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CHARACTERIZATION OF IMMOBILIZED ENZYME SYSTEMS

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ABSTRACT

Recently methods have been developed to characterize immobilized enzyme systems. These methods have been discussed extensively in Progress in Industrial Microbiology [1] and consist in procedures to determine the effective diffusion coefficient and the intrinsic values for the maximum reaction rate V_m and the Michaelis-Menten constant K_m. In this article a short review will be given, followed by a description of the experiments that have been conducted in order to test the methods.

Spheres of gelatine and sodium alginate were prepared with and without the enzyme invertase, and subsequently a crosslinking with glutaraldehyde and $CaCl_2$ respectively was performed.

From the results of the experiments with sucrose as substrate it can be concluded that the effective diffusion is, within limits, proportional to the radius of the spheres and is about a factor two to three slower than it is in pure water.

Experiments concerning the kinetic parameters learn that immobilization of invertase in alginate spheres hardly effects the enzyme, but that immobilization in gelatine causes a 85% loss in activity due to the destroying crosslinking with glutaraldehyde.

1. INTRODUCTION

For the design of reactors, filled with immobilized enzyme systems, information is needed about three important parameters:

- The effective diffusion coefficient	D_e
- The maximum reaction rate	V_m
- The Michaelis-Menten constant	K_m

Many investigators have tried to solve the problem of determining the kinetic constants, resulting in difficult computerprograms or poor approximation methods and even "initial rate methods", yielding apparent values for V_m and K_m.

The difficulties originate from the fact that there is no analytical solution to the differential equation that describes the diffusion and reaction process in an enzyme containing particle.

In the following an approximate solution will be discussed, which makes it possible to determine V_m and K_m from only a few experiments. Because the applied equations contain values for the effective diffusion coefficient also a method for determining these values had to be develloped. This method will also be briefly explained in the next chapter.

2. DESCRIPTION OF THE METHODS

2.1 Determination of the effective diffusion coefficient

When spheres of a carrier material (without enzyme) are mixed with a substrate containing liquid phase, the substrate penetrates into the spheres, resulting in a decrease in concentration in the liquid phase. Using this change in concentration with time it is possible to determine the effective diffusion coefficient of the substrate in the carrier material.

The concentration inside the spheres is place- and time dependent; the liquid phase is assumed to be ideally mixed and external mass transfer resistance is neglected (vigorous mixing). The diffusion inside the spheres is radial and the diffusion coefficient is supposed to be constant.

Because a solid and a liquid phase are involved in the mass transfer a partition coefficient must be introduced.

The change in concentration of the substrate is described by the following set of differential equations:

- inside the spheres:

$$\frac{\partial c_b}{\partial t} = D_e \left\{\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2 \frac{\partial c_b}{\partial r}\right)\right\}$$

starting condition : $t = 0 \quad c_b = 0$

boundary conditions : $r = R \quad c_b = \frac{c_f}{m}$

$r = 0 \quad c_b = \text{finite}$

- in the liquid phase:

$$-4 \pi R^2 n D_e \left.\frac{\partial c_b}{\partial r}\right|_{r=R} = V_f \frac{\partial c_f}{\partial t} \qquad \left(n = \frac{V_b}{\frac{4}{3}\pi R^3}\right)$$

starting condition : $t = 0 \quad c_f = c_{f,o}$

This set of equations can be solved using Laplace transformation and the result is presented graphically in figure 1 [2].

The following procedure is to be used to determine the effective diffusion- and the partition-coefficient:

-1- Add a known quantity of spheres of the carrier material to a known quantity of substrate solution.

-2- Follow the decrease in concentration of the substrate in the liquid phase as a function of the time.

-3- Calculate the partition coefficient from the equilibrium concentrations in both phases.

-4- Calculate the factor $(1 + \frac{mV_f}{V_b})(\frac{c_{f,o} - c_f}{c_{f,o}})$ at different times.

-5- Determine from figure 1 the corresponding values for the factor $\frac{D_e . t}{R^2}$.

-6- Calculate the effective diffusion coefficient by inserting time and radius of the spheres into the factor $\frac{D_e . t}{R^2}$.

It is remarkable that the effective diffusion coefficient in the solid phase can be determined by observing the change in concentration of the substrate in the liquid phase.

2.2 Determination of the Maximum Reaction Rate V_m and the Michaelis-Menten constant K_m.

The general equation for the reaction of a substrate in a spherical carrier charged with an enzyme can be written for stationary conditions as:

$$D_e \{\frac{1}{r^2} \frac{d}{dr} (r^2 \frac{dc}{dr})\} = \frac{V_m c}{K_m + c}$$

with boundary conditions: $r = R \quad c = c_o$

$r = 0 \quad \frac{dc}{dr} = 0$

The substrate is assumed to be converted in accordance with Michaelis-Menten's kinetics.

The above differential equation cannot be solved analytically. Exact solutions are known, however, for the two kinetic extremes of zero- and first order reaction.

Yamada [3] developed a method, the so-called "method of moments", to obtain approximate solutions for non-linear differential equations.

This method consists in principal in replacing the original differential equation by a zero-, first- or n^{th} moment equation, into which a carefully chosen function, containing an unknown constant, is introduced.

The unknown constant can subsequently be determined by solving the moment-equation.

The zero-, first- or n^{th} moment-equation is obtained by multiplying the original differential equation by one of the dependent parameters to the zero-, first- or n^{th} power and subsequently integrating the result over the range, covered by that parameter.

In cases in which extremes of the general differential equation give analytical solutions it is often simple to chose a function, which adequately describes the general equation.

Besides, the extremes provide a possibility of verifying the general approximate solution.

It is a known fact that the exact solution for the zero-order system has an abrupt transition point.

For the general solution problems are due to arise unless a form is taken that resembles the solution for the zero-order extreme.

Consequently the following form has been chosen as a basis:

$$y = 1 + p \left\{ \tfrac{1}{2}(x^2 - 1) + x_o^3 \left(\frac{1}{x} - 1\right) \right\}, \qquad x_o > 0$$

$$\text{with} \quad p = \left(x_o^3 - \frac{3}{2} x_o^2 + \frac{1}{2}\right)^{-1} \qquad (p > 2)$$

$$y = 1 + p \left\{ \frac{1}{2}(x^2 - 1) \right\} \quad , \; x_o = 0 \qquad (p \leq 2)$$

where $y = \dfrac{c}{c_o}$, $x = \dfrac{r}{R}$ and $x_o = \dfrac{r_o}{R}$

The following proves to give a very good solution:

$$P = \frac{1}{3\,\frac{2(E + 6)}{E(E + 12)} + \frac{1}{A}}$$

with $E = \frac{V_m R^2}{D_e K_m}$ and $A = \frac{V_m R^2}{D_e c_o}$

or: $$\frac{1}{p} = \frac{6(E + 6)}{E(E + 12)} + \frac{3 D_e}{V_m R^2} \cdot c_o$$

The following procedure can be derived from this solution to obtain values for V_m and K_m from a limited number of experiments in a continuously stirred tank reactor (CSTR), where external mass transfer resistance is neglected:

-1- Determine $\emptyset_m$ from experiments in a CSTR, where $\emptyset_m$ is defined by:

$$\emptyset_m = \frac{R}{c_o} \left.\frac{dc}{dr}\right|_{r=R} = \frac{q\, m\, R^2 (c_{in} - c_f)}{3\, D_e\, c_f (1 - \varepsilon) V}$$ (see also figure 2).

-2- Determine p:

if $\emptyset_m \leq 2$ than $p = \emptyset_m$
if $\emptyset_m > 2$ than

$$1 - \frac{\emptyset_m}{p} - \frac{3}{2}\left(1 - \frac{\emptyset_m}{p}\right)^{2/3} + \frac{1}{2} = \frac{1}{p}$$

or:

$$p = \frac{1}{12}\left(\emptyset^2_m + 8\,\emptyset_m + 4\right) + \frac{1}{12}\left\{\frac{1}{3}\left(3\,\emptyset_m^{\;4} - 16\,\emptyset_m^{\;3} + 24\,\emptyset_m^{\;2} - 16\right)\right\}^{\frac{1}{2}}$$

-3- Plot $\frac{1}{p}$ versus c_o $\left(c_o = \frac{c_f}{m}\right)$

By changing the flow rate q different values for $\emptyset_m$ and for c_o are obtained, resulting in a graph like figure 3.

-4- Determine from fig. 3:

$$\tan \alpha \text{ and } \frac{1}{p_o}$$

-5- Calculate V_m from:

$$V_m = \frac{3\ D_e}{\tan \alpha\ .\ R^2}$$

-6- Calculate E from:

$$\frac{6(E + 6)}{E(E + 12)} = \frac{1}{p_o}$$

$$\text{or } E = 3\ p_o - 6 + \{(3p_o - 6)^2 + 36\ p_o\}^{\frac{1}{2}}$$

-7- Calculate K_m from:

$$K_m = \frac{V_m\ R^2}{E\ .\ D_e}$$

With the above procedure the real, intrinsic values for V_m en K_m are determined.

3. EXPERIMENTS

The experimental work has been performed with two types of carrier material e.g. gelatine spheres, crosslinked with glutaraldehyde and alginate spheres, stabilized by exchanging the original sodium ions with bivalent calcium ions.

3.1 Determination of effective diffusion- and partition coefficients

Using the technique, described in section 2.1, effective diffusion- and partition coefficients were calculated from the decrease in concentration in the liquid phase after mixing the spheres with a solution of substrate.
The results of the experiments are shown in table 1. In all cases 50 ml of spheres, without enzyme, were mixed at room temperature with 50 ml solution, containing 100 kg/m^3 sucrose. Fractions with uniform diameter were obtained by wet sieving.

gelatine/sucrose			calciumalginate/sucrose		
average diameter [m]	D_e $[m^2/s]$	m [-]	average diameter [m]	D_e $[m^2/s]$	m [-]
2.58×10^{-3}	3.4×10^{-10}	1	3.075×10^{-3}	3.8×10^{-10}	1
2.18×10^{-3}	3.3×10^{-10}	1	2.18×10^{-3}	2.7×10^{-10}	1
1.85×10^{-3}	2.7×10^{-10}	1	1.55×10^{-3}	2.5×10^{-10}	1
1.20×10^{-3}	2.5×10^{-10}	1			

Table 1 Effective diffusion- and partition coefficients for different diameters.

Diffusion of sucrose in calciumalginate is somewhat slower than in gelatine. The diffusion coefficient of sucrose in water is about twice as high (5.2×10^{-10} m^2/s).
The results are shown graphically in fig. 4.
Remarkeble is the decreasing value for the diffusion coefficient with decreasing diameter of the particles. This tendency can be explained from the fact that for all diameters the same crosslinking conditions have been used.

3.2 Determination of the kinetic parameters V_m and K_m

The kinetic experiments were conducted with spherical particles containing the enzyme invertase.
Fractions with uniform diameter, obtained by wet sieving, were used for determination of the intrinsic values for the kinetic parameters V_m and K_m, following the procedure, explained in section 2.2.
The experiments with gelatine as carrier material can be divided into two parts e.g.:

- change in enzyme content at constant diameter of spheres,
- change in diameter of spheres at constant enzyme concentration.

In the case of the alginate spheres the influence of the enzyme content was investigated at three diameters.

All experiments were conducted at room temperature. A buffered solution of 100 kg/m^3 sucrose in water (buffer: acetate at pH = 4.8) was pumped from a container into a well stirred vessel by means of a peristaltic pump. In case of the experiments with calcium alginate addition of 1 mM $CaCl_2$ to the substrate solution was necessary in order to stabilize the spheres.

Continuous analysing was performed with the aid of an autoanalyser.

The results of the experiments with gelatine as carrier material are shown in the figures 5 and 6.

From the calculated values for the kinetic parameters (from fig. 5) the relationship between V_m and the enzymeconcentration can be deducted.

This relation is shown graphically in figure 7 and from this it follows:

$$V_m = 0.028 \times [E] \quad kg/m^3.s$$

The average value for K_m appears to be:

$$K_m = 10.2 \ kg/m^3$$

The results of the experiments with calciumalginate are shown in the figures 8, 9 and 10.

Figure 11 shows the relationships between V_m and the enzyme concentrations for the three diameters.

For the average values of V_m and K_m the following applies:

$$V_m = 0.17 \times [E] \ kg/m^3.s$$
$$K_m = 11.5 \ kg/m^3$$

4. CONCLUSIONS

The diffusion rate of sucrose in wet spherical particles of gelatine appears to be slightly better than in same-sized particles of calcium-alginate, both being about a factor two to three slower than the diffusion rate of sucrose in pure water.

The effective diffusion coefficient for sucrose in both materials have found to be a function of the size of the particles, caused by the crosslinking, which primarely takes place at the outside shell of the particle and which is of relatively more influence in case of smaller particles.

The partition coefficients have the value 1 for these kinds of wet carrier particles.

The results of the kinetic experiments are resumed in table 2.

	invertase in gelatine	invertase in calciumalginate	invertase free
V_m $[kg/m^3.s]$	0.028 x [E]	0.17 x [E]	0.21 x [E]
K_m $[kg/m^3]$	10.2	11.5	11.5

Table 2 Results of the kinetic experiments.

The maximum reaction rate V_m of invertase, immobilized in calciumalginate, is almost equal to that of the free invertase. There is only a small loss in activity, due to immobilization.

The situation for the invertase, immobilized in gelatine, is, however, totally different.

The immobilization, followed by crosslinking with gluteraldehyde, causes a loss in activity of more than 85%.

The values for the Michaelis-Menten constant K_m are hardly influenced by the immobilization. This is what should be expected, because the undamaged enzyme molecules will still have the same K_m as the free enzyme molecules have, the immobilization just being an inclusion without interaction with the carrier material.

As a concluding remark it can be stated that the suggested methods for the characterization of immobilized enzyme systems are very suited to obtain values for parameters that are important in describing and designing reactors with immobilized enzymes.

LIST OF SYMBOLS

c	concentration of substrate	kg/m^3
c_o	concentration at the wall of a sphere	kg/m^3
c_b	concentration inside sphere	kg/m^3
c_f	concentration in liquid	kg/m^3
$c_{f,o}$	concentration in liquid at $t = 0$	kg/m^3
c_{in}	inlet concentration	kg/m^3
m	partition coefficient	-
q	flow rate	m^3/s
r	radial coordinate	m
r_o	radius in sphere where $c_b = 0$	m
t	time	s
D	diameter of sphere	m
D_e	effective diffusion coefficient	m^2/s
$[E]$	enzyme concentration	kg/m^3
K_m	Michaelis-Menten constant	kg/m^3
R	radius of sphere	m
V	volume of reactor	m^3
V_b	volume of spheres	m^3
V_f	volume of liquid phase	m^3
V_m	maximum reaction rate	$kg/m^3.s$
ε	liquid fraction	-
ϕ_m	conversion factor	-

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LEGENDS

Fig. 1 Determination of D_e through concentration measurement in the liquid phase.

Fig. 2 Continuously stirred tank reactor in stationary state.

Fig. 3 Relationship between $\frac{1}{p}$ and c_o.

Fig. 4 Diffusion coefficient versus diameter.

Fig. 5 Influence of enzyme concentration on V_m and K_m (gelatine, $D = 2.18 \times 10^{-3}$ m).

Fig. 6 Influence of diameter on V_m and K_m (gelatine, $[E] = 10 kg/m^3$).

Fig. 7 Relationship between V_m and enzyme concentration (gelatine).

Fig. 8 Influence of enzyme concentration on V_m and K_m (calciumalginate, $D = 1.55 \times 10^{-3}$ m).

Fig. 9 Influence of enzyme concentration on V_m and K_m (calciumalginate, $D = 2.18 \times 10^{-3}$ m).

Fig.10 Influence of enzym concentration on V_m and K_m (calciumalginate, $D = 3.075 \times 10^{-3}$ m).

Fig.11 Relationship between V_m and enzyme concentration (calciumalginate).

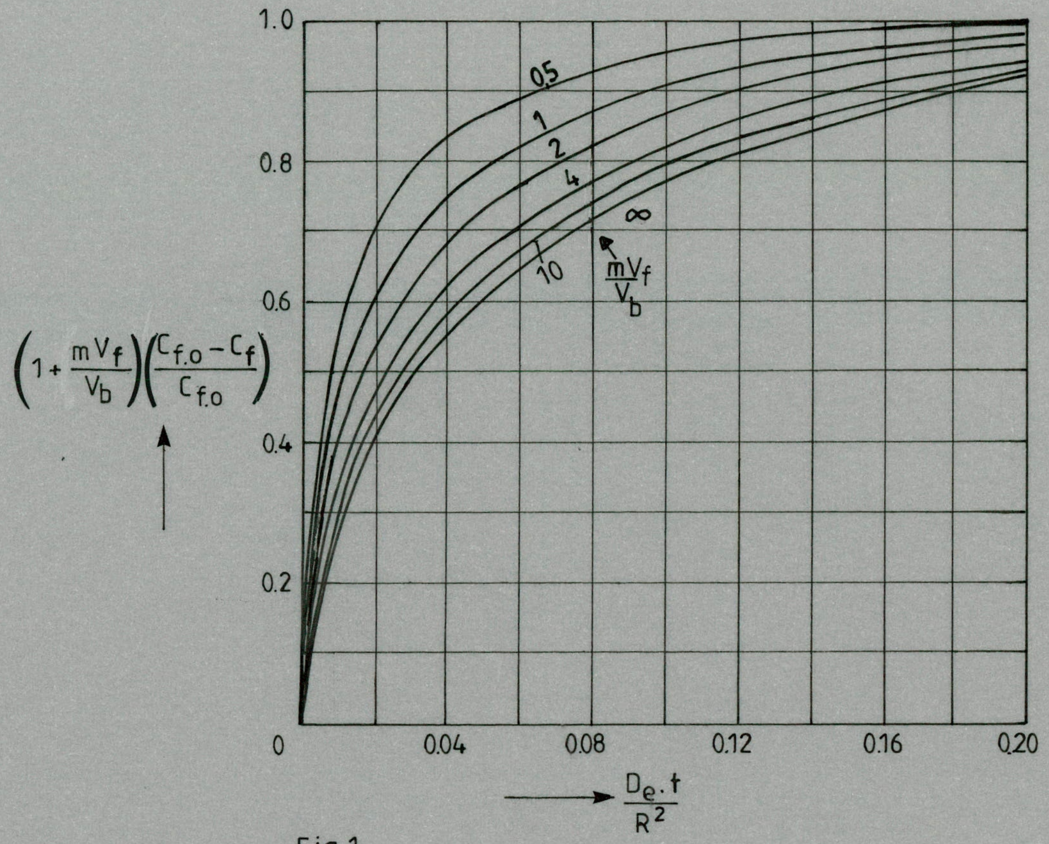

1.0
0.8
0.6
0.4
0.2
0
0.04
0.08
0.12
0.16
0.20
0.5
1
2
4
10
∞
$\frac{mV_f}{V_b}$
$\left(1+\frac{mV_f}{V_b}\right)\left(\frac{C_{f.o}-C_f}{C_{f.o}}\right)$
$\frac{D_e \cdot t}{R^2}$

Fig.1

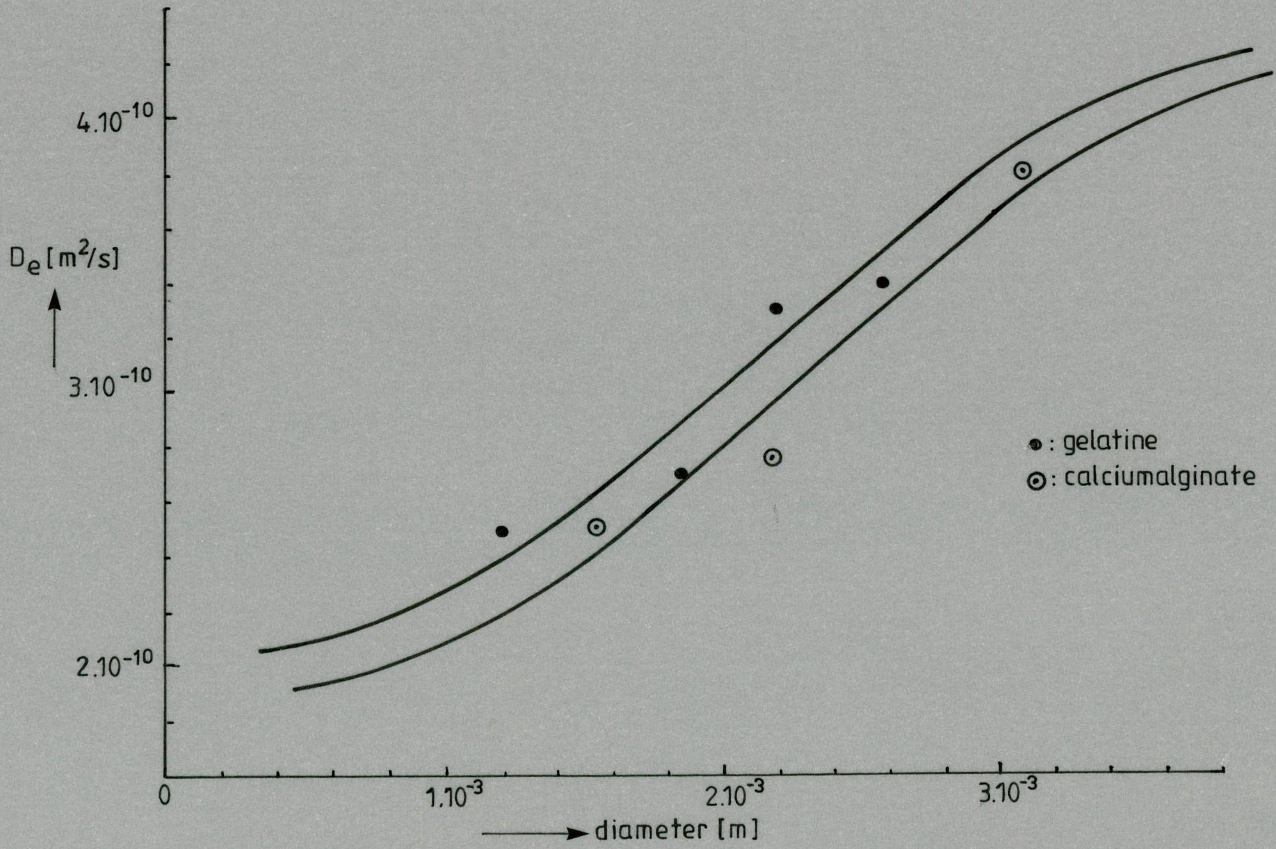
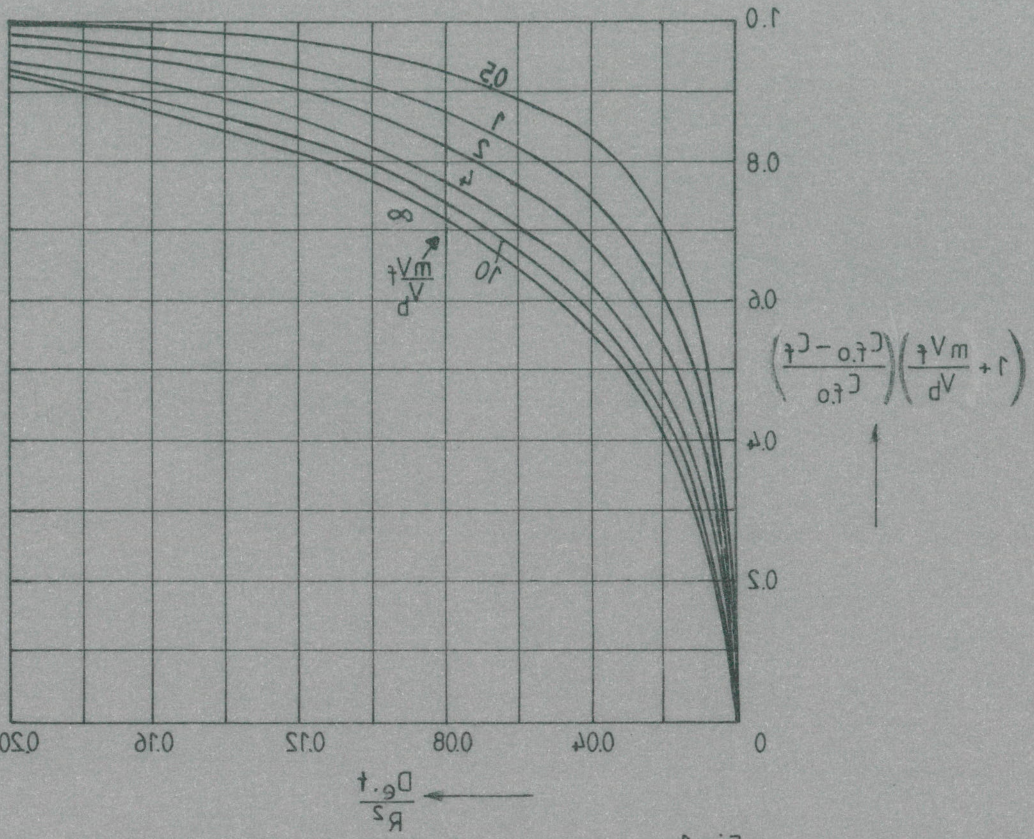

4.10^{-10}
3.10^{-10}
2.10^{-10}
D_e [m^2/s]
0
1.10^{-3}
2.10^{-3}
3.10^{-3}
diameter [m]
● : gelatine
⊙ : calciumalginate

Fig.4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0.8
0.6
0.4
0.2
0
0.04
0.08
0.12
0.16
0.20
0.5
1
2
4
∞
10
$\frac{mV_f}{V_b}$
$\left(1+\frac{mV_f}{V_b}\right)\left(\frac{C_{f.o}-C_f}{C_{f.o}}\right)$
$\frac{D_e \cdot t}{R^2}$

Fig.1

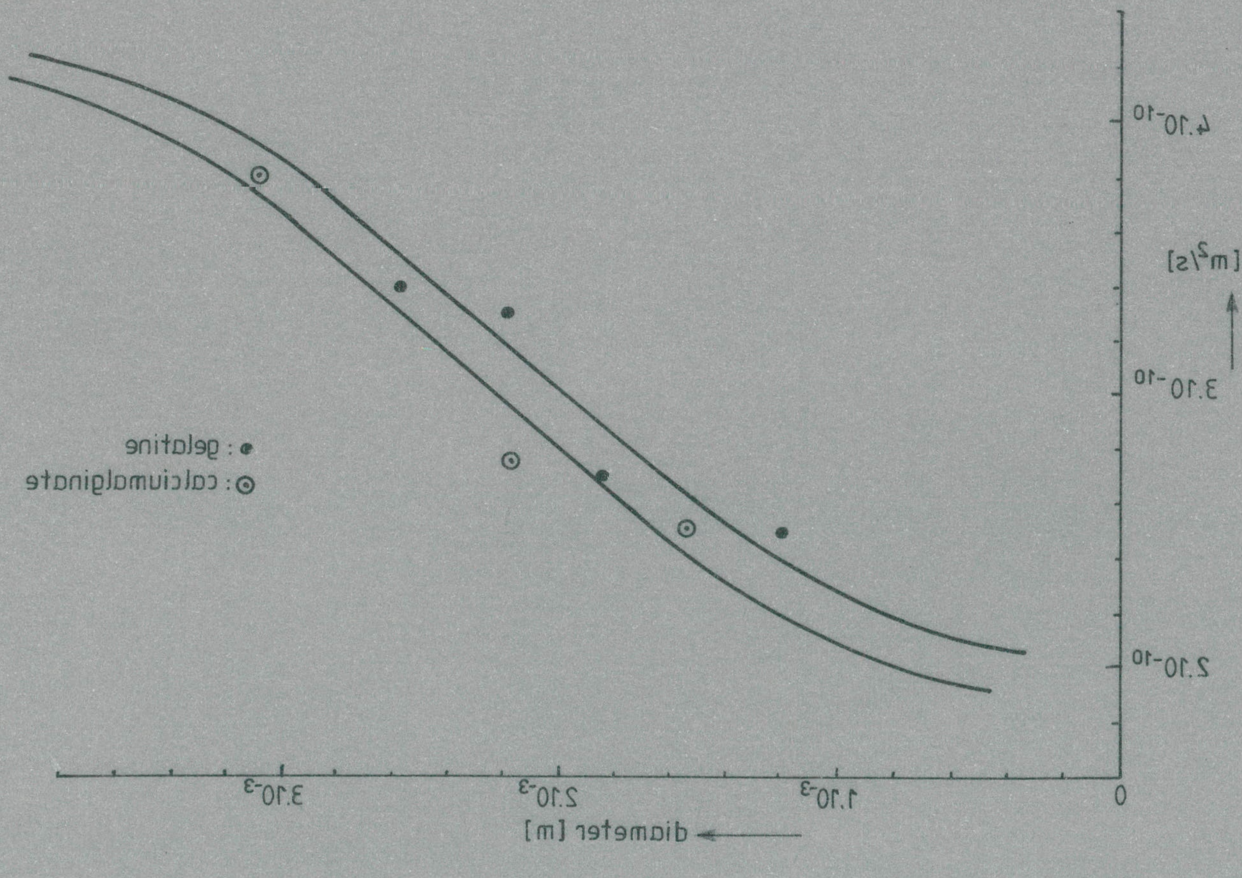
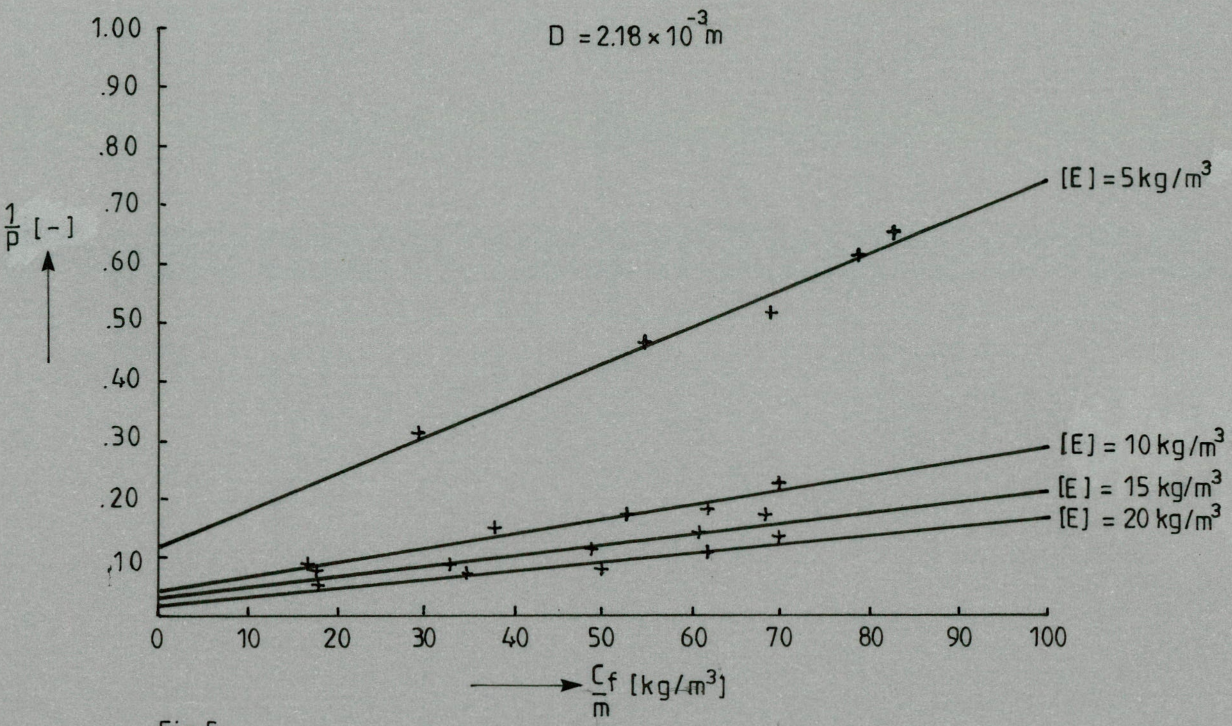
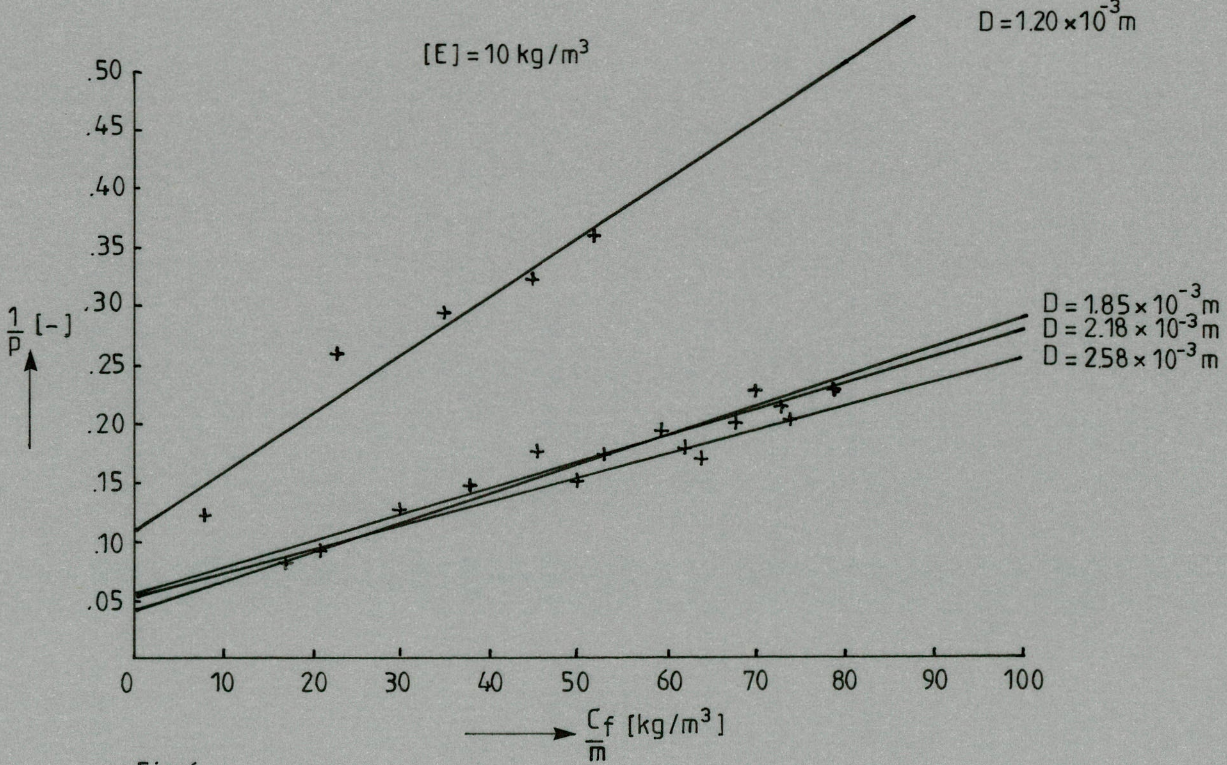

4.10⁻¹⁰
3.10⁻¹⁰
2.10⁻¹⁰
D_e [m²/s]
0
1.10⁻³
2.10⁻³
3.10⁻³
diameter [m]
● : gelatine
⊙ : calciumalginate

Fig.4



$D = 2.18 \times 10^{-3}$ m
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
$\frac{1}{P}$ [-]
$[E] = 5$ kg/m^3
$[E] = 10$ kg/m^3
$[E] = 15$ kg/m^3
$[E] = 20$ kg/m^3
0
10
20
30
40
50
60
70
80
90
100
$\frac{C_f}{m}$ [kg/m^3]

Fig.5



$[E] = 10$ kg/m^3
$D = 1.20 \times 10^{-3}$ m
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
$\frac{1}{P}$ [-]
$D = 1.85 \times 10^{-3}$ m
$D = 2.18 \times 10^{-3}$ m
$D = 2.58 \times 10^{-3}$ m
0
10
20
30
40
50
60
70
80
90
100
$\frac{C_f}{m}$ [kg/m^3]

Fig.6

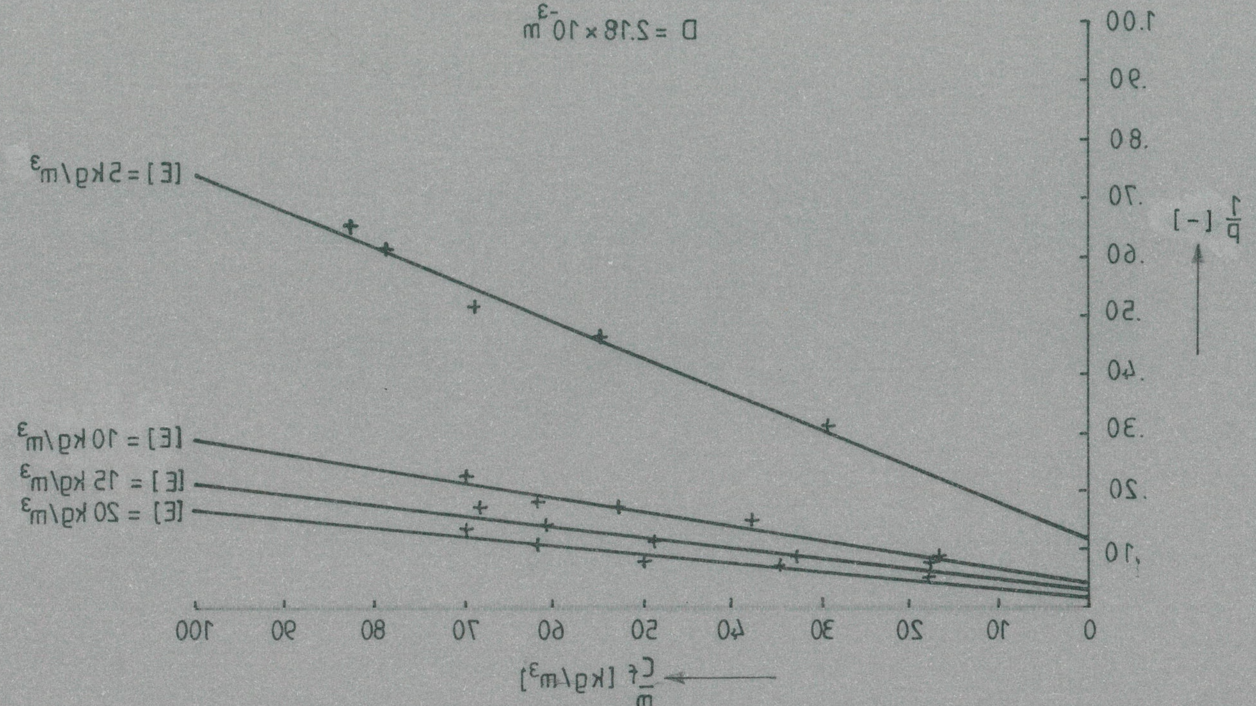
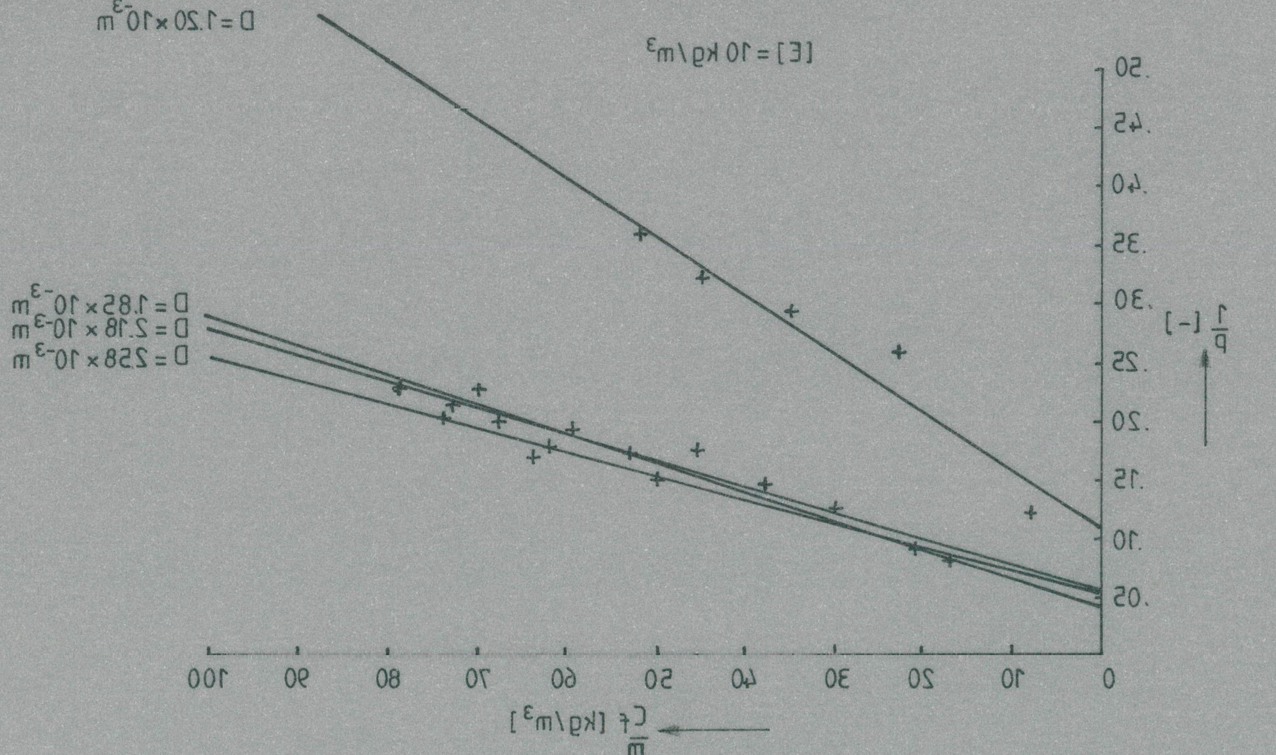

D = 2.18 × 10^{-3} m
$\frac{1}{p}$ [-]
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
[E] = 5 kg/m^3
[E] = 10 kg/m^3
[E] = 15 kg/m^3
[E] = 20 kg/m^3
0
10
20
30
40
50
60
70
80
90
100
$\frac{C_f}{m}$ [kg/m^3]

Fig.5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[E] = 10 kg/m^3
D = 1.85 × 10^{-3} m
D = 2.18 × 10^{-3} m
D = 2.58 × 10^{-3} m
$\frac{1}{p}$ [-]
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0
10
20
30
40
50
60
70
80
90
100
$\frac{C_f}{m}$ [kg/m^3]

Fig.6

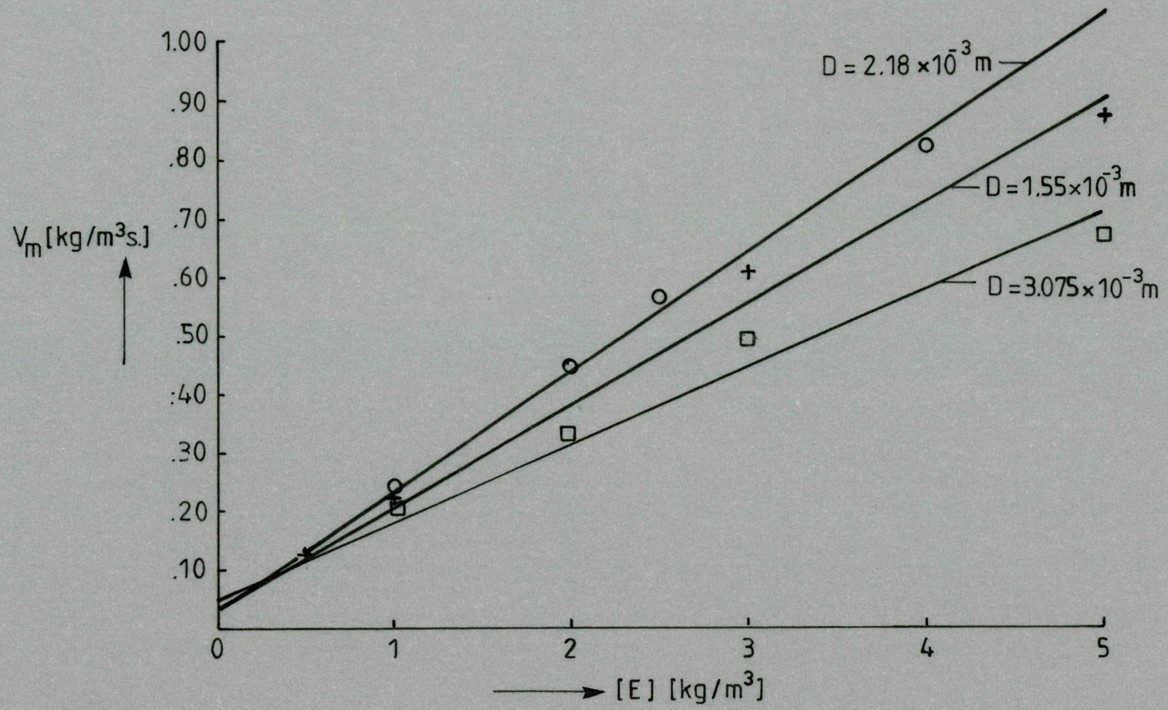


Fig.11

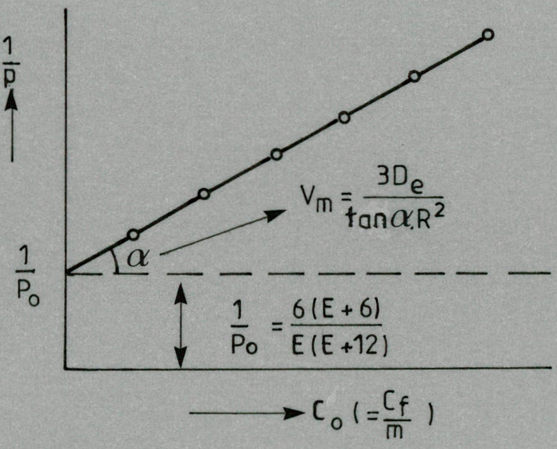


Fig.3

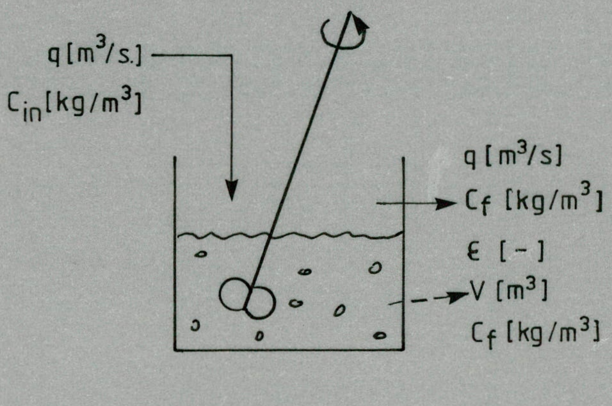
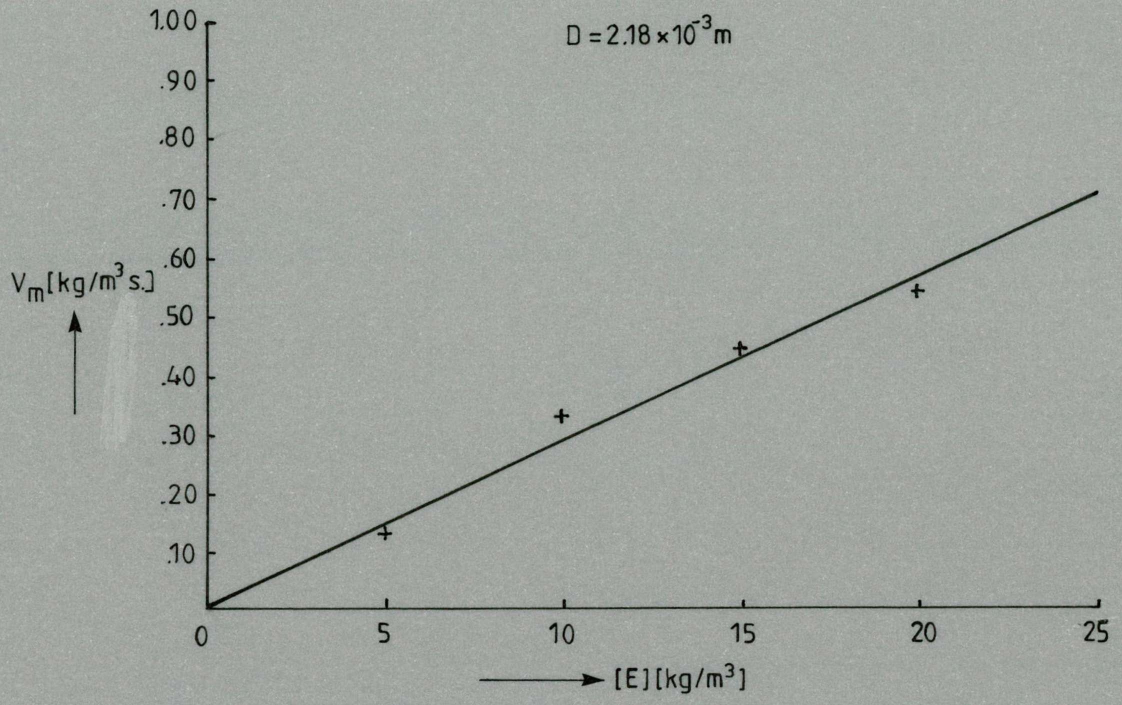


Fig. 2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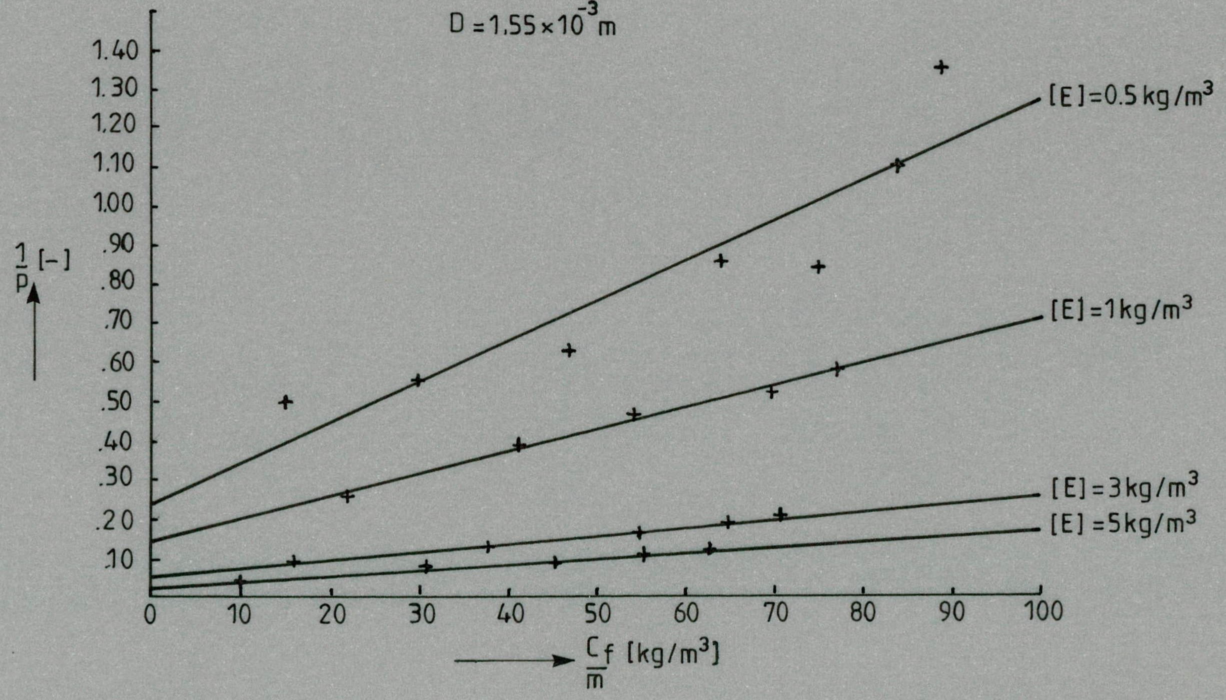
V_m [kg/m³ s.]
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0
5
10
15
20
25
[E] [kg/m³]

Fig. 7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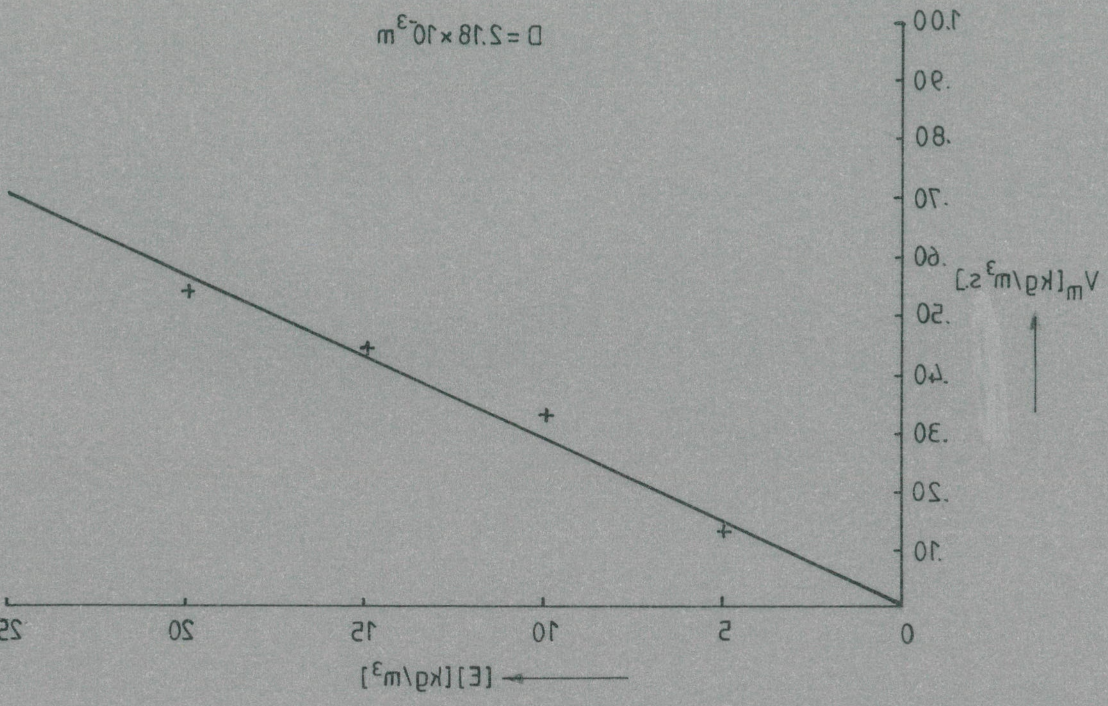
1/p [–]
1.40
1.30
1.20
1.10
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0
10
20
30
40
50
60
70
80
90
100
C_f/m [kg/m³]
[E] = 0.5 kg/m³
[E] = 1 kg/m³
[E] = 3 kg/m³
[E] = 5 kg/m³

Fig. 8


D = 2.18 × 10^{-3}m
V_m [kg/m^3 s.]
1.00
.90
.80
.70
.60
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.30
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.10
0
5
10
15
20
25
[E] [kg/m^3]

Fig. 7

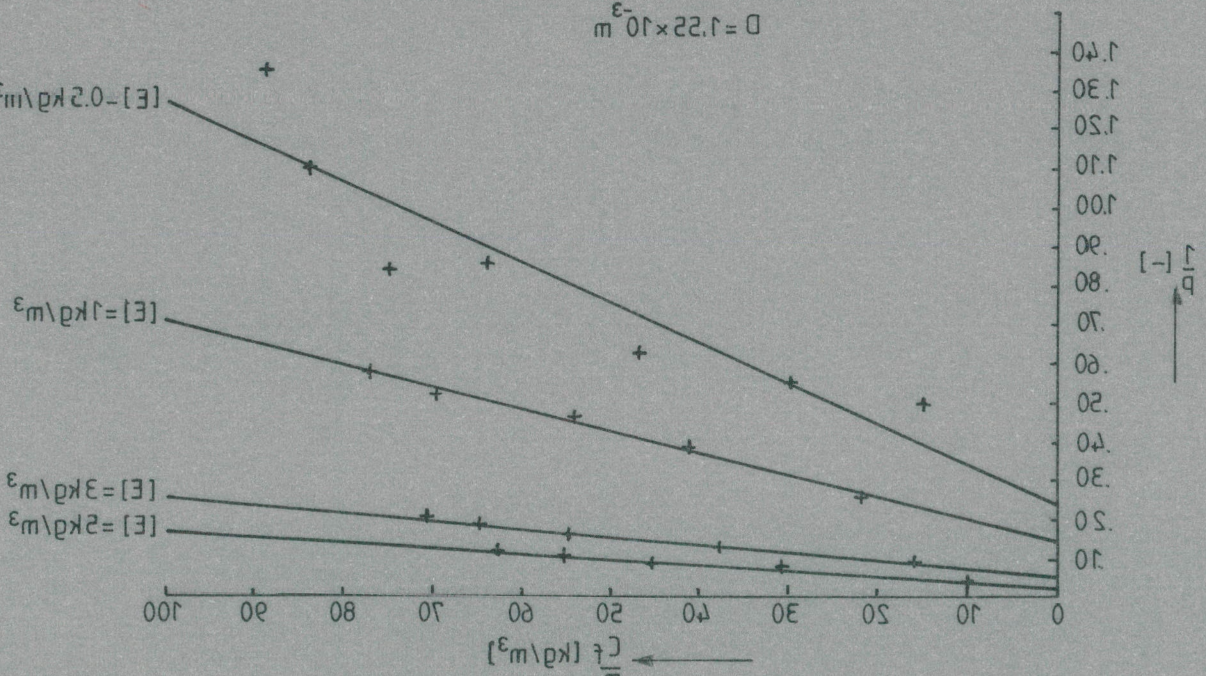
D = 1.55 × 10^{-3}m
$\frac{1}{p}$ [–]
1.40
1.30
1.20
1.10
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
[E] -0.5 kg/m^3
[E] = 1 kg/m^3
[E] = 3 kg/m^3
[E] = 5 kg/m^3
0
10
20
30
40
50
60
70
80
90
100
$\frac{C_f}{m}$ [kg/m^3]

Fig. 8

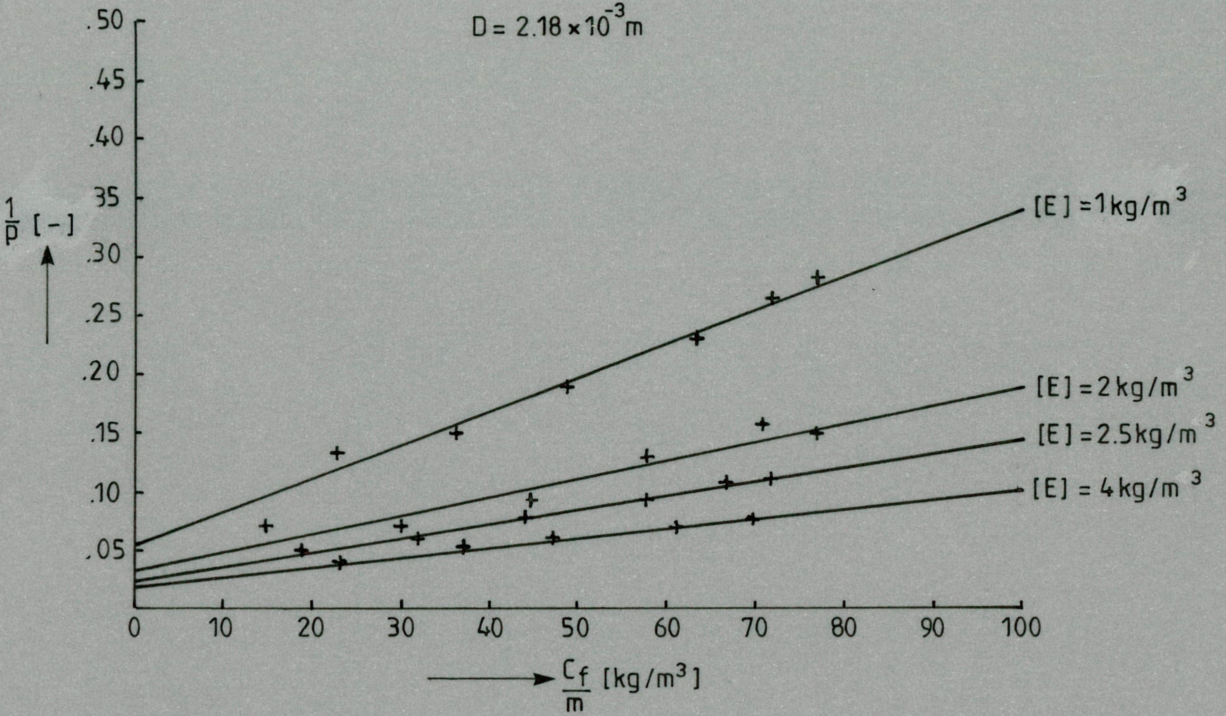
D = 2.18 × 10⁻³ m
1/p [-]
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0
10
20
30
40
50
60
70
80
90
100
Cf/m [kg/m³]
[E] = 1 kg/m³
[E] = 2 kg/m³
[E] = 2.5 kg/m³
[E] = 4 kg/m³

Fig. 9

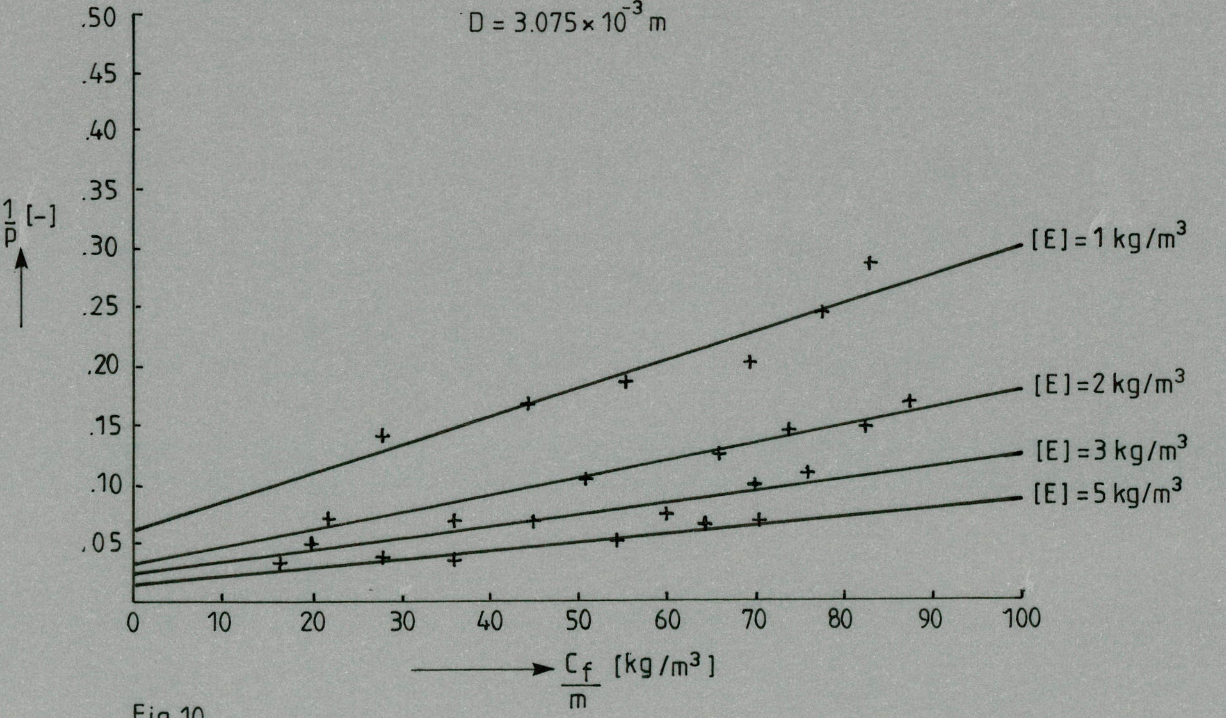
D = 3.075 × 10⁻³ m
1/p [-]
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0
10
20
30
40
50
60
70
80
90
100
Cf/m [kg/m³]
[E] = 1 kg/m³
[E] = 2 kg/m³
[E] = 3 kg/m³
[E] = 5 kg/m³

Fig. 10