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A RAPID METHOD FOR DETERMINING VOLTAGE–CONCENTRATION RELATIONS ACROSS MEMBRANES

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SUMMARY

1. A rapid method is described for obtaining curves relating the potential difference (p.d.) across a membrane to solute concentration gradients. The experiment consists of measuring the p.d. as a function of time while the concentration at the membrane is continuously increasing from 0 to C_o (or decreasing from C_o to 0) owing to diffusion through an unstirred layer.

2. The voltage trace is then analysed with a piece of transparent paper, on which lines corresponding to solutions of the diffusion equation convert the time axis of the voltage trace into a concentration axis. Superposition of this paper on an experimental voltage trace permits one to read off the p.d. corresponding to any concentration between 0 and C_o.

3. The relation between diffusion potentials and NaCl concentration, and between streaming potentials and concentrations of impermeant non-electrolytes, has been reconstructed by this procedure in rabbit gall-bladder. The method yields non-linear voltage–concentration curves in good agreement with those obtained by conventional methods, but requires much less time and experimental manipulation. The conditions that must exist for the method to be valid are discussed.

4. The thicknesses of the unstirred layers in rabbit gall-bladder are measured, and are shown to have a negligible effect upon steady-state streaming potentials and NaCl diffusion potentials.

5. The changes of permeability with osmolarity described in rabbit gall-bladder must be complete within a fraction of a second, in agreement with the interpretation that they represent swelling and shrinking of the cell membrane itself.

INTRODUCTION

Interest in the dependence of electrical potential differences (p.d.'s) upon solute concentration gradients across membranes arises in connexion with many biological problems. Examples of such problems are measure-

ments of ion permeability by means of diffusion potentials, of water flow by streaming potentials, and of transport rates by the p.d.'s of ion transport mechanisms. The usual method of obtaining voltage–concentration relations is to apply various concentration gradients, measure the p.d.'s produced, and fit a curve to the resulting graph of p.d. against concentration. In practice, a source of variability in experimental values obtained in this way is that they have been measured on the same preparation at different times, or on different preparations. Furthermore, if the voltage–concentration relation is moderately non-linear, numerous changes of experimental solutions are required to provide enough concentration gradients for accurately reconstructing the relation.

An alternative procedure would be to follow the change in p.d. as the concentration increased continuously from zero to its maximal value. In this way, the whole voltage–concentration relation could be reconstructed from one experimental trace, and problems arising from variability and numerous changes of solutions could be minimized. In the present paper, a continuous increase in concentration is achieved by taking advantage of the unstirred layer which inevitably separates a membrane from the well-stirred bulk solution. Because of this unstirred layer, a solute added to the bulk solution does not appear at the membrane instantaneously. Rather, its concentration at the membrane builds up gradually from zero to the final value, with a half-time inversely proportional to its diffusion coefficient and directly proportional to the square of the thickness of the unstirred layer. At any time during the transient, the concentration at the membrane may be computed by solving the diffusion equation. Hence a strip of transparent paper may be prepared which converts the time axis of an experimental voltage–time record into a concentration axis. In practice, then, the method consists of recording the change in p.d. after a change in solute concentration, placing the prepared strip of transparent paper on the voltage record, and reading off the voltage at any concentration.

As outlined above, the method assumes that: (*a*) the thickness of the unstirred layer, and diffusion constants in it, are independent of the composition of the solution; and (*b*) any concentration-dependent changes in permeability with time occur very rapidly in comparison with diffusion half-times. The validity of these assumptions must be confirmed for each tissue to which the method is to be applied. This paper establishes the validity of the method for concentration changes in the solution bathing the mucosal surface of rabbit gall-bladder, and shows that the resulting voltage–concentration curves are non-linear, in agreement with those obtained by conventional procedures. The experimental results yield a value for the thickness of the unstirred layers, knowledge of which is

essential in order to assess quantitatively the contribution of unstirred layers to non-linear osmosis (Diamond, 1966). Finally, the results also indicate the rapidity of the water permeability changes described in the previous paper.

METHODS

Techniques for measuring p.d.'s across cannulated rabbit gall-bladders *in vitro* were described in the previous two papers (Diamond & Harrison, 1966; Diamond, 1966). All experiments involving transient p.d. changes following a change of the mucosal bathing solution were carried out on everted gall-bladders, i.e. with the mucosal surface facing outwards. P.d. transients following changes in the serosal bathing solution were carried out on gall-bladders in the natural orientation, i.e. with the serosal surface facing outwards. Hence changes in solution were accomplished simply by lifting out from below the beaker of solution in which the gall-bladder rested, and substituting a fresh beaker, the procedure requiring less than a second. The time interval over which different parts of the gall-bladder became exposed to the new solution was far less than this, since most of the second was spent in lifting out the old beaker and lifting the new beaker from the laboratory bench up to the gall-bladder. Since half-times proved to be about 10 and 100 sec for mucosal and serosal transients, respectively, exposure of the whole gall-bladder to a new solution may be regarded as instantaneous. The delay of the recording system was also negligible compared to these half-times. Stirring by means of a gas bubbler was maintained at approximately the same rate in all experiments. All measurements were carried out at room temperature (17–21° C). NaCl-Ringer's solution had the composition (in mM): NaCl 148, KCl 6, $CaCl_2$ 0·25, Na_2HPO_4 2·125, NaH_2PO_4 0·375. To obtain isotonic solutions with lower NaCl concentrations, NaCl Ringer's solution was mixed with an otherwise identical solution in which 267 mM sucrose had been substituted for the NaCl. Hypertonic solutions were obtained by incorporating various concentrations of sucrose into NaCl Ringer's solution.

THEORY

The experiment consists of recording the p.d. when the solution bathing the gall-bladder is suddenly changed. As solute diffuses from the well-stirred bulk solution through the unstirred layer, its concentration immediately adjacent to the gall-bladder wall gradually builds up, and it is this 'wall' concentration rather than the bulk concentration which is related to the p.d. In this section the method for calculating this 'wall' concentration from the diffusion equation is derived.

While a gall-bladder is of course round rather than flat, the thickness of the unstirred layer proves to be negligible (*ca.* 0·01 cm) compared to the diameter of the organ (*ca.* 1·5 cm), and the problem may be regarded as one of diffusion up to an infinite plane. If the x-axis is drawn perpendicular to the gall-bladder, then the diffusion equation reads as follows:

$$\frac{\partial C(x,t)}{\partial t} = \frac{D\partial^2 C(x,t)}{\partial x^2}, \tag{1}$$

where t is time, D the diffusion coefficient of the solute, and $C(x,t)$ the concentration of solute at any time and distance from the gall-bladder. The gall-bladder wall is taken as $x = 0$, the thickness of the unstirred layer

is l, and the well-stirred bulk solution extends from $x = l$ to $x = \infty$. No term for the effect of water flow is included in eqn. (1), and this omission is justified on two grounds. First, when a water flow term was included and experimental values of D, l, and water flow were inserted, computation with the IBM 7094 computer showed that half-times of p.d. transients were changed by less than 1 %. Secondly, it will be demonstrated experimentally (p. 90) that the effect of maximal rates of water flow upon p.d.'s is too small to detect.

The boundary conditions for a build-up transient (solute concentration in bathing solution suddenly changed from 0 to C_o) are: $t = 0$, $C = 0$, $0 < x < l$; $C = C_o$, $x \geqslant l$; $D = 0 = \partial C/\partial x$, $x = 0$. The last condition states that there is negligible leakage of solute across the gall-bladder wall itself over the duration of the transient, an assumption which is valid for the relatively impermeant molecules used in this study (sucrose and NaCl). We want to obtain $C(0,t)$, the solute concentration immediately adjacent to the gall-bladder wall. The solution to this problem (Crank, 1956, p. 45) is

$$C(0,t) = C_o - \frac{4C_o}{\pi} \sum_{n=0}^{\infty} [\exp\{-D(2n+1)^2\pi^2 t/4l^2\}] \frac{(-1)^n}{(2n+1)}. \qquad (2)$$

The boundary conditions for a decay transient (solute concentration in bathing solution suddenly changed from C_o to 0) are: $t = 0$, $C = C_o$, $0 < x < l$; $C = 0$, $x \geqslant l$; $D = 0 = \partial C/\partial x$, $x = 0$. The solution for the solute concentration at the gall-bladder wall is

$$C(0,t) = \frac{4C_o}{\pi} \sum_{n=0}^{\infty} [\exp\{-D(2n+1)^2\pi^2 t/4l^2\}] \frac{(-1)^n}{(2n+1)}. \qquad (3)$$

Equations (2) and (3) are shown graphically in Fig. 1, where it is apparent that either is simply an upside-down version of the other. The half-times for build-up and decay of the concentration (time for $C(0,t)$ to build up or decay to $\frac{1}{2}C_o$) are both $0{\cdot}38l^2/D$. Hence, under circumstances where the p.d. across the gall-bladder wall is directly proportional to concentration, p.d. transients will have the form of Fig. 1, and half-times for build-up and decay of the p.d. will be the same. Should the voltage–concentration relation not be linear, the form of p.d. transients will deviate from Fig. 1, and build-up and decay half-times will be unequal.

Suppose the thickness of the unstirred layer (as indicated by the half-time for p.d. transients following a given concentration change) were absolutely invariant. Then one need only construct a ruler on which time units were marked off at regular intervals, and write beside each time unit the value of $C(0,t)$ prevailing at that time according to eqns. (2) or (3). Such a 'ruler' (Fig. 2*a*, above) could be placed on the time axis of an experimental voltage–time trace, and the p.d. corresponding to any value

of $C(0,t)$ read off. While this procedure would give approximately correct values in the gall-bladder, it is more accurate to take account of the observation that half-times for transients following a given concentration change are not perfectly reproducible but vary by 10 %, probably because of unavoidable slight variations in stirring rates and unstirred layer thicknesses. Hence, Fig. 2*b* was constructed, providing a graded series of such rulers to cover a range of half-times. Any horizontal line across Fig. 2*b* is divided in the same proportion by the diagonals and thus forms a ruler corresponding to a shorter half-time than Fig. 2*a*.

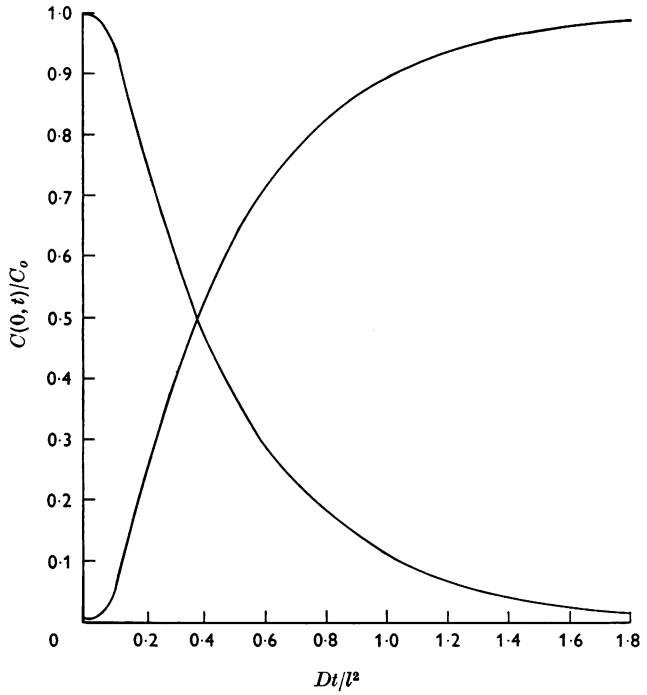

Fig. 1. Theoretical curves (from eqns. (2) and (3)) for build-up and decay of the solute concentration at a membrane ($C(0,t)$) separated from a well-stirred solution by an unstirred layer of thickness l in which solute has the diffusion coefficient D. The rising curve gives the relative concentration at the membrane after the concentration in the well-stirred solution has suddenly been raised from 0 to C_o. The falling curve gives the relative concentration at the membrane after the concentration in the well-stirred solution has suddenly dropped from C_o to 0. The abscissa is proportional to time.

The analysis of the experimental voltage–time record proceeds as follows:

(1) The point in the voltage trace is located corresponding to $t = 0$, when the gall-bladder entered the new solution. This is marked by an

artifact in the trace as the gall-bladder passes from air back into fluid and the electrical continuity of the recording circuit is restored. Figure 2*b* is prepared on a transparent piece of paper and placed over the voltage trace so that the left hand edge of Fig. 2*b* is lined up with the zero-time artifact.

(2) At some previous time the steady-state p.d. corresponding to half the concentration step now being tested was measured. The point where the voltage trace attains this p.d. corresponding to $C(0,t)/C_o = 0{\cdot}5$ is located. Figure 2*b* is moved up and down until this point on the trace is intersected by the diagonal $C(0,t)/C_o = 0{\cdot}5$ on Fig. 2*b*. A horizontal line is drawn on Fig. 2*b* through this intersection. In effect, this step chooses a 'ruler' corresponding to the appropriate unstirred layer thickness.

(3) To find the p.d. corresponding to any given value of $C(0,t)/C_o$, one locates the point on the horizontal 'ruler' just drawn where it is intersected by the diagonal corresponding to the given $C(0,t)/C_o$. The required p.d. is

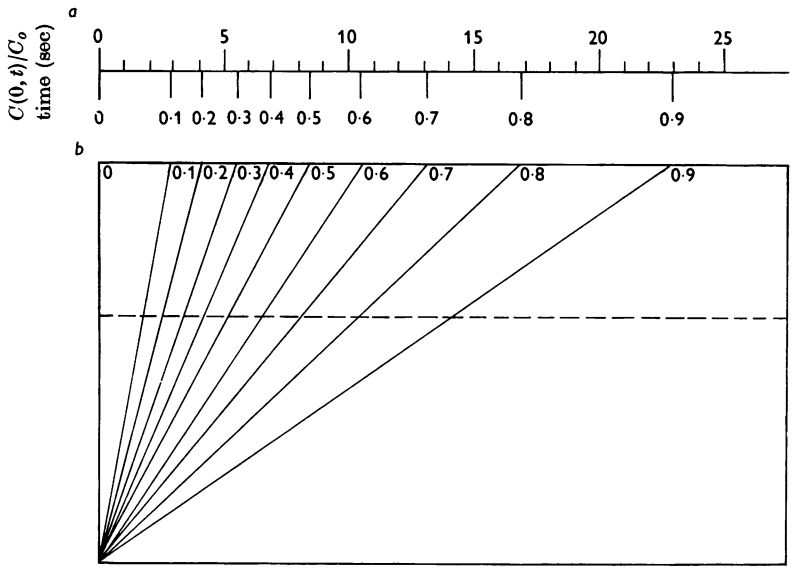

Fig. 2. Graphs for converting the time axis of a p.d. build-up transient into a concentration axis. When these figures are prepared on transparent paper and superimposed on experimental p.d. traces, they permit one to read off the relative solute concentration at the membrane ($C(0,t)/C_o$) at any time following an increase of solute concentration from 0 to C_o in the bulk solution. In the 'ruler' above (Fig. 2*a*), the value of $C(0,t)/C_o$ from eqn. (2) is written below the corresponding value of t for $D/l^2 = 0{\cdot}044$ sec^{-1}. Figure 2*a* would be the correct ruler for a transient resulting from diffusion of sucrose ($D = 4{\cdot}4 \times 10^{-6}$ cm^2/sec) through a layer of thickness 10^{-2} cm. In the chart below (Fig. 2*b*) any horizontal line (e.g. the interrupted line) is divided in the same proportions by the diagonals and would represent a similar 'ruler' corresponding to a shorter time constant. Graphs for decay transients are identical except that values of $C(0,t)/C_o$ are reversed, decreasing from 1 to 0·1 from left to right.

then the value of the voltage trace vertically above or below this point. In effect, this step locates the time along the voltage trace when the given value of $C(0,t)/C_o$ prevails at the membrane.

The entire voltage–concentration relation may be reconstructed by repeating the third step for different values of $C(0,t)/C_o$. This procedure will become clearer from the examples in the Results section.

RESULTS

Constancy of the mucosal unstirred layer

The proposed method requires that the properties of the mucosal unstirred layer (its thickness, and diffusion coefficients in it) be unaffected by the changes in osmolarity, salt concentration, and water flow occurring during transients. The present section demonstrates experimentally that this requirement is satisfied.

To determine whether the properties of the mucosal unstirred layer were affected by the osmolarity of the bathing solutions, diffusion potential transients were measured at two different osmolarities. With [NaCl] of the serosal solution maintained at 200 mM, the half-times ($t_{\frac{1}{2}}$'s) for build-up and decay of the p.d. were determined when [NaCl] of the mucosal solution was lowered from 200 to 100 mM by isosmotic replacement with sucrose, then brought back to 200 mM. Half-times for the same diffusion potential were measured again after the osmolarities of both bathing solutions had been raised from 387 to 589 m-osM by addition of 200 mM sucrose. The $t_{\frac{1}{2}}$'s with which the diffusion potential built up and decayed were 5·9 and 3·9 sec, respectively, at 387 m-osM; and 6·3 and 4·2 sec at 589 m-osM. The slightly greater $t_{\frac{1}{2}}$'s at 589 m-osM do not reflect a change in unstirred layer thickness, but rather a decrease in D_{NaCl} because of the greater viscosity associated with higher sucrose concentrations. Changes of osmolarity were also found to have no effect upon unstirred layer thicknesses as measured by diffusion potential transients resulting from two other concentration gradients (200–50 mM-NaCl, and 200–0 mM-NaCl).

Analogous experiments were performed to rule out any effect of salt concentration upon the unstirred layer. At different values of [NaCl] in the bathing solutions, $t_{\frac{1}{2}}$'s were determined for build-up and decay of the streaming potential resulting from addition of 100 mM sucrose to the mucosal solution. Half-times proved to be the same within $\pm 10\%$ in one gall-bladder at 50, 95, 148, 210, and 300 mM-NaCl. In two other gall-bladders $t_{\frac{1}{2}}$'s were the same at 10, 25, 50, 75, 100, and 200 mM-NaCl. This observation that the unstirred layer is unaffected by salt or sucrose concentration is supported by the observation (p. 98) that its thickness comes out the same whether calculated from sucrose streaming potential transients or from NaCl diffusion potential transients.

Because of effects related to viscosity, increasing concentrations of sucrose decrease the diffusion coefficients of both salt (Steel, Stokes & Stokes, 1958) and sucrose (Gosting & Morris, 1949), while the diffusion coefficient of salt also decreases with salt concentration (Harned & Owen, 1958). Diffusion coefficients in the unstirred layer are therefore not strictly independent of time during transients, but the effect is not large enough to be serious over the range of concentrations used here. One may calculate that the sucrose diffusion coefficient at any time during a streaming potential transient will differ from the average value by at most 9·2 %, if sucrose concentrations up to 350 mM are used. During diffusion potential transients NaCl is being replaced isotonically by sucrose, and the effects of increasing [sucrose] and decreasing [NaCl] upon D_{NaCl} are opposite. For [NaCl] changes between 0 and 150 mM, one may calculate that D_{NaCl} at any time in a diffusion potential transient will differ from the average value during the transient by at most 4·8 %.

The following experiment was carried out to determine whether osmotic water flow through the unstirred layer could affect solute concentrations at the gall-bladder wall (in principle, water flow from the wall into the layer could reduce wall concentrations by dilution, if the unstirred layer were sufficiently thick and the water flow high enough). First, a diffusion potential was measured when mucosal [NaCl] was lowered from 200 to 50 mM by isosmotic replacement with sucrose, then brought back to 200 mM. Since the mucosal and serosal solutions had the same osmolarity, there was no water flow. Secondly, the experiment was repeated but with the difference that the mucosal solution was hypertonic to the serosal by 200 mM sucrose throughout the whole sequence, so that there was a constant rate of osmotic water flow into the mucosal solution. Finally, the same diffusion potential measurement was repeated but with the mucosal solution hypertonic by 400 mM sucrose, which yields close to the maximum obtainable rate of osmotic water flow into the mucosal solution. In the first case the diffusion potential built up from 0 mV, while in the second and third cases it was superimposed upon streaming potentials of 3·5 and 5·1 mV, respectively. However, in all three cases the steady-state diffusion potentials were virtually the same: 7·4, 7·65, and 7·65 mV, in that order. The effect of water flow through the mucosal unstirred layer upon wall concentrations and p.d.'s is therefore quantitatively negligible. The same conclusion was reached by calculating the effect of water flow from the diffusion equation.

Since the layer of epithelial cells in the gall-bladder is in direct contact with the mucosal solution, the unstirred layer at the mucosal surface is simply a layer of free solution. It is therefore not surprising that the properties of this unstirred layer at constant stirring rate are independent

of bathing solution composition—a necessary condition if voltage–concentration curves are to be reconstructed from diffusion transients.

Streaming potentials

Figure 3 shows a build-up transient for a streaming potential, produced by immersing the gall-bladder at $t = 0$ in a mucosal solution hypertonic by 350 mM sucrose. The streaming potential attained a steady-state value of +15·0 mV. Half of the maximal voltage change, +7·5 mV, was attained after 7·2 sec. However, when an isotonic mucosal solution was restored and the decay transient was measured (not shown), half of the total voltage drop was not attained until 12·6 sec. Hence, the relation between concentration and voltage cannot be linear, since half of the maximal concentration change is attained at the same time in both a build-up and a decay transient. The steady-state streaming potential resulting from a mucosal solution hypertonic by 175 mM sucrose (half the final concentration in the transient of Fig. 3) was then measured and found to be +11·6 mV.

To reconstruct the voltage–concentration relation by the method outlined in the Theory section, Fig. 2*b* on a piece of transparent paper was superimposed upon the transient of Fig. 3. The caption to Fig. 3 describes the alignment procedure and the details of determining graphically the sucrose concentration next to the gall-bladder wall at any time. By reading off the p.d. on the voltage trace of Fig. 3 at times corresponding to various concentrations, the complete voltage–concentration relation for sucrose concentrations from 0 to 350 mM was reconstructed. This is presented as the continuous curve of Fig. 4, where the experimental points are streaming potentials obtained from steady-state p.d. measurements with seven different concentration gradients in the same gall-bladder. Both the transient and the steady-state method yield the same non-linear relation between streaming potential and concentration, confirming the validity of the transient method for mucosal streaming potentials. The explanation for this non-linear relation was discussed in the preceding paper (Diamond, 1966). Figure 4 is similar to Fig. 3 of the preceding paper, in which the steady-state streaming potential method and direct, gravimetric measurements of water flow were compared in an analogous experiment. These three methods for measuring water flow thus yield the same results, and the main difference between them is the time required. The experiment of Fig. 4 would have taken 11 hr by the gravimetric method, whereas it required 42 min by means of steady-state streaming potentials and 3 min by the transient method.

In all, 530 streaming potential transients following osmolarity changes in the mucosal solution have been recorded. Reconstruction of voltage–

concentration curves from either build-up or decay transients by the method described above consistently yielded non-linear relations similar to those of Fig. 4. These non-linear relations may be conveniently summarized in terms of half-times for the transients. As mentioned in the

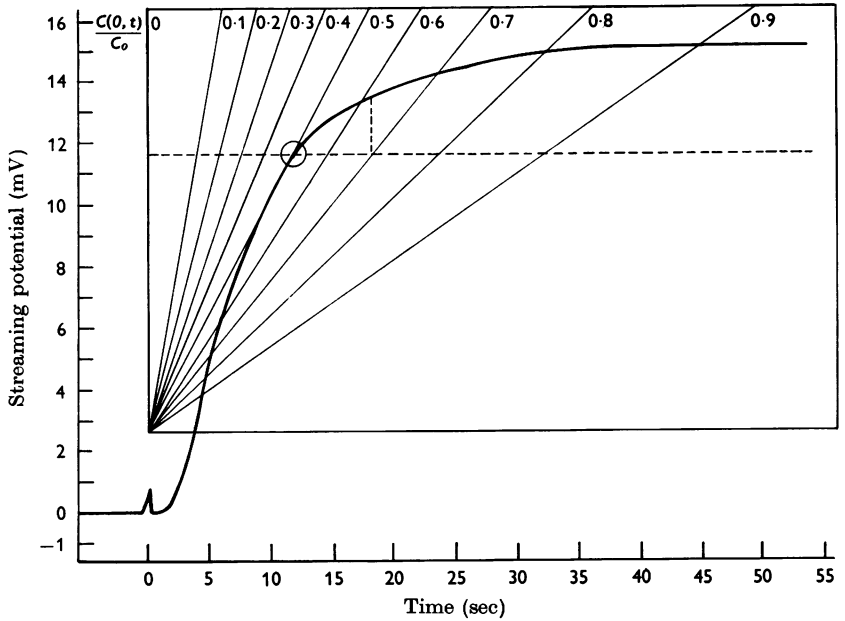

Fig. 3. Build-up transient for a streaming potential in an everted rabbit gall-bladder (heavy curve). Until $t = 0$ the mucosal and serosal solutions were both NaCl Ringer's solution. At $t = 0$ the mucosal solution was replaced by an otherwise identical solution made hypertonic with 350 mM sucrose. Before $t = 0$ there was an asymmetry potential of $-0{\cdot}2$ mV, and the whole trace has been shifted by this amount to give a base line of zero for convenience. The deflexion at $t = 0$ is an artifact from the change of solutions.

Figure 2*b* (lighter lines) has been superimposed upon the transient so as to permit the voltage–concentration relation to be read off directly. To align Fig. 2*b* properly, its left-hand edge was lined up with the zero-time artifact. The transient of Fig. 3 attained 11·6 mV (steady-state p.d. corresponding to $C(0,t)/C_o = 0{\cdot}5$) after 12·0 sec, and this point is circled. At that time the concentration at the wall ($C(0,t)$) must have been half the final value (C_o). Hence Fig. 2*b* was moved up and down, maintaining alignment of the left-hand edge with the zero-time artifact, until the diagonal marked 0·5 intersected 11·6 mV on the underlying voltage trace. A horizontal interrupted line was drawn through this point, and the intersections of the diagonals with this horizontal line then give the time at which each value of $C(0,t)/C_o$ prevailed at the gall-bladder wall. For instance, at the time when the horizontal line is intersected by the diagonal $C(0,t)/C_o = 0{\cdot}7$, the sucrose concentration next to the gall-bladder wall must have been (0·7) (350) = 245 mM. The p.d. at this time on the voltage trace (located by the vertical interrupted line) is 13·5 mV, and this must be the streaming potential resulting from 245 mM sucrose.

Theory section, the same time is required to complete half the total concentration change at the membrane during both build-up and decay transients, which would therefore have the same $t_{\frac{1}{2}}$'s if the relation between p.d. and concentration were linear. Experimentally, build-up and decay $t_{\frac{1}{2}}$'s were nearly the same only for streaming potential transients following small changes of osmolarity. For example, in one gall-bladder the streaming potential resulting from addition of the same small concentration of sucrose (50 mM) to the mucosal solution was measured six times. The $t_{\frac{1}{2}}$ for build-up of the streaming potential was $102 \pm 9\%$ (average value and standard deviation) of the $t_{\frac{1}{2}}$ for decay of the streaming potential on removal of sucrose.

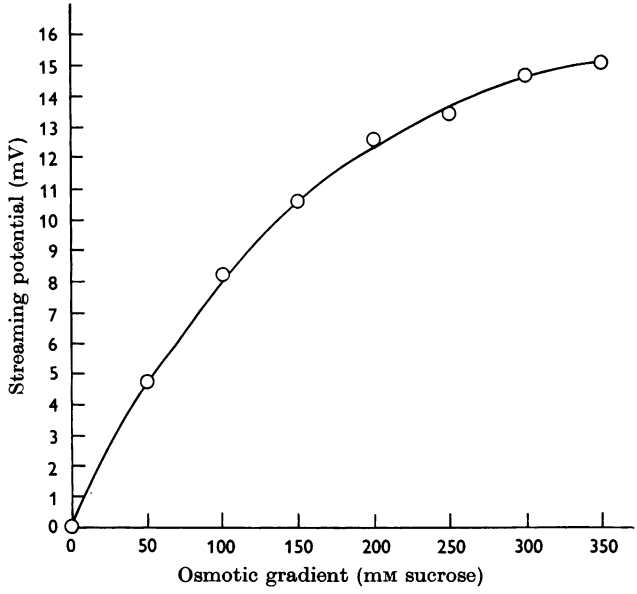

Fig. 4. Relation between streaming potentials (mV) and osmotic gradient (mM sucrose) in an everted rabbit gall-bladder. The continuous curve was obtained from analysis of the p.d. transient of Fig. 3 by the graphical procedure described in the text. The experimental circles were obtained by measurements of steady-state streaming potentials.

When larger osmotic gradients were tested, $t_{\frac{1}{2}}$'s were consistently greater for the mucosal solution going hypotonic than hypertonic (Table 1). This finding is an expression of the non-linear relation between concentration (or osmotic gradient) and streaming potentials (or water flow) in the gall-bladder. The permeability to water (water flow divided by the osmotic gradient) is lower in hypertonic than in hypotonic solutions. Hence, when a streaming potential is produced by a hypertonic solution, the lower concentrations of sucrose that reach the membrane during the first half

of a build-up transient give proportionately more water flow (greater streaming potentials) than do the higher concentrations during the last half of the transient. Half the total p.d. change is therefore attained before half the total concentration change with a solution going hypertonic, while the reverse is true for a solution going hypotonic.

TABLE 1. Half-times of mucosal streaming potential transients

[sucrose] (mM)					t (sec)	
Serosal	Mucosal				Build-up	Decay
0	0	→	200	→ 0	9·3	11·2
0	0	→	400	→ 0	7·1	12·5
0	0	→	600	→ 0	5·8	14·4
600	600	→	450	→ 600	11·0	11·1
600	600	→	100	→ 600	12·1	8·0
600	600	→	0	→ 600	12·9	6·4

The figures in the right-hand two columns are the half-times for build-up and decay of the streaming potential when the mucosal sucrose concentration was altered and then restored to the original value. The electrolyte composition of both bathing solutions remained identical (NaCl Ringer's solution). The upper three rows of values were obtained from one gall-bladder, the lower three from another. Note that $t_{\frac{1}{2}}$'s are greater for the mucosal solution going hypotonic than hypertonic.

When streaming potentials were produced with different impermeant molecules (erythritol, mannitol, sucrose, raffinose), half-times increased with increasing molecular weight (decreasing diffusion coefficient). In fourteen gall-bladders the average value of $t_{\frac{1}{2}}$ was found to be 10·3 sec for streaming potentials resulting from small concentration gradients of sucrose (< 100 mM). With these small gradients, build-up and decay $t_{\frac{1}{2}}$'s are nearly equal, and p.d.'s nearly proportional to concentrations. From Fig. 1, $t_{\frac{1}{2}}$ for a change of concentration is $0{\cdot}38l^2/D$. Setting $t = 10{\cdot}3$ sec and $D = 4{\cdot}4 \times 10^{-6}$ cm^2/sec (the free-solution value for D_{sucrose} at 50 mM, 19° C), one obtains $l = 108\ \mu$. This is accordingly the average thickness of the mucosal unstirred layer under the standard conditions of stirring employed. This value is somewhat larger than one would expect for a smooth surface under the stirring conditions used, because of the presence of occasional ridges of tissue, which protect an additional layer of fluid from the effects of stirring.

Whereas the epithelial cells of the gall-bladder are in direct contact with the mucosal solution, they are separated from the serosal solution by about 300 μ of connective tissue. For six gall-bladders the average $t_{\frac{1}{2}}$ for build-up of the streaming potential resulting from addition of 100 mM sucrose to the serosal solution was 102 sec. Substituting $t = 102$ sec, $l = 300\ \mu$ into $Dt_{\frac{1}{2}}/l^2 = 0{\cdot}38$, one obtains $D = 3{\cdot}4 \times 10^{-6}$ cm^2/sec for the diffusion coefficient of sucrose in the serosal connective tissue. This is about 23 %

below the value in free solution, probably because the presence of connective tissue fibres makes the diffusion path tortuous and longer.

Diffusion potentials

The procedure for reconstructing diffusion potential transients differs from that employed for streaming potentials in one respect: a correction must be made for distortion of the voltage trace by junction potentials.

Figure 5 shows the change in p.d. when all the NaCl in the mucosal solution (150 mM) was replaced isosmotically with sucrose. The p.d. immediately went negative to −11·6 mV, then crossed zero and gradually approached a steady-state level of +21·2 mV. The time to rise half-way from −11·6 to +21·2 mV was 9·9 sec. When all the NaCl in the mucosal solution was restored, the p.d. (not shown) immediately went more positive by 11·6 to +32·8 mV, then decayed to zero with a half-time of 3·4 sec. Since the $t_{\frac{1}{2}}$'s for build-up and decay transients are unequal, the voltage–concentration relation cannot be linear. The steady-state p.d. corresponding to half this concentration gradient (75 mM-NaCl) was then measured and found to be +7·6 mV, 35·8 % of that resulting from the full gradient (21·2 mV).

The 11·6 mV step at the beginning of the transient corresponds in magnitude to the junction potential measured between NaCl Ringer's solution and sucrose Ringer's solution. When the gall-bladder is transferred to sucrose Ringer's solution, this junction potential is immediately set up between the new sucrose solution and the unstirred layer of NaCl Ringer's solution still surrounding the gall-bladder. As the concentration of NaCl next to the gall-bladder wall gradually drops, this junction potential decays, while the diffusion potential across the gall-bladder builds up. The decay of the junction potential and the build-up of the diffusion potential have approximately the same time course, because both depend upon the same NaCl concentration at the gall-bladder wall. Hence the effect of the junction potential is equivalent to shifting the base level for the diffusion potential from 0 to −11·6 mV, and exaggerating the diffusion potential with respect to this base level at any time during the transient by 55 % (21·2 + 11·6 mV is 55 % larger than 21·2 mV).

To reconstruct the voltage–concentration relation, Fig. 2*b* on a piece of tracing paper was superimposed upon the transient of Fig. 5. The caption to Fig. 5 describes the alignment procedure and the details of determining graphically the NaCl concentration at the gall-bladder wall at any time. By reading off the p.d. on the voltage trace of Fig. 5 at times corresponding to various NaCl concentrations, the voltage–concentration relation illustrated in Fig. 6 was derived. For comparison, Fig. 6 also gives the p.d. computed from the constant-field equation, which was found to give a

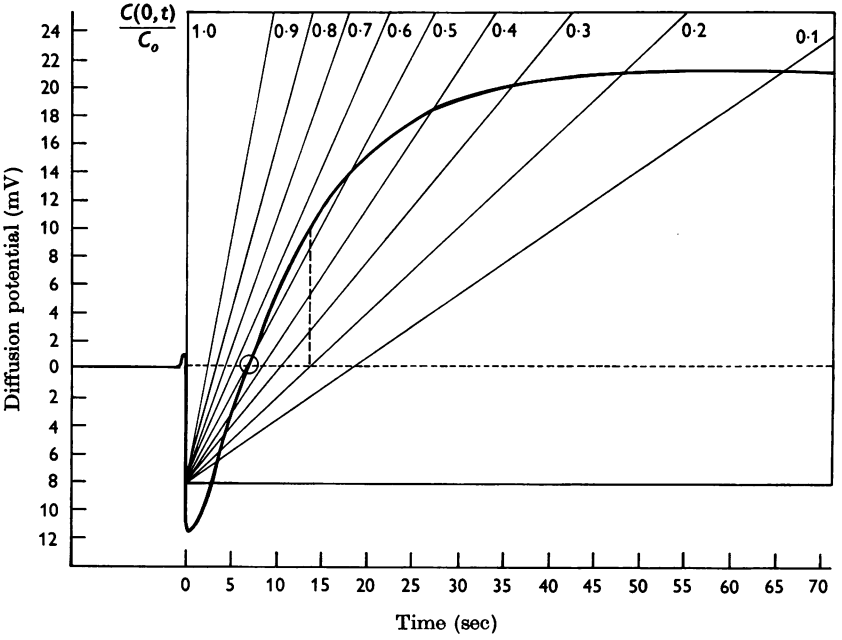

Fig. 5. Diffusion potential transient in an everted rabbit gall-bladder (heavy curve). Until $t = 0$ the mucosal and serosal solutions were both NaCl Ringer's solution. At $t = 0$ the mucosal solution was replaced by an otherwise identical solution in which [NaCl] had been lowered from 150 to 0 mM by isosmotic replacement with sucrose. Before $t = 0$ there was an symmetry potential of $-0{\cdot}4$ mV, and the whole trace has been shifted by this amount to give a base line of zero for convenience. Figure 2*b* (lighter lines) has been superimposed upon the transient so as to permit the voltage–concentration relation to be read off directly. To align Fig. 2*b* properly, its left-hand edge was lined up with the zero-time artifact. Half the total concentration gradient had been found to produce 35·8% of the final p.d., and the voltage trace had achieved a value 35·8% of the way from $-11{\cdot}6$ to $+21{\cdot}2$ mV, or $+0{\cdot}2$ mV, after 6·8 sec (circled point). Hence at this time the concentration at the wall must have been half of the initial value, i.e. $C(0,t)/C_o = 0{\cdot}5$. Figure 2*b* was moved up and down until the diagonal 0·5 intersected $+0{\cdot}2$ mV on the underlying voltage trace, and an interrupted horizontal line was drawn through this point. For any desired value of $C(0,t)/C_o$, the intersection of the corresponding diagonal and this interrupted horizontal line was located, in effect locating the time when this value of $C(0,t)$ prevailed at the membrane. The value of the voltage trace vertically above or below this point was read off. To compensate for the shift of origin and scale caused by the junction potential, the difference between this value of the voltage and $-11{\cdot}6$ mV was divided by 1·55, yielding the diffusion potential corresponding to this value of $C(0,t)/C_o$. For example, the voltage trace reads 9·8 mV at the point vertically above the intersection of the diagonal $C(0,t)/C_o = 0{\cdot}2$ with the horizontal interrupted line. $[9{\cdot}8 - (-11{\cdot}6)]/1{\cdot}55$ is 13·8 mV, which is therefore the diffusion potential corresponding to 30 mM-NaCl ($0{\cdot}2 \times$ 150 mM).

close fit to p.d.'s measured by the steady-state method (Diamond & Harrison, 1966). The two methods give p.d.'s agreeing within 0·8 mV over the whole concentration range, confirming the validity of the transient method for mucosal diffusion potentials.

In all, 164 diffusion potential transients following [NaCl] changes in the mucosal solution have been recorded. Voltage–concentration relations reconstructed from these transients were consistently non-linear, as Fig. 6.

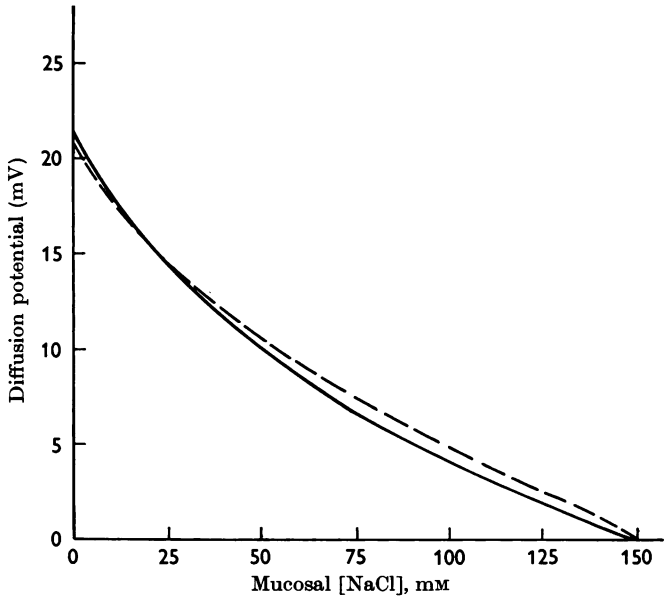

Fig. 6. Relation between diffusion potentials (mV) and mucosal [NaCl] in an everted rabbit gall-bladder, when serosal [NaCl] is maintained at 150 mM. The interrupted curve was obtained from analysis of the p.d. transient of Fig. 5 by the graphical procedure described in the text. The solid curve represents the p.d. given by the constant-field equation with relative Na, Cl, and K permeabilities of 1·00, 0·36, and 1·84, the average values calculated from steady-state diffusion potentials in this gall-bladder.

TABLE 2. Half-times of mucosal diffusion potential transients

[NaCl] (mM)					$t_{\frac{1}{2}}$ (sec)	
	Mucosal					
Serosal					Build-up	Decay
200	200	→	100	→ 200	6·1	4·1
200	200	→	50	→ 200	6·9	3·4
200	200	→	0	→ 200	8·4	2·7

The figures in the right-hand two columns are the half-times for build-up and decay of the diffusion potential when the mucosal NaCl concentration was lowered and then restored to 200 mM. The bathing solution was NaCl Ringer's solution modified to contain 200 mM-NaCl, and mucosal [NaCl] was lowered by isosmotic replacement with sucrose.

This non-linearity is reflected in asymmetry of the half-times, which were always longer for build-up transients (decreases in mucosal [NaCl]) than for decay transients (increase of [NaCl] back to the original value). As illustrated in Table 2, asymmetry of the transients increased with increasing concentration gradient. This asymmetry arises from the fact that steady-state diffusion potentials increase more rapidly than the concentration gradient as mucosal [NaCl] is lowered (reading Fig. 6 from right to left). Thus, at the time when half of the final concentration drop has been completed at the membrane during a build-up transient, the p.d. is still less than half its final value. Conversely, in a decay transient, the p.d. has declined more than half-way back to zero by the time the concentration has risen half-way back towards the original value.

In eight gall-bladders the average value of $t_{\frac{1}{2}}$ was found to be 4·3 sec for diffusion potentials resulting from small concentration gradients of NaCl. Substituting $t = 4{\cdot}3$ sec and $D = 12{\cdot}4 \times 10^{-6}$ cm^2/sec (the free-solution value for D_{NaCl} at 125 mM, 19° C) into $Dt_{\frac{1}{2}}/l^2 = 0{\cdot}38$, one obtains $l = 119\mu$ for the thickness of the mucosal unstirred layer. This is in good agreement with the value of 108 μ deduced from sucrose streaming potential transients.

DISCUSSION

Validity of reconstructing voltage–concentration relations from p.d. transients

The method described here for obtaining voltage–concentration relations has the advantages of (*a*) being rapid; (*b*) avoiding numerous changes of solutions; and (*c*) avoiding problems of comparing different preparations or of correcting for changes in one preparation with time, by collecting all the experimental information on one preparation within a short time. One purchases these advantages at the expense of acquiring three other kinds of difficulties. While the good agreement between results of the transient method and the conventional steady-state method (Figs. 4 and 6) shows that none of these difficulties proved serious in applying the method to the mucosal surface of rabbit gall-bladder, they must be borne in mind in extending the technique to other tissues.

First, the method requires that the properties of the unstirred layer be independent of bathing solution composition, as was proved to hold for the mucosal unstirred layer in rabbit gall-bladder. This condition will probably be satisfied whenever cells are in direct contact with the bathing solution, since the unstirred layer will then be simply a layer of free solution and will be affected solely by the stirring rate. However, when cells are separated from the bathing solution by a layer of connective tissue, the properties of the connective tissue may well be found to change with

bathing solution composition, altering the diffusion path and invalidating the method. For example, preliminary observations indicated that the method gave poor results for the serosal surface of rabbit gall-bladder, because of changes in the serosal connective tissue layer with solute concentration.

Secondly, since the method assumes constancy of the diffusion coefficient D throughout a transient, one is restricted to a range of solutions in which variations of D are small. The principle source of variation is the viscosity-related decrease in D with increasing concentrations of non-electrolytes, and in the present paper this was minimized by reconstructing transients involving sucrose concentrations of 350 mM or less. This effect could be reduced further by using smaller non-electrolytes as mannitol or erythritol, solutions of which are less viscous than sucrose solutions.

Finally, any changes of permeability with concentration must occur in small times compared with the time scale of the transient. Otherwise, the p.d. at a given moment in the transient will not be a unique function of the concentration prevailing then. In rabbit gall-bladder this problem does not arise in diffusion potential transients because there are no changes of ion permeability with NaCl concentration. In streaming potential transients large changes of water permeability with osmolarity certainly do occur (Diamond, 1966). However, the agreement between the transient method and steady-state method is as good for streaming potentials as for diffusion potentials. Furthermore, when one allows for the differing diffusion coefficients of NaCl and sucrose, there is no prolongation of streaming potential transients with respect to diffusion potential transients, such as would have been caused by slow changes in permeability. Evidently, then, the large changes of water permeability with osmolarity in rabbit gall-bladder are complete in a fraction of a second. This would be in keeping with the interpretation that they depend upon swelling and shrinking of the membrane itself. Since osmotic shrinkage of the cells has a half-time of several minutes, streaming potential transients would have been enormously prolonged if the permeability changes had been related to shrinkage of the cell rather than just of the membrane.

Effect of unstirred layers upon steady-state p.d.'s

In the previous paper (Diamond, 1966) the effect of unstirred layers was considered as a possible explanation for the fall-off of osmotic water flow with large osmotic gradients. The osmotic flow of water into a hypertonic solution would reduce by dilution the concentration of solute at the membrane, to an extent depending upon the thickness of the unstirred layer and upon the linear velocity of water flow. This explanation was rendered implausible by two experimental observations: (1) the fall-off of water flow

is the same whether the anisotonic solution is applied to the mucosa or serosa, although the serosal unstirred layer is three times as thick as the mucosal, and diffusion half-times in it ten times as long; (2) the diffusion potential produced by a given mucosal [NaCl] is the same whether osmotic water flow is zero or maximal (p. 90), showing that the dilution effect on NaCl at the membrane is negligible.

The estimates of unstirred layer thicknesses obtained in this paper permit one to calculate this effect quantitatively. In the steady state, the rate at which solute is swept away from the membrane by water flow must equal the rate at which solute diffuses back down the resulting concentration gradient through the unstirred layer: $D\mathrm{d}^2C/\mathrm{d}x^2 = v\mathrm{d}C/\mathrm{d}x$, with the boundary conditions $C = C_o$ at $x = l$, $D = \mathrm{d}C/\mathrm{d}x = 0$ at $x = 0$. v is the linear velocity of water flow (cm/sec), and the other symbols have the same meaning as in the Theory section. The solution for the concentration of solute at the membrane ($C(0)$) is

$$C(0) = C_o e^{-vl/D},$$

$e^{-vl/D}$ is thus the factor by which water flow through the unstirred layer dilutes the concentration of solute at the membrane.

The maximum linear velocity of osmotic water flow in rabbit gall-bladder (with gradients of 400–600 m-osM) is about 10^{-5} cm/sec. At the mucosal surface D is simply the diffusion coefficient of sucrose in free solution ($4{\cdot}4 \times 10^{-6}$ cm^2/sec). Averaging the values from streaming potentials and diffusion potentials, the thickness l of the unstirred layer was found to be 113 μ. Substituting these values gives: $e^{-vl/D} = 0{\cdot}975$. In the serosal connective tissue D is about 23 % below its free-solution value (i.e. about $3{\cdot}4 \times 10^{-6}$ cm^2/sec), and l is 300 μ = 3×10^{-2} cm. Substituting these values gives: $e^{-vl/D} = 0{\cdot}915$. Thus, the effect of the unstirred layer could at most account for a 2·5 % reduction in water flow for high sucrose concentrations in the mucosal solution, and an 8·5 % reduction for sucrose in the serosal solution. Experimentally, the reduction below linearity was 64–76 % for either solution. The effect of unstirred layers is therefore much too small to explain the fall-off of water flow at large osmotic gradients.

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