DIFFUSION COEFFICIENTS OF GLUCOSE AND ETHANOL IN CELL-FREE AND CELL-OCCUPIED CALCIUM ALGINATE MEMBRANES

Betty Jane Miller Hannoun

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Department of Chemical Engineering
California Institute of Technology
Pasadena, California 91125

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ABSTRACT

The diffusivities of glucose and ethanol in cell-free and cell-occupied membranes of calcium alginate were measured in a diffusion cell. The lag time analysis was employed. Diffusivities decreased with increasing alginate concentration and were comparable to those in water for a 2% alginate membrane. Glucose and ethanol concentrations had no effect on the respective diffusion coefficients. The ratio of ethanol diffusivity to glucose diffusivity in 2% and 4% alginate agreed closely to the inverse ratio of the hydrodynamic radii for the two molecules in water indicating that the hydrodynamic theory of diffusion in liquids may be applicable to diffusion in dilute alginate gels. Also the presence of 20% dead yeast cells had no effect on the diffusivities. The data reported can be used to study reaction and diffusion in immobilized cell reactors and cell physiology under immobilized conditions.

INTRODUCTION

The immobilization of cells in calcium alginate has been widely studied for the production of biochemicals. Alginate is a naturally-occurring polymer consisting of β-D-mannuronic and α-L-guluronic acid units linked by 1,4-glycosidic bonds[1]. Monovalent alginate salts are water soluble, while polyvalent cations cause cross-linking between macromolecular chains. By diffusing calcium ions into an alginate solution, a polymer gel is formed. Several factors have contributed to the interest in cell immobilization in calcium alginate. By immobilizing cells dense cell cultures can be established leading to faster overall reaction rates. The cells are retained in the reactor and therefore used for a longer period of time, reducing the need for new biomass synthesis. Increased yields have been reported for growth and nongrowth associated products[2-3]. Furthermore, a cell-free product stream simplifies downstream processing. Finally, a high percentage of the cells remain viable during calcium alginate immobilization[4], and the activity of the cells persists for long periods of time.

Despite the potential of immobilized cell processes for improved efficiency, practical problems have often prevented the benefits from being realized. An important disadvantage of immobilized cells is that the transport of nutrients and products to and from the cells can become rate limiting, decreasing the cells' overall productivity[5]. Additionally, rapid cell growth occurs in a small outer shell of alginate beads[6-7], decreasing product yields. Due to diffusion limitations and cell growth in the outer shell, as little as 10% of the alginate bead may contain active cells. Furthermore, rapid growth near the gel surface leads to cell leakage into the product stream and breakup of the support.

Investigations on the behavior of calcium alginate immobilized cells have been hindered by concentration gradients and cell growth in the alginate. Experimental complexities have prevented researchers from determining the cell count and concentrations of substrates and products near the cells. Measuring substrate and product concentrations in the liquid phase does not adequately describe intramatrix concentrations, as is often assumed. Thus it is difficult to determine the effects of substrate and product concentrations on specific rates of growth, substrate utilization, and product synthesis for immobilized cells.

Experiments were conducted to determine the rates of glucose and ethanol diffusion through calcium alginate under various conditions. Diffusion coefficients have been measured previously by other investigators for a few substrates in matrices suitable for immobilized cells. Table 1 summarizes the results. Oxygen and sucrose in 2% agar were measured to diffuse at 70% and 72%, respectively, of their diffusion rates in water[8-9]. The diffusion coefficient of glucose was determined to be the same in 2% calcium alginate as it is in water[10]. Oxygen, however, was reported to have a diffusion coefficient in 2% barium alginate of only 25% of its diffusion coefficient in water[5].

Diffusion coefficients under various conditions are needed to model reaction and diffusion in immobilized cell reactors. The reactor operating conditions can then be optimized for productivity using reaction-diffusion models. For example the feed substrate concentration and residence time may be manipulated to control cell growth. Some growth may be desirable to prevent enzyme deactivation, while excess growth causes cell leakage, support breakup, and reduced product yields. The accumulation of toxic products inside

TABLE 1

Substrate	Matrix	Diffusion coefficient (cm^2/sec)	Fraction of diffusivity in water	Reference
glucose	2% calcium alginate	6.8×10^{-6}	1.0	10
sucrose	2% agar	6.7×10^{-6}	0.72	8
oxygen	2% agar	1.9×10^{-5}	0.70	9
oxygen	2% barium chloride	7.0×10^{-6}	0.25	5

Substrate diffusion coefficients in immobilization matrices

the matrix can be prevented by maintaining a production rate equal to the rate of product diffusion out of the alginate. Since steep concentration gradients are present in alginate beads, the effect of concentration on the diffusion coefficients needs to be investigated. Additionally, the presence of cells in the gel may alter some properties of the alginate, including diffusion coefficients. In Saccharomyces cerevisiae metabolism the Crabtree effect, Pasteur effect, and product inhibition are controlled by glucose, oxygen, and ethanol concentrations, respectively. The first step in analyzing and explaining these metabolic patterns for immobilized cells is to determine the diffusivities of the effectors under various conditions. The results are reported here for glucose and ethanol diffusion in calcium alginate.

THEORY

The diffusion coefficients of glucose and ethanol in calcium alginate were determined using the lag time analysis. An alginate membrane of thickness 4.2 mm is suspended between two well mixed chambers of concentrations c_1 and c_2 in the component whose diffusivity is to be measured. The system is shown schematically in Figure 1. Assuming no film mass transfer resistance between the bulk fluids in the two chambers and the membrane, the transient diffusion process inside the membrane is governed by the partial differential equation:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \tag{1}$$

where c is the concentration in the membrane,

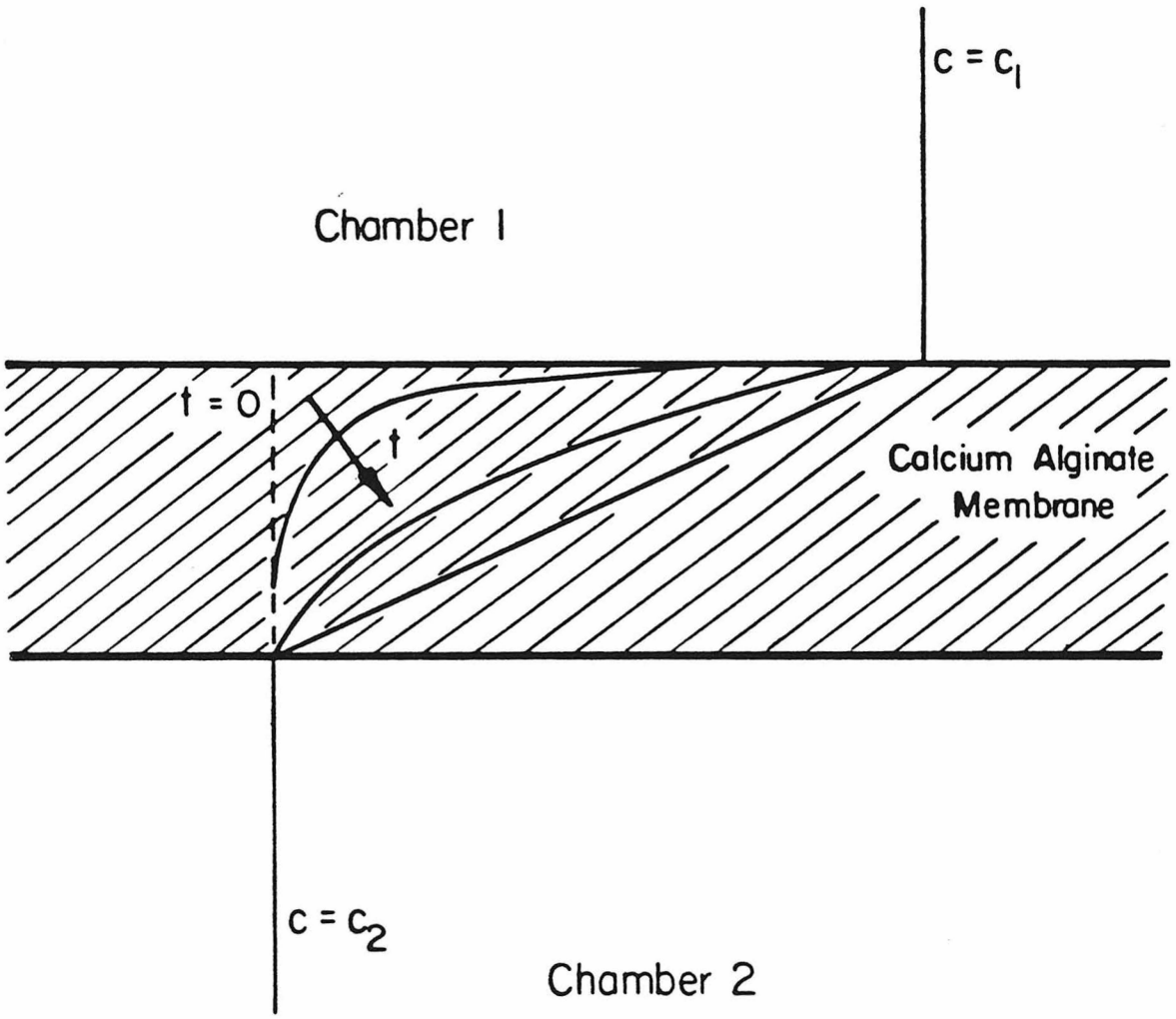
D is the diffusion coefficient,

t is time,

and x is distance,

subject to the boundary conditions:

Figure 1: Concentration gradient in a calcium alginate membrane.



$$c = c_1 \quad \text{at} \quad x = 0$$

$$c = c_2 \quad \text{at} \quad x = \ell \tag{2}$$

During the experiment solute diffuses from the chamber where $c = c_1$ through the membrane into the chamber where $c = c_2$. Thus the concentrations in the two chambers are not precisely constant as is assumed in the solution of the differential equation. For large enough chambers, however, the changes in concentration over the time of the experiment are small and as a result the derived solution fits the experimental results well.

The experiment can be designed so that none of the diffusing component is present initially in the membrane. Then the initial condition to equation (1) is:

$$c = 0 \qquad 0 \leq x \leq \ell \qquad \text{at } t = 0 \tag{3}$$

The solution to equation (1) with boundary conditions (2) and initial condition (3) is given by[11]

$$c = c_1 + (c_2 - c_1)\frac{x}{\ell} + \left\{ \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{c_2 \cos n\pi - c_1}{n} \sin \frac{n\pi x}{\ell} \exp\left(\frac{-Dn^2\pi^2 t}{\ell^2}\right) \right\} \tag{4}$$

Daynes applied the partial differential equation solution (4) to gas diffusion through a rubber membrane, developing the lag time analysis to measure gaseous diffusion coefficients[12]. The solution (4) is simplified by making the diffusing component concentration zero in one of the chambers ($c_2 = 0$). Then we can write

$$c = c_1 \left(\frac{\ell - x}{\ell}\right) + \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{-c_1}{n} \sin \frac{n\pi x}{\ell} \exp\left(\frac{-Dn^2\pi^2 t}{\ell^2}\right) \tag{5}$$

It is convenient to measure the concentration of the diffusing component as a function of time in the chamber where c_2 is assumed to be zero. As previously

mentioned, the actual deviation from zero is small so that the experimental data fit the theoretical results well. By differentiating equation (5) we can determine the instantaneous flux into the chamber

$$F|_{x=\ell} = -D\left(\frac{\partial c}{\partial x}\right)_{x=\ell} = \frac{Dc_1}{\ell}\left[1 + 2\sum_{n=1}^{\infty}(-1)^n \exp\left(\frac{-Dn^2\pi^2 t}{\ell^2}\right)\right] \tag{6}$$

Integrating equation (6) with respect to t from t = 0 to t = t_s and multiplying by the membrane area A determines $Q|_{t_s}$, the total amount of solute transferred through the membrane at the time of sampling. This amount of course is the product of the measured concentration in the chamber where c_2 = 0 and the chamber volume. Thus we find

$$Q\Big|_{t_s} = \frac{ADc_1 t_s}{\ell} + \frac{2\ell A}{\pi^2}\sum_{n=1}^{\infty}\frac{c_1(-1)^n}{n^2}\left\{1-\exp\left(\frac{-Dn^2\pi^2 t_s}{\ell^2}\right)\right\} = (V_c)\Big|_{c_2,t_s} \tag{7}$$

Equivalently,

$$Q|_{t_s} = \frac{ADt_s c_1}{\ell} - \frac{Ac_1\ell}{6} - \frac{A2\ell c_1}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(\frac{-Dn^2\pi^2 t_s}{\ell^2}\right) \tag{8}$$

For sufficiently large times the summation term on the right hand side of equation (8) becomes insignificant. In this case the total amount of solute transferred increases linearly with time

$$Q|_{t_s} = \frac{ADc_1}{\ell}\left(t_s - \frac{\ell^2}{6D}\right) \tag{9}$$

A graph of Q versus t approaches a straight line, which intercepts the time axis at t = $\frac{\ell^2}{6D}$. Figures 2 and 3 are experimental graphs of Q versus t for the diffusion of glucose and ethanol, respectively, in 2% calcium alginate. The intercept of the linear part of the curve is referred to as the lag time.

Figure 2: Lag time for glucose diffusion in 2% calcium alginate. C_1 = 10 g/ℓ, t_o = 81.4 min

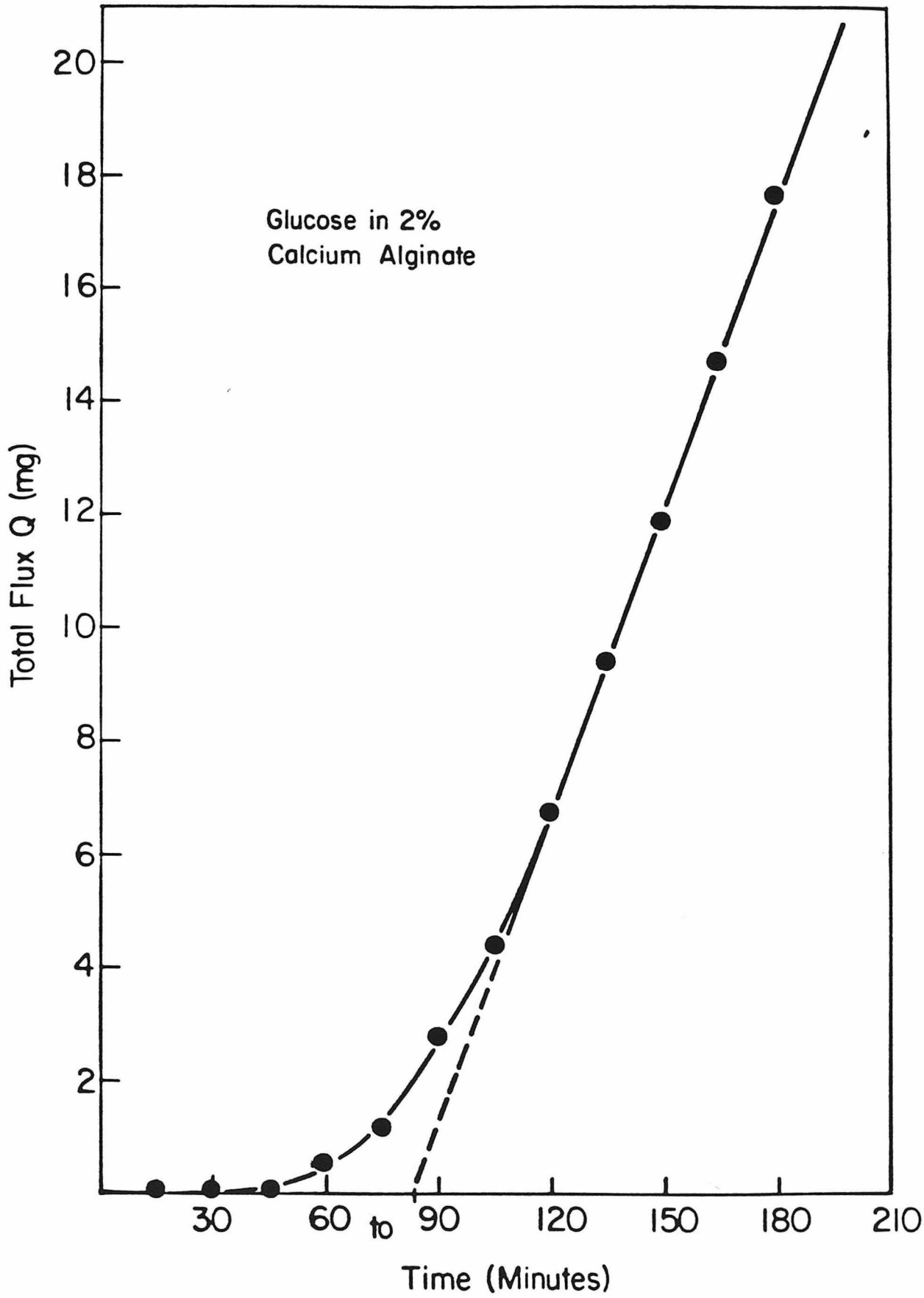
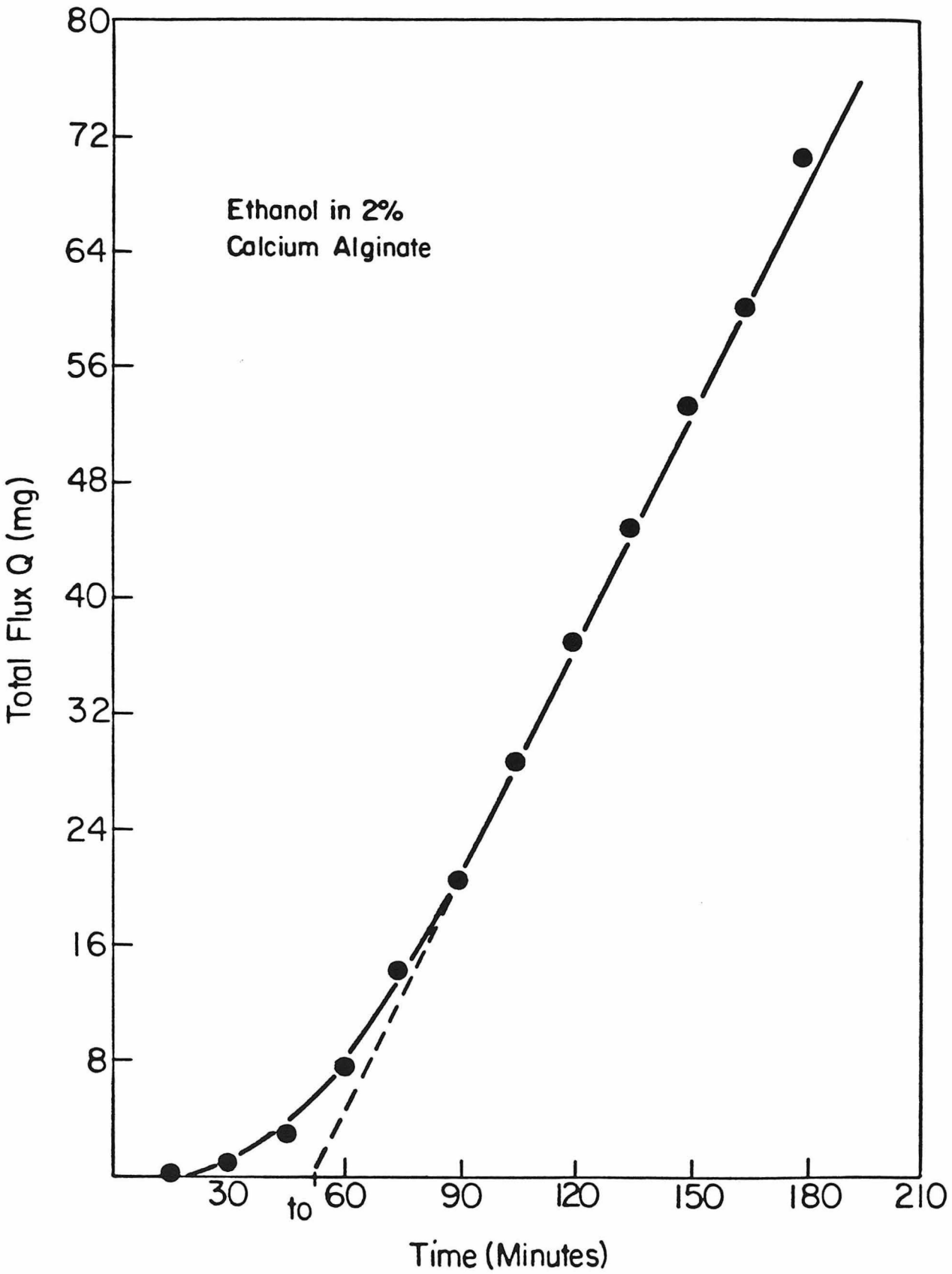


Figure 3: Lag time for ethanol diffusion in 2% calcium alginate. C_1 = 10 g/ℓ, t_o = 51.1 min



Diffusion coefficients are calculated from the lag time and the membrane thickness.

Rogers, et al.[13] took only the leading term of the rapidly converging series in equation (6) and obtained the equation

$$\ln (t^{1/2}F) = \ell n \{2c_1(\frac{D}{\pi})1/2\} - \frac{\ell^2}{4Dt}$$

Plotting $\ln(t^{1/2}F)$ versus $1/t$ results in a straight line of slope $-\frac{\ell^2}{4D}$. Thus the diffusion coefficient is measured from the slope of the line. Determining F experimentally requires differentiating a graph of Q versus t at various points since $F = \frac{1}{A}(\frac{dQ}{dt})$, where A is the membrane area. Differentiation of the Q versus t graph introduced additional error, however, which led to significant scattering of the data. Therefore, Roger's method of measuring the diffusion coefficients was not applied to the experimental data.

In addition to the lag time analysis and Roger's, method diffusion coefficients are measured by the steady-state method[14]. At steady-state equation (1) becomes

$$0 = D \frac{d^2c}{dx^2} \tag{9}$$

Applying the boundary conditions (2) to equation (9) gives the steady-state concentration profile

$$c = c_1 + (c_2-c_1) \frac{x}{\ell} \tag{10}$$

The flux at steady state is given by

$$F = -D(\frac{dc}{dx}) = \frac{D((c_1-c_2))}{\ell} = \frac{1}{A}(\frac{dQ}{dt}) \tag{11}$$

The slope of the straight line in the Q versus t plot is simply $(\frac{dQ}{dt})$ at steady-state. Therefore,

$$D = \frac{\ell}{(c_1-c_2)A} \left(\frac{dQ}{dt}\right)_{ss} \quad (12)$$

The disadvantage of the steady-state method is that more parameters are required. In addition to the membrane thickness, it is necessary to know the concentration difference across the membrane and the area of the membrane. For solute partition coefficients, K_p, other than 1.0, the concentration gradient across the membrane equals K_p (c_1-c_2) rather than (c_1-c_2). Measuring the concentration difference in the chambers and the partition coefficient introduced additional error in the diffusion coefficient. Furthermore, since a wire mesh was used to support the membrane, the surface area for transport was not known precisely. The steady-state method was found to give less consistent results than the lag time analysis for the experimental system used.

MATERIALS AND METHODS

Solutions of 1%, 2% and 4% sodium alginate were made by dissolving 1g, 2g, and 4g respectively of sodium alginate (Fisher Scientific, Pittsburgh, PA), in 100 ml of deionized water. The solutions were then autoclaved to prevent contamination. Membranes of 4.2 mm thickness and 10.9 cm diameter were cast in a metal ring between two porous glass plates and hardened in a bath of 2% (w/v) calcium chloride. 1% and 2% alginate membranes were hardened for 2-1/2 hours. 4% alginate membranes required 3 hours in the bath before hardening was complete. Since the diffusion coefficient measured by the lag time analysis is proportional to the square of the membrane thickness, the accuracy of the experimental diffusion coefficients depends on the measurement of the membrane thickness to a large degree. In order to make suitable and consistent membranes for the experiments the metal ring used to mold the alginate membranes must be uniformly thick and the glass plates must be flat.

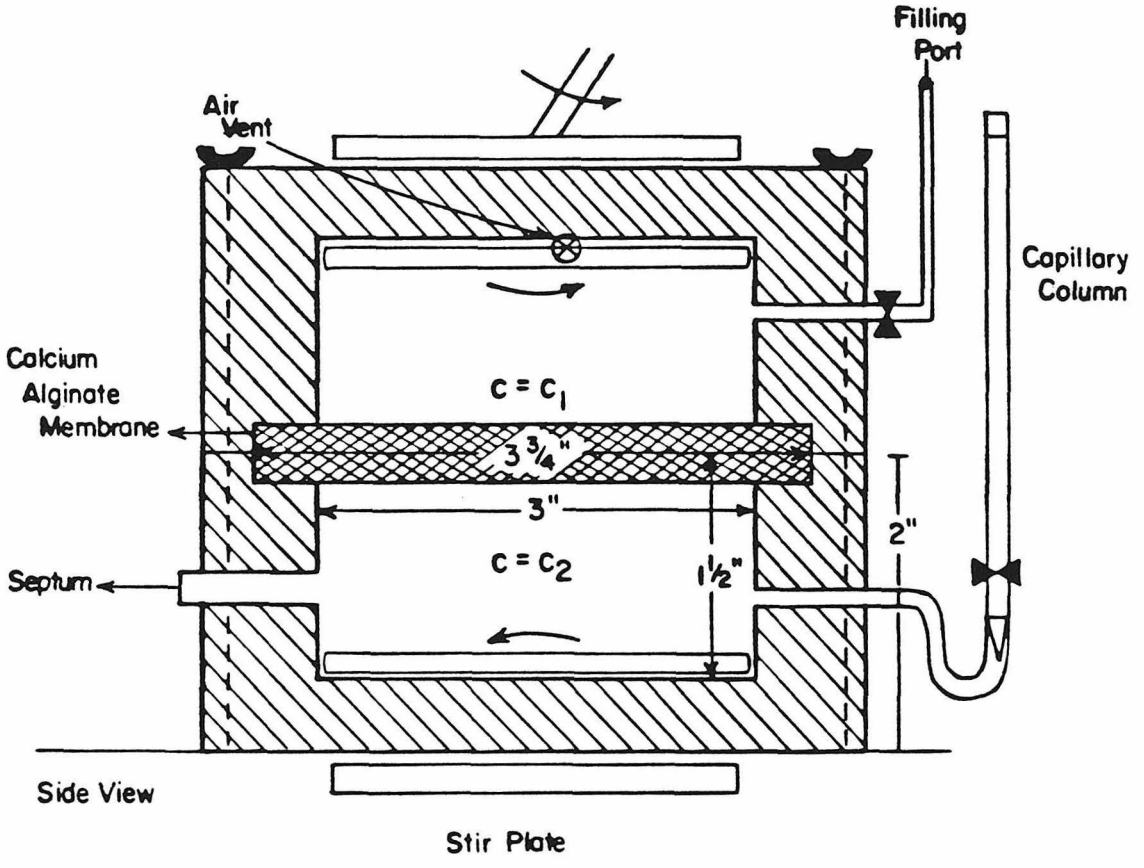
Slightly warped plates were found to produce nonuniform membranes which introduced reproducibility problems in the measured values of the diffusivities. Using a micrometer the membrane thickness could not be measured consistently because the gel compresses under very light pressure. The membrane thickness was best controlled by weighing an appropriate amount of the alginate solution before casting between the glass plates.

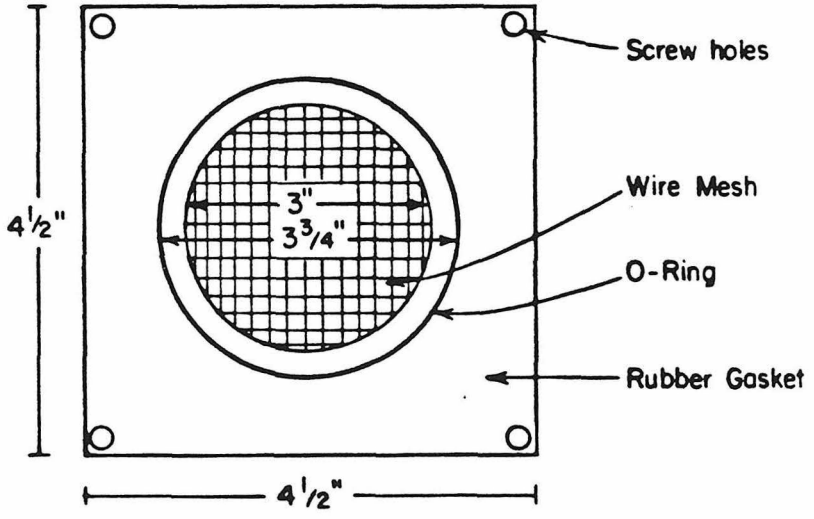
Membranes containing S. cerevisiae cells were prepared as follows. A dense cell suspension was washed with 50% ethanol to kill the yeast. Staining with methylene blue showed cell death to be complete without lysis of the cells. The yeast were centrifuged, then washed with water and centrifuged again. Membranes containing 2% alginate plus yeast were prepared by mixing 20% (wt.) cells, 40% (wt.) of 4% alginate solution, and 40% (wt.) deionized water. The required amount of the mixture was cast to produce a membrane 4.2 mm thick. Membranes of 1% alginate with cells were similarly prepared.

Diffusion Cell

Diffusion experiments were performed in a plexiglass cell consisting of two half-cells. The apparatus is shown in Figure 4. Each half-cell contained a cylindrical chamber of 152 ml volume. The half-cells were held together with screws so that the chambers connected. A calcium alginate membrane supported by a wire mesh was placed between the chambers and sealed with o-rings. The area around the chambers was sealed with a rubber gasket. The lower chamber, filled initially with deionized water was connected to a capillary column. Samples were withdrawn from the lower chamber through a septum. The upper chamber contained a solution of glucose or ethanol. Both chambers were magnetically stirred. The capillary column provided a reservoir of water to replace liquid removed during sampling. Diffusion experiments

Figure 4: Diffusion Cell.





Top View of Bottom Half Cell

were conducted at 22-26°C.

Analyses

Glucose concentrations were assayed using hexokinase/glucose-6-phosphate dehydrogenase enzyme kits (Sigma Chem. Co., St. Louis, MO). Ethanol concentrations were determined by gas chromatography on a Chromabsorb 102 column (Varian Associates, Walnut Creek, CA).

RESULTS AND DISCUSSION

The diffusion coefficient of glucose in 2% calcium alginate is 6.1 x 10^{-6} cm^2/sec. Under the same conditions the diffusion coefficient of ethanol is 1.0 x 10^{-5} cm^2/sec. Both values are only slightly lower (9% lower) than the respective diffusion coefficients in water, as shown in Table 2. Tanaka, et al., reported similar results for glucose diffusion into and from 2% calcium alginate beads[10]. In dilute water solutions, ethanol with a hydrodynamic radius of 2.25 Å diffuses 1.6 times faster than glucose with a hydrodynamic radius of 3.61 Å. Similarly, the ratio of ethanol diffusivity to glucose diffusivity in 2% calcium alginate is 1.6. The consistency of the diffusivity ratio indicates that the hydrodynamic theory of diffusion in liquids may be applicable to diffusion in dilute alginate gels.

The diffusion coefficients of glucose and ethanol in 1%, 2% and 4% alginate are shown in Figure 5. Increasing the alginate concentration from 1% to 4% leads to a significant decrease in the ethanol diffusion coefficient. The diffusion coefficient of glucose also decreases with increasing alginate concentration. Klein, et al measured the maximal pore size by inverse size exclusion chromatography and determined that calcium alginate gels have maximal pore diameters on the order of 150 Å for 3% Manugel DLB alginate and 7% Manucol LD alginate[1]. Scherer, et al. reported the diameter of calcium

TABLE 2

Substrate	Diffusion coefficients (cm^2/sec)		
	2% calcium alginate membrane	2% calcium alginate beads	water
Glucose	6.1×10^{-6}	6.8×10^{-6} (a)	6.7×10^{-6} (b)
Ethanol	1.0×10^{-5}	---	1.1×10^{-5} (c)

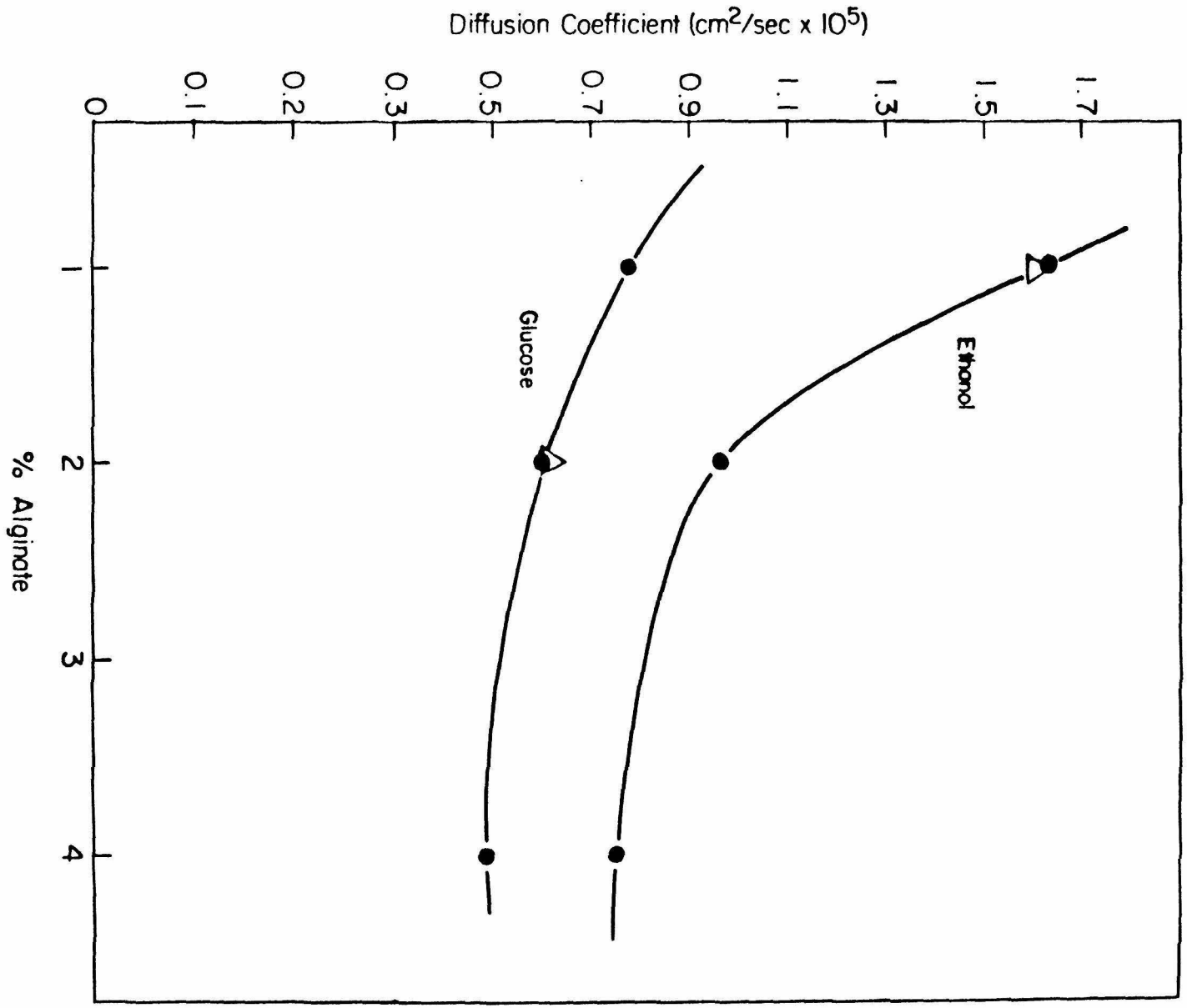
(a) Reference 10
(b) Reference 15
(c) Reference 16

Glucose and ethanol diffusion coefficients in 2% alginate and water

Figure 5: Effect of alginate concentration on glucose and ethanol diffusion coefficients.

● cell-free membrane
△ cell-occupied membrane

C_1 = 10 g/ℓ



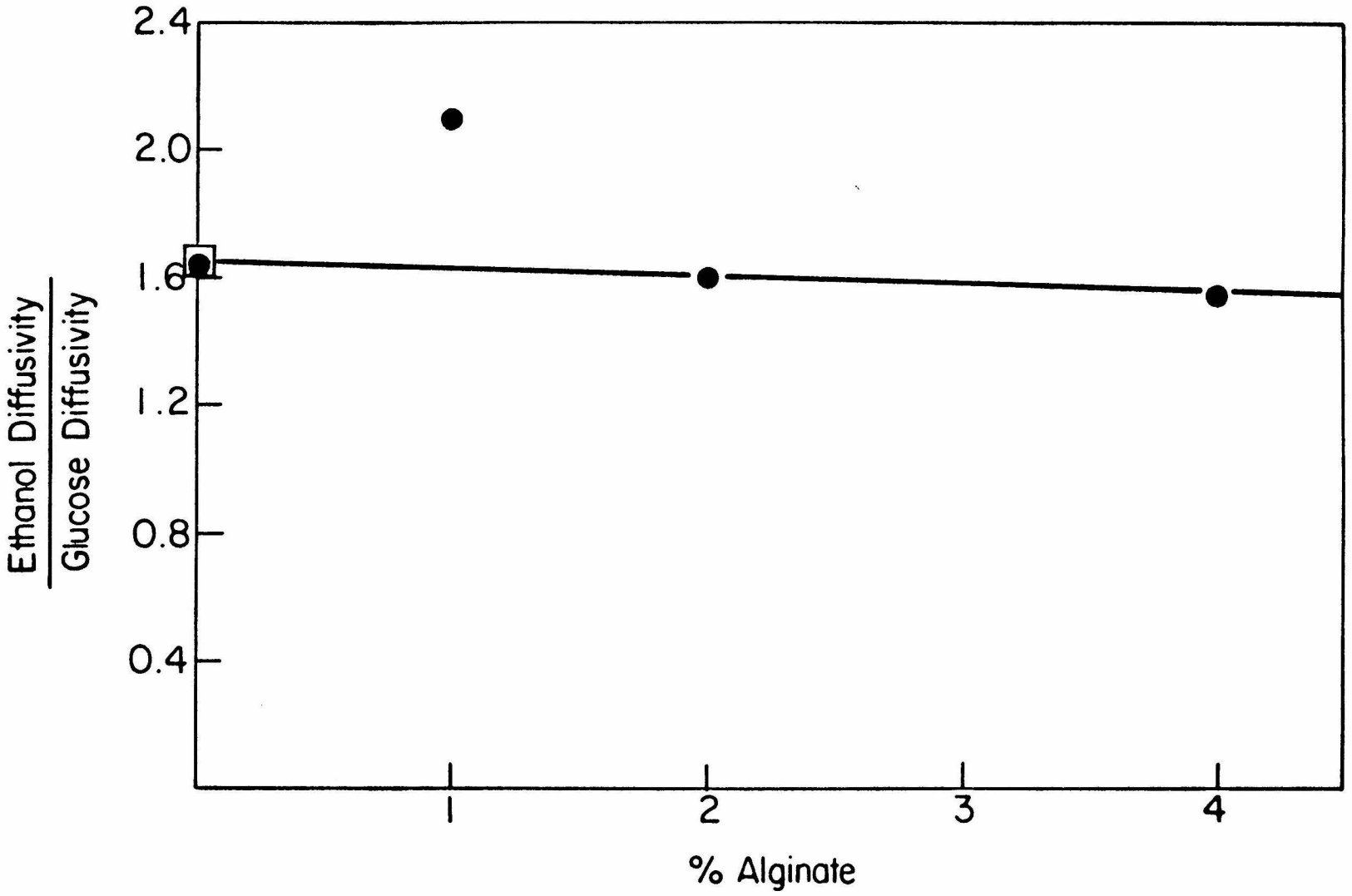
alginate pores measured by scanning electron microscopy to be on the order of 10 μm[17]. Klein, et al. explained the discrepancy by observing that the surface structure of alginate gels have a lower porosity than the bulk phase[1]. Glucose and ethanol with diameters of 7.2 Å and 4.5 Å, respectively, are an order of magnitude smaller than the surface pores and four orders of magnitude smaller than the bulk pores. Therefore, slower diffusion rate of glucose and ethanol in more concentrated alginate gels is probably due to a decrease in the number and length of the pores rather than a decrease in the pore diameters. In 1% and 2% alginate glucose and ethanol diffusion is mainly controlled by the rate at which the solute diffuses through the solvent occupying the pores (i.e. water). At 4% alginate concentration the diffusion rate is further restricted by the increased pathlength required for diffusion through the gel.

The ratio of ethanol diffusivity to glucose diffusivity is plotted as a function of alginate concentration in Figure 6. At 1% alginate concentration the ratio deviates significantly from the value of 1.6 for dilute water solutions, 2%, and 4% alginate gels. For 1% calcium alginate membranes convective transport may not be negligible as assumed in the lag time analysis. The two chambers of the diffusion cell were well-stirred so solute molecules could have been carried by convection at the edges of the membrane. The effective diffusion distance through the membrane is decreased, leading to an overestimate of the diffusion coefficient. Thus partial convective transport in 1% alginate can account for the deviation from the hydrodynamic theory.

Concentrations of glucose in the range of 2 g/ℓ to 100 g/ℓ and ethanol in the range of 10 g/ℓ to 80 g/ℓ did not affect the diffusion coefficients. The

Figure 6: *Effect of alginate concentration on the ratio of ethanol diffusivity to glucose diffusivity.*

● cell-free membrane
□ water



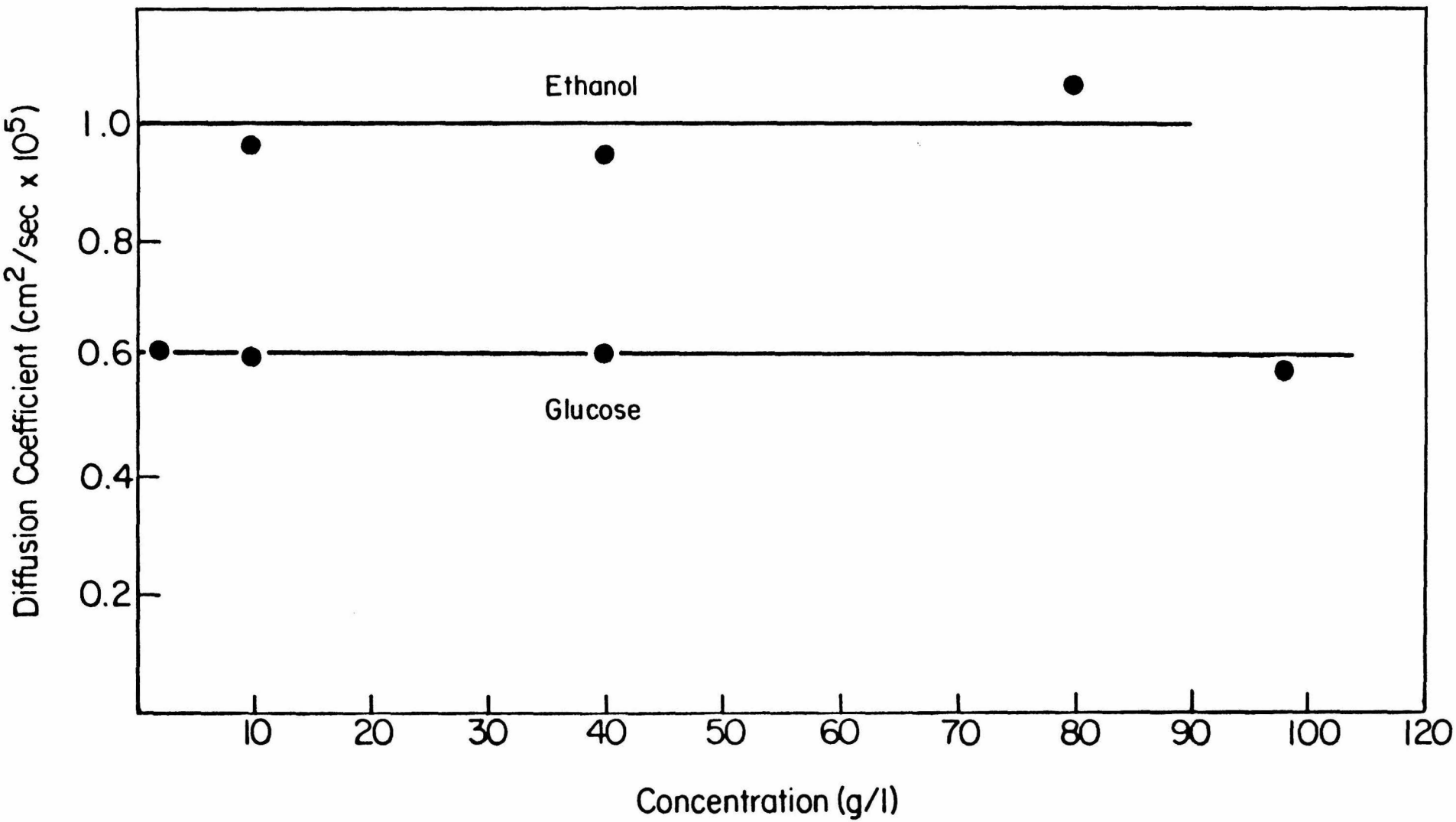
experimental results are shown graphically in Figure 7.

Yeast cells immobilized in 2% calcium alginate had no effect on the diffusion coefficient of glucose at a cell concentration of 20% by weight. Similarly the diffusion coefficient of ethanol in 1% alginate was unaffected by immobilized cells. Figure 5 illustrates these results. Cell concentrations denser than 20% may reduce the diffusion rate by increasing the tortuosity of the gel. Higher cell loadings were attempted; however, they weakened the membrane, causing deterioration before the flux became linear with time. Cells immobilized in calcium alginate and then allowed to reproduce may significantly affect diffusion coefficients even at low overall cell loadings. Instead of being well distributed throughout the gel, immobilized growing cells are found in clumps. Since most cell growth occurs near the gel surface, the cells may form a dense microbial layer slowing the rate of mass transport. Additionally, cells killed by 50% ethanol have permeabilized cell membranes which may allow glucose and ethanol diffusion through the cell. The intact membrane of living cells presents an additional barrier to solute diffusion

CONCLUSION

Although alginate gels allow fairly rapid diffusion of small molecules, the productivity of immobilized cells may still be limited by the presence of the alginate matrix. Cells immobilized in calcium alginate obtain nutrients solely by diffusion, while in suspended cultures nutrients are carried by convective flow. Diffusion is a significantly slower process than convective transport in a well-stirred reactor. Cell reproduction in the outer shell of alginate beads illustrates the lack of substrate inside the bead. This situation can be described as a reaction front. The rates of cell growth and product synthesis depend on the substrate concentration immediately

Figure 7: Effect of glucose and ethanol concentrations (c_1) on the diffusion coefficients in 2% alginate.



surrounding the cell. For immobilized cells the substrate concentration falls below a critical value for growth and possibly product synthesis close to the bead's surface. The position of the reaction front, where the limiting substrate concentration falls below a critical value, depends on the substrate diffusion coefficient and the rate of substrate utilization by the cells. To operate an immobilized cell reactor efficiently it is desirable to extend the reaction front to the center of the beads, resulting in a smoother concentration gradient. Rapid cell growth in the outer shell and cell death in the inner core could thus be prevented. Controlling the concentration gradient for productivity optimization requires diffusion coefficients as well as models for the rates of immobilized cell growth, substrate utilization, and product synthesis under varying substrate and product concentrations. Developing the necessary immobilized cell models is a complicated endeavor. The simplest solution is to try to apply suspended cell models to immobilized cells. Metabolic changes may be induced by entrapping the cells in a polymer, however, making suspended cell models invalid. Another problem is that suspended cell models are usually developed under steady-state growth conditions and may not apply to the transient conditions of immobilized cells. Modelling the rates of growth, substrate utilization, and product synthesis for immobilized cells will be aided by knowing diffusion coefficients under various conditions to account for diffusion due to concentration gradients which cannot easily be eliminated.

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NOTATION

A	membrane area (cm^2)
C	concentration (g/cm^3)
C_1	concentration at $x = 0$ (g/cm^3)
C_2	concentration at $x = \ell$ (g/cm^3)
D	diffusion coefficient (cm^2/sec)
F	flux (g/cm^2 sec)
K_p	partition coefficient of solute between gel and aqueous phases
ℓ	membrane thickness (cm)
Q	total flux or amount of solute transferred (g)
t	time (sec)
t_o	lag time (min)
t_s	time of sampling (sec)
x	distance (cm)
V	volume of chamber (cm^3)

REFERENCES

1. J. Klein, J. Stock, and K.-D. Vorlop, Eur. J. Appl. Microbiol. Biotechnol., 18, 86 (1983).
2. A Shinmyo, H. Kimura, and H. Okada, Eur. J. Appl. Microbiol. Biotechnol., 14 (1982).
3. J. M. Navarro and G. Durand, Eur. J. Appl. Microbiol. Biotechnol., 4, 243 (1977).
4. I. Nilsson, S. Ohlson, L. Häggstrom, N. Molin, and K. Mosbach, Eur. J. Appl. Microbiol Biotechnol., 10, 261 (1980).
5. H. Hiemstra, L. Dykhuizen, and W. Harder, Eur. J. Appl. Microbiol. Biotechnol., 18, 189 (1983).
6. H. Bettmann and H. J. Rehm, Appl. Microbiol. Biotechnol., 20, 285 (1984).
7. H. Eihmeier, F. Westmeier, and H. J. Rehm, Appl. Microbiol. Biotechnol., 19, 53 (1984).
8. K. Sato and K. Toda, J. Ferment. Technol., 61(3), 239 (1983).
9. K. Toda and M. Shoda, Biotechnol. Bioeng., 17, 481 (1975).
10. H. Tanaka, M. Matsumura, and I. A. Veliky, Biotechnol. Bioeng., 26, 53 (1984).
11. H. S. Carslaw, Fourier's Series and Integrals, The Macmillar Co., New York, 1906, p. 263.
12. H. A. Daynes, Proc. R. Soc., 97A, 286 (1920).
13. W. A. Rogers, R. S. Buritz, and D. Alpert, J. Appl. Phys., 25, 868 (1954).
14. J. Crank and G. S. Park, ed., Diffusion in Polymers, Academic Press, New York, 1968, p. 4.
15. L. G. Longsworth, J. A. Chem. Soc., 75, 5705 (1953).
16. P. A. Johnson and A. L. Babb, Chem. Revs., 56, 387 (1956).

17. P. Scherer, M. Kluge, J. Klein, H. Sahm, Biotechnol. Bioeng., 23, 1057 (1981).