The generation of non-linear solute gradients for chromatography by using only simple apparatus

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It is shown that by using only a series of tubes with constant cross-sectional area and a single-channel peristaltic pump, it is possible to produce solute gradients with time profiles that are concave, convex, sigmoid or even with turning points. The general theory for predicting gradients, by using tubes in series that are either open or closed to the atmosphere, is presented, and the equations using three compartments are given. Experiments are described that support the usefulness and accuracy of such a theoretical treatment.

There is an ever-present need in biochemical laboratories to produce gradients of salt, pH or nutrients. Perhaps ion-exchange and affinity chromatography are the best known examples, and here there is frequently a requirement for non-linear solute gradients. Usually gradients with convex or sigmoid time profiles are required, but, sometimes the experimentalist wishes to have concave gradients or even more complicated gradients with turning points. Of course, there are commercial instruments for this purpose, but these are expensive and not always freely available, and so we have investigated the nature of gradients that can be produced by using only a single-channel pump and standard laboratory equipment. Because of the widespread interest in this subject, there is a large literature dealing with multi-compartment Varigrad devices (Peterson & Sober, 1959; Burns *et al.*, 1965; Smith & Stahman, 1962), and great ingenuity has been devoted to designing vessels of special shapes to produce non-linear gradients (Baker *et al.*, 1966; Horton, 1967; Bock & Ling, 1954; Margolis, 1969). Paper cut-outs have found application (Keck, 1971), and sometimes nomograms or formulae have been given for predicting gradient profiles as functions of volume delivered (Kocent, 1961; Lakshmanan & Lieberman, 1954; Bock & Ling, 1954; Schwab *et al.*, 1957). However, there does not appear to have been an exhaustive treatment of this question: what solute gradients are possible by using only glass vessels of constant cross section, flexible connecting tubes, a magnetic stirrer and a single-channel peristaltic pump? In the present paper we provide the theory behind two such systems that can be used to produce complicated gradients. Equations are cal-

culated, and experiments are reported, that demonstrate good agreement between predicted solute gradients and actual behaviour.

Outline of the systems under consideration and the nomenclature to be employed

We consider the situation depicted in Fig. 1, where there is a starting vessel open to the atmosphere with volume V_{00} , concentration of solute C_{00} and amount of solute M_{00} at time $t = 0$. This is then connected to a series of n further vessels, each with volume V_{i0} , concentration of solute C_{i0} and amount of solute M_{i0} at zero time ($t = 0$). The n th vessel is connected to a single-channel pump, which delivers at a constant flow rate, q , whereupon the volumes, concentration of solute and total amount of solute as functions of time in the i th compartment will be denoted by $V_i(t)$, $C_i(t)$ and $M_i(t)$. Naturally we suppose that consistent units have been chosen for V_i , C_i , M_i , q and t . It should be noted that:

$$C_i(t) = M_i(t)/V_i(t)$$

and the problem is, given C_{i0} , V_{i0} and q , to predict $C_i(t)$. The outflow from the n th compartment will have concentration $C_n(t)$, and this is the concentration of solute that will be applied to the chromatography column as a function of time. In other words, $C_n(t)$ is the time profile of the solute gradient. Since the total volume delivered by the pump, v , may be thought by some to be a more appropriate independent variable, we note that:

$$v(t) = \int_{u=0}^{u=t} q(u) du$$

u being a dummy variable of integration, and since we are assuming that the flow rate, $q(t)$, is constant at q , and $v(0) = 0$, we simply have that $v = qt$, and so the substitution $t = v/q$ may be applied if functions of v , the total volume delivered, are preferred to functions of t , the time elapsed from commencing the gradient.

There are two distinct ways of operating this

$$C_0(t) = C_{00}$$

$$C_1(t) = C_{10} + (C_{00} - C_{10})(q/V_{10})t$$

$$C_2(t) = C_{20} + 2(C_{10} - C_{20})(q/V_{20})t + [(C_{20} - C_{10}) + (V_{20}/V_{10})(C_{00} - C_{10})](q/V_{20})^2 t^2$$

simple device to generate gradients. In Fig. 1(a) we depict all of the vessels open to the atmosphere and we shall show how this arrangement generates gradients that are polynomials in time, t (or alternatively volume delivered, v). However, in Fig. 1(b) we illustrate another possible arrangement, in which all of the vessels except the first one are closed to the atmosphere, and we shall demonstrate that this arrangement leads to gradients that are multi-exponentials in time, t (or alternatively volume delivered, v).

Generation of polynomial gradients

Consider an assembly such as is illustrated in Fig. 1(a), in which there is an unstirred starting vessel connected in series to n other well-stirred vessels, the gradient emerging from the n th of these vessels at a constant rate of flow q . Since all the vessels are at atmospheric pressure, the rate of flow through the tube out of vessel i will be $(i+1)q/(n+1)$ as long as hydrostatic and hydrodynamic effects are neglected and no correction is made for differences in relative density due to differences in concentration of solute. The net rate of flow from the i th vessel will be $q/(n+1)$. If the further assumption is made that mixing is instantaneous and so all the vessels are homogeneous in concentration of solute throughout the time course of the experiment, then we can describe the assembly by a system of differential equations as follows:

$$\frac{dM_0}{dt} =$$

$$\frac{dM_1}{dt} = qC_{00}/(n+1)$$

$$\frac{dM_2}{dt} = -2M_1/[t - (n+1)(V_{10}/q)]$$

...

$$\frac{dM_n}{dt} = -nM_{n-1}/[t - (n+1)(V_{n-1,0}/q)] + (n+1) M_n/[t - (n+1)(V_{n,0}/q)]$$

The first equation can be integrated directly and the result substituted into the second, which can then be integrated by use of an integrating factor. Continuing in this way, expressions from $M_i(t)$ can be obtained for $i = 0, 1, 2, \dots, n$ and formulae for $C_i(t)$ can then be calculated. $C_i(t)$ will be a polynomial of degree i in the variable t and we now give the results for the first three compartments:

Obviously the concentration in the first, open, unstirred vessel is constant at C_{00} , whereas the second vessel has a linear gradient with positive or negative slope, but evidently the gradient in the third compartment is of the parabolic type. Adding a further compartment would give a cubic gradient, and so on.

The time interval during which this treatment is valid is $0 \leq t \leq V_{10}/q$, where V_{10} is the smallest starting volume of any of the vessels, and it should be remembered that all of the vessels are assumed to have the same cross-sectional area. If this is not assumed, the result is no longer a polynomial in time t . In the assemblies depicted in Fig. 1, the vessels are shown with the same height. It is possible to have different starting volumes (V_{10}) by using vessels of different heights, even though the cross-sectional areas are the same. If this were done, then, to avoid pressure effects, it is necessary to start with the meniscus at a constant level as in the arrangement shown in Fig. 1(a). One inconvenience of this approach is that if the starting volumes (V_{10}) are very different, it may be necessary to use more than one magnetic stirrer.

With one starting compartment and two further compartments, we can produce gradients that are concave, linear or convex and one turning point is possible at positive zeros of dC_2/dt , i.e. if:

$$t = (C_{20} - C_{10})/[(C_{20} - C_{10}) + (V_{20}/V_{10})(C_{00} - C_{10})](q/V_{20})$$

$$- \quad qC_{00}/(n+1)$$

$$+ \quad 2M_1/[t - (n+1)(V_{10}/q)]$$

$$+ \quad 3M_2/[t - (n+1)(V_{20}/q)]$$

Fig. 1. *Experimental assemblies discussed in the text for generating solute gradients*

The vessels are numbered consecutively 0, 1, ... n and the pump, labelled P, delivers fluid at a constant rate, q . The horizontal bars in compartments 1, 2, ... n represent magnetic-stirring followers and all the compartments are joined by narrow flexible tubing, clamps being removed at time $t = 0$ when the pump is switched on. (a) In this arrangement, the vessels are all open to the atmosphere, at the same pressure at time $t = 0$ and of the same cross-sectional area, although the total volumes may differ by having different heights of vessels but with constant levels for each meniscus. Note that q_i , the rate of flow into the i th vessel, is given by $q_i = iq/(n+1)$. The gradient is a polynomial in time. (b) This is an alternative arrangement, differing from (a) in that rubber bungs, labelled B, are used to seal compartments 1, 2, ... n from the atmosphere, so that these vessels have no volume change until vessel 0 is exhausted. In this set-up, the volumes and cross-sectional areas of the tubes 0, 1, 2, ... n are immaterial to the mathematical analysis. The gradient is a multi-exponential function in time or a combination of exponentials and polynomials in time or total volume delivered if any of the vessels have identical volumes.

is positive and realized experimentally. It can be shown that, when $C_{00} > C_{10} > C_{20}$ and $(C_{20} - C_{10}) + (V_{20}/V_{10})(C_{00} - C_{10}) < 0$, then the gradient is always increasing and the turning point is not actually reached before the smallest vessel empties. More complicated gradients with several turning points are, of course, possible with additional compartments.

Generation of multi-exponential gradients

Here we analyse the situation depicted in Fig. 1(b), in which only one vessel is open to the

atmosphere, the others being closed. Because of this restriction, there is no volume change in vessels other than the open one. So, if the pump rate is constant at q , this will also be the rate of flow out of and into all compartments, and the mathematical analysis to be given is valid in the time range $0 \leq t \leq V_{00}/q$, i.e. until the open vessel is exhausted. The range can be extended if need be after the open vessel is exhausted, because then vessel 1 becomes open. Note that in this analysis it is not necessary to have constant cross-sectional area in the vessels.

The system is described by this set of linear differential equations:

$$\begin{bmatrix} \frac{dM_1}{dt} \\ \frac{dM_2}{dt} \\ \frac{dM_3}{dt} \\ \dots \\ \frac{dM_n}{dt} \end{bmatrix} = \begin{bmatrix} -q/V_{10} & 0 & 0 & \dots & 0 \\ q/V_{10} & -q/V_{20} & 0 & \dots & 0 \\ 0 & q/V_{20} & -q/V_{30} & \dots & 0 \\ \dots & \dots & \dots & \dots & \dots \\ 0 & 0 & 0 & \dots & -q/V_{n0} \end{bmatrix} \begin{bmatrix} M_1 \\ M_2 \\ M_3 \\ \dots \\ M_n \end{bmatrix} + \begin{bmatrix} qC_{00} \\ 0 \\ 0 \\ \dots \\ 0 \end{bmatrix}$$

Table 1. Gradients possible with one open and two closed compartments

When $V_{10} = V_{20} = V$ in the arrangement in Fig. 1(b), then the equation describing the solute-gradient-concentration profile as a function of time is:

$$C_2(t) = C_{00} + [(C_{20} - C_{00}) + (q/V)(C_{10} - C_{00})t] \exp(-q/V)t$$

Alternatively, the substitution $t = v/q$ may be used to express the profile as a function of v , the total volume delivered by the pump. When $t = 0$, then $C_2(0) = C_{20}$ and other specific values are as follows. When $t = V_{00}/q$, the open vessel empties and:

$$C_2(V_{00}/q) = C_{00} + [(V_{00}/V)(C_{10} - C_{00}) + (C_{20} - C_{00})] \exp(-V_{00}/V)$$

If $t = (C_{10} - C_{20})V/(C_{10} - C_{00})q$, there is a turning point and:

$$C_2 = C_{00} + (C_{10} - C_{00}) \exp-(C_{10} - C_{20})/(C_{10} - C_{00})$$

If $t = (2C_{10} - C_{00} - C_{20})V/(C_{10} - C_{00})q$, there is a point of inflexion and:

$$C_2 = C_{00} + 2(C_{10} - C_{00}) \exp-(2C_{10} - C_{00} - C_{20})/(C_{10} - C_{00})$$

This information allows a classification of possible gradients into one of the six types shown in Fig. 2. The inequalities involved are now listed.

Inequalities and equalities required	Turning point	Inflexion point	Shape in Fig. 2
$C_{00} > C_{10} > C_{20}$	No	No	1
	No	Yes ($t = 0$)	1
	No	Yes ($0 < t < V/q$)	2
$C_{00} < C_{10} < C_{20}$	No	Yes ($0 < t < V/q$)	3
	No	Yes ($t = 0$)	4
	No	No	4
$C_{10} \geq C_{00}$ and $C_{10} \geq C_{20}$	Maximum ($t > V/q$)	Yes ($t > 2V/q$)	5 (i)
	Maximum ($t = V/q$)	Yes ($t = 2V/q$)	5 (ii)
	Maximum ($0 < t < V/q$)	Yes ($V/q < t < 2V/q$)	5 (iii)
	Maximum ($t = 0$)	Yes ($t = V/q$)	3
$C_{10} \leq C_{00}$ and $C_{10} \leq C_{20}$	Minimum ($t > V/q$)	Yes ($t > 2V/q$)	6 (iii)
	Minimum ($t = V/q$)	Yes ($t = 2V/q$)	6 (ii)
	Minimum ($0 < t < V/q$)	Yes ($V/q < t < 2V/q$)	6 (i)
	Minimum ($t = 0$)	Yes ($t = V/q$)	2

Fig. 2. Gradients possible with one open and two closed compartments

A common experimental arrangement would be that illustrated in Fig. 1(b), in which one unstirred vessel is open to the atmosphere and this is connected in series to two further closed vessels containing an equal volume, i.e. $V_{10} = V_{20} = V$, which remains constant for $0 < t < V_{00}/q$. The gradient commences at C_{20} , when $t = 0$, and continues at $C_2(t)$ until $t = V_{00}/q$, when the open vessel empties. Six gradients are possible as follows: 1, concave increasing gradients; 2, sigmoid increasing gradient; 3, sigmoid decreasing gradient; 4, convex decreasing gradient; 5, gradient with a maximum; 6, gradient with a minimum. Note that gradients 5 and 6 can finish either (i) above C_{20} , (ii) at C_{20} or (iii) below C_{20} , and the inequalities that are necessary and sufficient for these gradient profiles will be found in Table 1. It is possible to replace time t as the independent variable by $v(t)/q$, where $v(t)$ is the total volume delivered by the pump.

with characteristic equation:

$$|A - \lambda I| = -(1)^n \prod_{i=1}^n (q/V_{i0} + \lambda)$$

The whole solution will be the n real negative exponentials corresponding to the eigenvalues $-q/V_{i0}$ representing the solution for the homogeneous system, plus the particular integral corresponding to the column vector. Identities such as $V_{i0} = V_{j0}$, $i \neq j$, lead to repeated eigenvalues, and the solution will then have polynomials of degree k in t multiplying the exponential representing the repeated eigenvalues of order $k+1$. This is discussed in standard textbooks dealing with differential equations. Where

$$C_2(t) = C_{00} + [(C_{20} - C_{00}) + (q/V)(C_{10} - C_{00})t] \exp(-q/V)t; \quad V = V_{10} = V_{20}$$

Fig. 3. Experimentally generated parabolic gradients

The gradients, shown as C_2 in units of A_{350} as a function of total volume, v , delivered by the pump, were generated by using the apparatus illustrated in Fig. 1(a), with vertical glass vessels of diameter 35mm, and with a flow rate (q) of $20 \text{ ml} \cdot \text{h}^{-1}$. The total volume delivered by the pump in time t is v , where $v = qt$ and all experiments were conducted until all vessels were empty, i.e. until $v = 3V_{00} = 600 \text{ ml}$. The flexible connecting tubes had a diameter of 4 mm, but each contained a plug with internal diameter 1 mm to act as a flow restricter. The volumes at time $t = 0$ (i.e. $v = 0$) were $V_{00} = V_{10} = V_{20} = 200 \text{ ml}$, and in each case the solid line was calculated by using the equation:

$$C_2(v) = C_{20} + 2(C_{10} - C_{20})(v/V_{20}) + [(C_{20} - C_{10}) + (V_{20}/V_{10})(C_{00} - C_{10})](v/V_{20})^2$$

The concentrations at time $t = 0$ (i.e. $v = 0$), measured as A_{350} , with sodium dichromate, were as follows: (i) $\circ$: $C_{00} = 1$, $C_{10} = 0.2$, $C_{20} = 0$; $\triangle$: $C_{00} = 1$ (in 1 M-NaCl), $C_{10} = 0.2$ (in 0.2 M-NaCl), $C_{20} = 0$. (ii) $\circ$: $C_{00} = 1.0$, $C_{10} = 0.8$, $C_{20} = 0$; $\triangle$: $C_{00} = 1.0$ (in 1 M-NaCl), $C_{10} = 0.8$ (in 0.8 M-NaCl), $C_{20} = 0$.

there is only one closed vessel the solution is given by:

$$C_1(t) = C_{00} + (C_{10} - C_{00}) \exp(-q/V_{10})t$$

but where there are two closed vessels, i.e. three vessels in all, then the same expression holds for $C_1(t)$ and the concentration of the solute emerging from vessel 2 as a function of time is:

$$C_2(t) = C_{00} + [V_{10}(C_{10} - C_{00})/(V_{10} - V_{20})] \exp(-q/V_{10})t + [C_{20} + (C_{00}V_{20} - C_{10}V_{10})/(V_{10} - V_{20})] \exp(-q/V_{20})t$$

In the special case when $V_{10} = V_{20} = V$, then the solution is found to be:

With one closed vessel it is only possible to obtain exponential types of gradients, but with two closed

Fig. 4. Experimentally generated gradients with turning points or of the sigmoid type

The gradients, shown as C_2 in units of A_{350} as a function of total volume, v , delivered by the pump, were generated by using the apparatus illustrated in Fig. 1(b) with vertical glass vessels of diameter 35mm, and with a flow rate (q) of $20 \text{ ml} \cdot \text{h}^{-1}$. The total volume delivered by the pump in time t is v , where $v = qt$, and all experiments were conducted until the unstirred vessel was empty, i.e. until $v = V_{00} = 400 \text{ ml}$. The flexible connecting tubes had a diameter of 4 mm, but each contained a plug with internal diameter 1 mm to act as a flow restricter. The volumes at $t = 0$ (i.e. $v = 0$) were $V_{00} = 400 \text{ ml}$, $V_{10} = V_{20} = 200 \text{ ml}$, and the solid line was calculated, by using the equation:

$$C_2(v) = C_{00} + [(C_{20} - C_{00}) + (C_{10} - C_{00})(v/V_{10})] \exp(-v/V_{10})$$

The concentrations at time $t = 0$ (i.e. $v = 0$), measured as A_{350} with sodium dichromate, were as follows: (i) $\circ$: $C_{00} = 1.68$, $C_{10} = 0.02$, $C_{20} = 0$; $\triangle$: $C_{00} = 1.68$ (in 1.68 M-NaCl), $C_{10} = 0.02$ (in 0.02 M-NaCl), $C_{20} = 0$. (ii) $\circ$: $C_{00} = 0$, $C_{10} = 1.6$, $C_{20} = 0.5$; $\triangle$: $C_{00} = 0$, $C_{10} = 1.6$ (in 1.6 M-NaCl), $C_{20} = 0.5$ (in 0.5 M-NaCl).

vessels a variety of curves is possible, including those of the sigmoid variety. Also, gradient profiles with turning points can be produced. To give some idea of the shapes possible, Table 1 presents a classification of all curves that can be realized with $V_{10} = V_{20}$, and these are illustrated in Fig. 2.

Evidently curves of great complexity are possible when additional closed vessels are employed.

Accuracy with which predicted gradients may be realized in the laboratory

Since the foregoing analysis was based on a neglect of differential hydrostatic effects and assumed instantaneous perfect mixing to homogeneity in all vessels, it seems important to have some idea of how well the predicted non-linear solute gradients can be actually generated with laboratory equipment. In particular, it is of interest to know how closely the predicted gradients are paralleled when it is attempted to produce solute gradients similar to those required in ion-exchange or affinity chromatography. A gradient varying from zero to, say, 1 or 2 M might be typical. We have, therefore, tested the equations experimentally by using solutions with no appreciable relative-density effects represented by dilute sodium dichromate solution, the gradient being measured by collecting fractions and reading the absorbance. Also, in order to ascertain the extent of any correction required when salt gradients are used, we have then repeated the same experiments with NaCl added to the vessels in proportion to the dichromate to bring the molarity to requirement in the open compartment. Corrections should, of course, be minimal with the closed compartments, but might be appreciable with open ones. The results of these experiments are shown in Fig. 3 for a concave and a convex gradient produced by using three open compartments, and in Fig. 4 a sigmoid gradient and one with a turning point produced by two closed vessels and one open

one are illustrated. Experimental details will be found in the legends to these Figures.

Conclusion

Theoretical calculations have shown how complex non-linear solute gradients can be expected with an apparatus consisting only of a number of vessels in series, flexible connecting tubing, a magnetic stirrer and a peristaltic pump operating at a constant flow rate. The governing differential equations have been given, the general method of solution is outlined and the integrated equations for up to three compartments have been calculated. It has been shown experimentally that the solute gradients actually obtained are extremely close to the profiles predicted theoretically and that corrections due to differential hydrostatic effects when salt gradients up to 1 M are used are minimal.

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