	0.000063	
0.71631	298.425	0.000062	
0.71568	298.820	0.000061	

responding values of H and β_n were set up about the theoretical count nearest to the experimental value and this is illustrated in Table II for $t = 16470$, Expt. 1 (Table I).

A smooth graph was drawn of H *versus* c and the value of H corresponding to the experimental count was interpolated from the curve and these are listed in Column 4 of Table I. Two different batches of lecethin were used in Expts. 1 and 2 and this accounts for different values of D_1.

TABLE III

$10^5 D_1 = 1.1160$ cm^2·sec^{-1}, $l = 2.1465$, $c_0 = 1229.0$ sec^{-1}.

Time (*sec*)	*Experimental count* (*counts/sec*)	*Calculated count* (*counts/sec*)
149 789	397.5	407.9
156 909	398.0	390.8
236 939	230.0	241.5
251 203	211.0	222.3
262 219	195.7	208.1
322 669	135.5	144.9
348 412	115.2	124.2

A slightly different experimental procedure from that described previously was adopted for the results reported in Table III. As previously described the phospholipid sol was layered on the albumin solution, and it was then allowed to stand for 6 h in order to form a membrane at the interface. The phospholipid solution was then very carefully removed so as not to disturb the boundary and was replaced with albumin solution of the same composition as that in the lower layer. From this point the experiment was carried out as before, the zero time being taken as the time when the capillary tip was immersed in the inactive 0.1 M NaCl–0.001 M $CaCl_2$ reservoir solution. Theoretical activities at times t were calculated according to Eqn. 26 (using term $n = 1$ only) and as there is very little difference from the experimental and calculated values the system corresponds to a finite layer of length 0 to l, and there is no resistance to diffusion in the middle of the tube. This seems to indicate that more than 6 h is required to form the membrane. Work is proceeding on semi-infinite systems and the results will be reported later.

The diffusion of the lecithin sol from the capillary to the reservoir occurs at a rate which is in the region of a hundred times slower than that of the Na^+, and therefore does not markedly alter the system over a period of 5 days. The position of the boundary relative to the tip of the capillary was measured with a kathetometer before and after each experiment and in all cases it did not move. It appears that osmosis does not affect the system to any great extent.

The method described in this paper has the following advantages: (i) It is possible to measure the change in permeability coefficient of the synthetic membrane with time. (ii) Each layer of the composite system only occupies 25 μl. (iii) Both ionic and non-ionic labelled substances can be used provided (a) the radiation will pass through the capillary wall to the counter, or (b) the activity in the capillary is sufficiently strong so that the isotope can be detected in the reservoir fluid[6].

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