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Comparative study of reaction kinetics in membrane and agarose bead affinity systems

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ABSTRACT

Compared to conventional agarose bead affinity supports, a microporous nylon membrane exhibits greatly improved reaction kinetics as quantified in the reaction between γ-globulin and immobilised protein A. The improvement is only observed when the solution of γ-globulin is forced through the membrane pores. In the absence of flow in the pores, it is possible to relate approximately the rate of uptake onto either type of affinity support to independently determined diffusion coefficients. In the presence of flow, the reaction rate is similar for membranes having 0.45 and 3.0 μm diameter pores, and considerably smaller than predicted by the Smoluchowski formula.

INTRODUCTION

As the analysis of biomolecular association processes became more quantitative, the mechanism of diffusion controlled reactions required deeper and more detailed study of the nature and properties of diffusion driven events.

Our interest in these problems stems, in part, from our efforts to develop an affinity separation system which overcomes the inherent limitations of systems based on beaded agarose gel. One such limitation, which is economically very significant in industrial-scale [1] and biomedical applications [2], is the slow reaction kinetics.

We have developed a system in which protein A (a ligand for plasma γ-globulins) is supported by a flat microporous nylon membrane. It has been found that, if γ-globulin solution is forced through the pores of the membrane, a much faster rate of uptake can be obtained than with an agarose bead affinity system.

Brandt *et al.* [3] have described a hollow fibre membrane affinity system. The fast reaction kinetics in this system were attributed to the elimination of the diffusional pathlength corresponding to the bead diameter in agarose affinity columns. Horstmann and Chase [4] have recently studied the rate of uptake of human γ-globulins onto agarose–protein A in stirred batch experiments. They used computed solutions to partial differential equations to model this system, and concluded that diffusion from the outer surface to the interior of the beads was the dominant rate controlling mechanism, as such an assumption produced the best fit with the experimental data.

In this paper we wish to present a more detailed comparison between a membrane-based and an agarose bead affinity system. The objective of our study is to

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investigate the importance of convective and diffusive processes which may limit the reaction rate in such systems. To this end, experimental data have been compared with various theoretical models.

EXPERIMENTAL

Materials

Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). Protein A was bound to Sepharose CL-4B using the cyanogen bromide coupling technique [5].

The membrane-based affinity system was developed in our laboratory. Covalent immobilisation of protein A within the porous matrix of a nylon Loprodyne membrane (Pall Bio Support, Portsmouth, U.K.) was achieved by modification of the 2-fluoro-1-methylpyridinium toluene-4-sulphonate (FMP) method [6]. Two types of membrane were used one with 0.45, the other with 3.0 μm diameter pores.

Human γ-globulin fraction was isolated from colleagues' blood using a Sepharose–protein A column according to the manufacturer's recommendations (Pharmacia). Porcine γ-globulin, Cohn fraction II, III was obtained from Sigma (Poole, U.K.). N-succinimidyl [2,3-^{3}H]propionate was obtained from Amersham (Little Chalfont, U.K.). Tritiation of human γ-globulin with N-succinimidyl [2,3-^{3}H]propionate was carried out according to the method by Bolton and Hunter [7].

All other reagents were analytical grade and commercially available.

Measurement of the reaction rate in the membrane

The rate of the reaction between γ-globulin and protein A, immobilised on the membrane, was measured by studying the time-course of this second order reaction with one of the reactants, γ-globulin, in excess.

Two different hydrodynamic conditions were used. In the first instance, γ-globulin solution was forced under pressure through the pores of the membrane. In the second type, samples of the membrane with attached protein A were immersed into the solution of γ-globulin allowing molecules to diffuse to binding sites on the protein A.

(i) Reaction rate with flow through the pores. In the measurement of reaction rate, the membrane holder was designed to use compressed air, of adjustable pressure, to drive the solutions through the membrane at a controlled flow-rate. This holder, illustrated in Fig. 1, held 6 discs of membrane–protein A (each having an expose surface area 13 mm in diameter) in such a way that they were perfused in parallel and under the same conditions. The multiplicity of the results thus obtained gave a better statistical significance. Both 0.45 and 3.0 μm pore size membranes were tested so that the two could be compared.

Four different concentrations (0.0125, 0.025, 0.05 and 0.1 mg ml^{-1}) of human γ-globulin in phosphate buffered saline, pH 7.2 (PBS), containing ^{3}H-labelled human γ-globulin (specific activity of 1.8 μCi mg^{-1}), were tested. The volume of γ-globulin solution forced through each membrane disc was chosen, according to the concentration, so that a constant amount of 2 mg was supplied. Pilot studies established the approximate capacity of the membrane–protein A to adsorb γ-globulin, on the basis of which this amount (2 mg) was chosen in order to provide an excess factor of at least 20. The reaction was timed and at least three durations in the range 20–600 s were used for every concentration. In order to control the flow-rate of fluid (between 3.0 and 55.0 ml

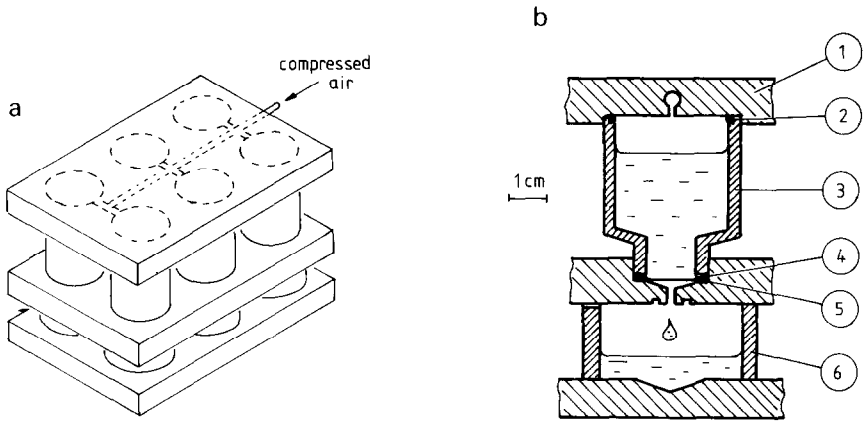

Fig. 1. Holder for six membrane discs as used in experiment to measure reaction rate with flow through the pores. (a) Whole device (Perspex). (b) Detail of individual cell: 1 = compressed air manifold; 2 = "O" ring seal, 3 – liquid reservoir; 4 = silicone washer; 5 = membrane–protein A disc; 6 = collecting well.

min^{-1} cm^{-2}) through the membrane, air pressure was varied from 0.03–0.7 bar. Membranes were washed afterwards with 80 ml of PBS at a flow-rate of 1.5 ml min^{-1} per cm^2 of membrane, sufficient for the measured radioactivity of the eluate to return to the background value. The amount of γ-globulin adsorbed on the protein A was estimated by elution with 0.1 *M* glycine–HCl (pH 3.0) and the radioactivity of the eluates and membranes was measured directly by counting for 10 min in the scintillation counter. By varying the ratio of radio-labelled to unlabelled γ-globulin it was also established than the method of labelling did not interfere with the adsorption by the membrane–protein A.

(ii) Reaction rate without flow. Membrane–protein A discs (20 mm in diameter) were immersed in solution of human γ-globulin in PBS, containing ^{3}H-labelled human γ-globulin (specific activity 1.8 μCi mg^{-1}). In this experiment, membrane of 0.45 μm pore size was used. Four different concentrations of γ-globulin were tested: 0.05, 0.1, 0.25 and 0.4 mg ml^{-1}. As above, an amount of 2 mg of γ-globulin was used for each disc. The discs were incubated under slow agitation at 25°C for various times: 15, 30, 45 min, 1, 2, 18 and 48 h. They were then removed and installed in a suitable membrane holder for washing and elution, which were performed as in (i) above. Counting efficiency studies established that the membrane discs did not quench the emission of radiation in a significant amount, and ^{125}I-labelled γ-globulin was successfully substituted by ^{3}H-labelled γ-globulin.

Measurement of the reaction rate in beads

In order to study the rate of uptake of porcine γ-globulins onto the Sepharose–protein A beads, a cell was constructed to contain approximately 1 ml of the gel. The cell was connected to reservoirs of liquid via tubing which passed through peristaltic pumps and solenoid valves controlled by a personal computer. This made accurate timing of the various perfusions possible. The small dead spaces at the top and bottom of the gel each had two ports, so that the liquid they contained could be exchanged

independently from that contained in the gel itself. Hence protein contained in these spaces was excluded from the measure of the amount of protein eluted from the gel. This made it possible to determine how much protein was desorbed at neutral pH.

Solutions of γ-globulin in PBS, of concentration 2 and 4 mg ml^{-1}, were allowed to flow through the cell at a rate of 32 ml min^{-1} for periods of time varying from 15 s to 128 min. This provided an excess factor of at least 5 over the amount which bound. Immediately after stopping the flow, the space above the gel was washed with PBS for 15 s, followed by similar washing of the space below the gel (including the connecting tubing containing protein solution). The gel was then washed with 20 ml of PBS, pH 7.2, over 10 min, followed by elution with 20 ml of 0.1 *M* glycine–HCl, pH 3.0, over 10 min. The eluates from these two buffers were collected separately, and their protein content measured spectrophotometrically at 280 nm, using a 1 cm pathlength. From a standard curve, the extinction coefficient, $E_{280}^{1\,cm}$, was found to be 1.23. The amount of γ-globulin collected in the pH 3.0 buffer was greater than in the pH 7.2; the two were added to give a total. A small deduction was made in this calculation, in order to account for the protein occupying the estimated 0.3 ml void space surrounding the particles. The gel was neutralized with 4 ml of PBS before beginning the next cycle of adsorption and elution.

By repeating runs, it was found that the amount of γ-globulin released remained at the same level, indicating that the adsorbent capacity of the Sepharose–protein A was maintained (as has also been reported elsewhere [8]) and that binding was completely reversed during elution. Hence the amount adsorbed during each cycle was equated to the amount subsequently released.

Effect of bead size on the reaction rate

The Sepharose–protein A beads were separated into two size-fractions by hydraulic elutriation [9]. The objective was to compare the rates of uptake by the two fractions. Unfortunately, the smaller beads formed a less permeable bed than the larger ones, and the necessary flow-rates to produce the excess factor of 5 referred to in the previous section could not be obtained, because, due to the deformability of these particles, increase of the applied fluid pressure does not bring about a more rapid flow once a certain limit (about 200 mmHg) is exceeded. An alternative to the perfusion method, namely a stirred-reservoir method after Chase [10], was therefore used. Suspensions of beads of each size, containing 1 ml of gel and 11 ml of PBS, as measured by centrifugation at 200 *g* for 5 min, were prepared. The suspensions were placed, in turn, in a magnetically stirred reservoir. A peristaltic pump collected fluid from the reservoir, via a strainer, and circulated it at a rate of 2 ml min^{-1} through a recording spectrophotometer set to 280 nm. The volume of the tubing and detection cell was 1.5 ml. A stock solution containing approximately 16 mg ml^{-1} of porcine γ-globulins was prepared. One ml of this solution was pipetted into the reservoir. The concentration recorded by the spectrophotometer rose sharply, and then fell at a rate dependent on the rate of uptake by the particles.

In a separate experiment, in which the reservoir contained 12 ml of PBS and no Sepharose–protein A, it was found that this system of monitoring the concentration responded within about 30 s of the γ-globulin entering the reservoir. This was sufficiently rapid compared to the rate of uptake by the beads. It was also noted that, with a suspension of Sepharose–protein A in the reservoir, and no γ-globulin added, no

protein was detected, which indicated that there was no significant leaching of protein A from the beads due to the stirring.

The above experiments were carried out with a stirrer speed of 900 rpm. Using unfractionated Sepharose–protein A beads, the effect on the rate of uptake of increasing the stirrer speed to 2900 rpm was investigated.

Measurement of the diffusion coefficient of γ-globulin in membrane

The diffusion coefficient of γ-globulin across the membrane was measured by creating an approximately constant gradient of concentration of γ-globulin between one side of membrane and the other and measuring the flux which ensued.

The diffusion apparatus consisted of two cylindrical glass chambers separated by the membrane under study (Fig. 2). The membrane was underivatised, *i.e.* no protein A was attached to it, and the 0.45 μm pore size was used throughout. Its thickness as measured with a micrometer was 161 ± 5 μm (mean ± S.D., 20 samples). A solution of porcine γ-globulin (100 ml) at known concentration in PBS, containing ^{3}H-labelled human γ-globulin (specific activity 1.8 μCi mg^{-1}) was placed in the lower chamber (donor chamber) in contact with the lower surface of the membrane. Care was taken to remove any air that was trapped beneath the membrane. The upper chamber was filled with 20 ml of PBS. At 5-min intervals this fluid was exchanged with fresh buffer. Solutions were slowly agitated by magnetic stirring in the lower, and an impeller in the upper chamber. The temperature during the experiment was maintained at 25°C.

Concentrations of the ^{3}H-labelled γ-globulin in the aliquots of the fluid removed from the upper chamber was measured by scintillation counting, enabling the diffusive flux to be calculated.

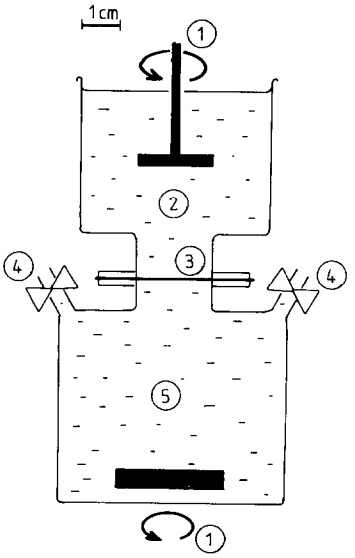

Fig. 2. Apparatus for measuring coefficient of diffusion of γ-globulin through underivatised membrane. 1 = Stirrers; 2 = upper chamber; 3 = membrane sample, 4 = taps, and 5 = donor chamber.

RESULTS

Comparison of the reaction rate and performance in the different affinity systems

The results of the reaction-rate studies measured with the techniques described in the Experimental are shown in the form of graphs of uptake of γ-globulin (in mg per cm^3 of the membrane or gel) against time multiplied by concentration (Fig. 3). These enable a comparison of the four different affinity systems:

(i) Sepharose–protein A with porcine γ-globulin;

(ii) 0.45 μm pore size membrane–protein A with human γ-globulin solution forced to flow through the pores under pressure;

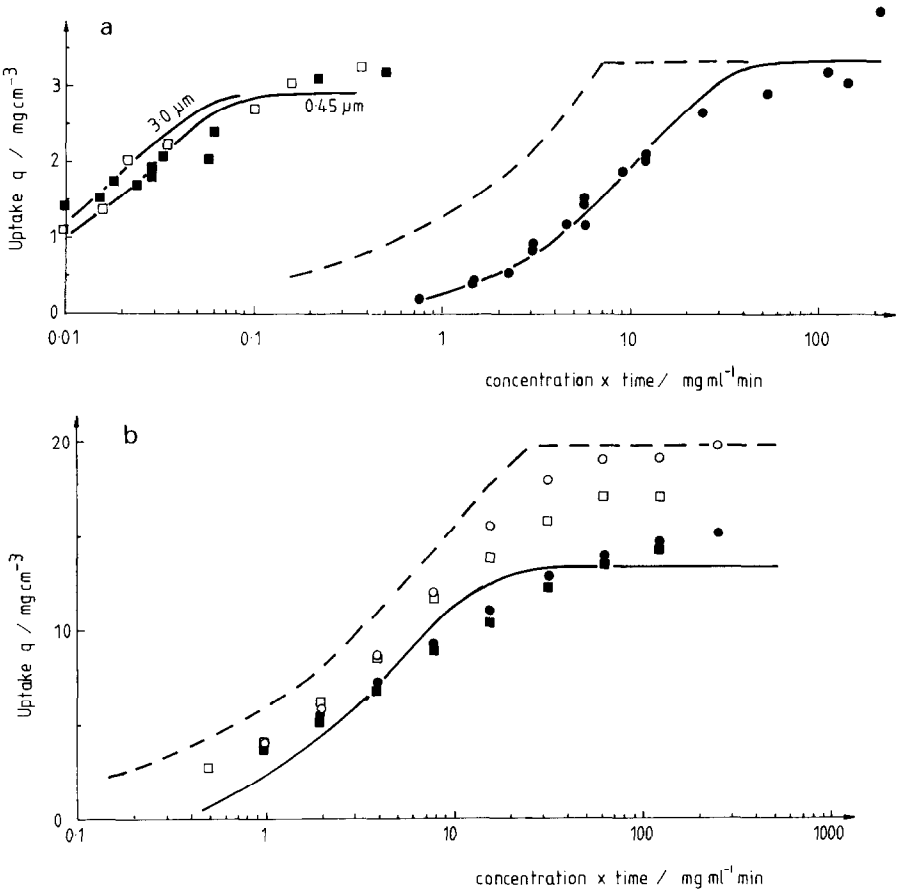

Fig. 3. (a) Uptake q of human γ-globulin onto membrane–protein A *versus* γ-globulin concentration (c) multiplied by time (t). Squares show results obtained with γ-globulin solution forced to flow through pores; ■, 0.45 μm pore-diameter membrane; □, 3.0 μm pore-diameter membrane. Circles show results obtained by immersing membrane (0.45 μm pore diameter) in the γ-globulin solution with no flow through the pores. ———, Curves given for each case by least square fit of eqn. 11. — — —, Theoretical curve for immersed membrane based on eqn. 6. (b) Uptake q of porcine γ-globulin onto Sepharose–protein A *versus* γ-globulin concentration (c) multiplied by time (t). Circles, c = 4 mg/ml; squares, c = 2 mg/ml. ● and ■, Values based on amount eluted with pH 3.0 buffer and ——— least square fit to eqn. 1. ○ and □, Values based on amount washed off with pH 7.2 buffer added to that eluted by pH 3.0 buffer and theoretical curve (— — —) for this case based on eqns. 4 and 5.

(iii) as (ii) but with 3.0 μm pore size membrane–protein A;

(iv) 0.45 μm pore size membrane–protein A simply immersed in human γ-globulin, with no flow through the pores.

The Sepharose–protein A system, (i), may be compared to the immersed membrane, (iv), in the sense that since there is no flow through the pores of the beads or membrane, diffusion is relied on to transport the γ-globulin molecules to the binding sites.

The comparison is aided by the use of the following equation derived from the laws of mass-action for a first-order, irreversible reaction:

$$q = q_m [1 - \exp(-k_{eff}\, ct)] \quad (1)$$

where q is the uptake after time t, q_m is the effective capacity for γ-globulin (supplied at concentration c) under the experimental conditions used, and k_{eff} is an effective forward reaction rate constant. This is only an approximate model, since it assumes that the various factors which could control the reaction may all be described by one constant k_{eff}. Also, the assumption that the reaction is irreversible is only approximately valid, as will be discussed later with regard to the theoretical modelling. Nevertheless, this equation gives a useful indication of the performance of each system. The values of q_m and k_{eff}, summarized in Table I, were obtained using a statistical computer program which gave a least-squares fit. We include in the measure of q only the γ-globulin eluted by the pH 3.0, and not by the pH 7.2. For the Sepharose system we are able to assess the importance of the protein released by the pH 7.2 washing: if included, values of $k_{eff} = 0.16\ mg^{-1}\ ml\ min^{-1}$ and $q_m = 17\ mg\ cm^{-3}$ result, respectively 16% lower and 31% higher than those shown in Table I (row 1). The amount of protein released by the pH 7.2 buffer was consistently more than could be accounted for purely in terms of protein occupying pores inside the beads, and it is estimated that up to 3 mg were chemically desorbed per ml of gel.

TABLE I

PARAMETERS DESCRIBING THE REACTION BETWEEN γ-GLOBULINS AND IMMOBILISED PROTEIN A IN THE DIFFERENT SYSTEMS

Values of q_m and k_{eff} were obtained by curve fitting. The values in parentheses show the likely variation on the basis of a 95% confidence limit. Ligant utilisation represents the average number of γ-globulin molecules binding to each molecule of protein A.

Support	Pore diameter (μm)	Flow conditions	Source of γ-globulin	Maximum capacity (q_m) ($mg\ cm^{-3}$)	Reaction rate coefficient (k) ($mg^{-1}\ ml\ min^{-1}$)	Ligand utilisation (mol/mol)
1. Sepharose	–	Immersion	Pig	13 (12–14)	0.19 (0.13–0.24)	1.6
2. Membrane	0.45	Flow-through	Human	2.9 (2.5–3.4)	39 (24–55)	1.5
3. Membrane	3.0	Flow-through	Human	2.9 (2.4–3.5)	51 (20–83)	1.5
4. Membrane	0.45	Immersion	Human	3.3 (3.2–3.5)	0.086 (0.072–0.101)	1.5

Our primary interest was in studying the adsorption of human γ-globulin, with relevance to medical applications, but the large quantities required for the experiments with Sepharose–protein A favoured the use of porcine γ-globulin as a model. Pilot studies indicated that the rates of adsorption onto Sepharose–protein A were comparable between the two species. We also note that values of $k_{eff} = 0.283$ mg^{-1}ml min^{-1} and $q_m = 14.5$ mg cm^{-3} have been reported for the uptake of human γ-globulin onto Sepharose–protein A [11], which are comparable to those shown in Table I (row 1) for porcine γ-globulin.

The value of k_{eff} in the case of the Sepharose, (i), was of comparable order to that obtained for the immersed membrane with no imposed flow, (iv). With flow through the pores, (ii) and (iii), however, the value of k_{eff} for the membrane was 200–300 times greater than for the Sepharose. The difference in pore size did not affect significantly the performance of the membrane system. The capacity q_m (in terms of amount of γ-globulin per unit volume of adsorbent) of the membrane was lower than for the Sepharose by a factor of about 4. This comparison may be affected by the differences in the concentrations of the γ-globulin solutions used, but unfortunately these differences were necessary in order to bring the timescales of the reactions into practical limits for experimentation. Ligand utilisation, expressed as a number of γ-globulin molecules bound per molecule of protein A, was comparable in all four instances.

Effect of bead size on reaction rate

The variations of the concentration of γ-globulins in the stirred reservoir with time, for the smaller and larger beads, are compared in Fig. 4. In the case of the smaller

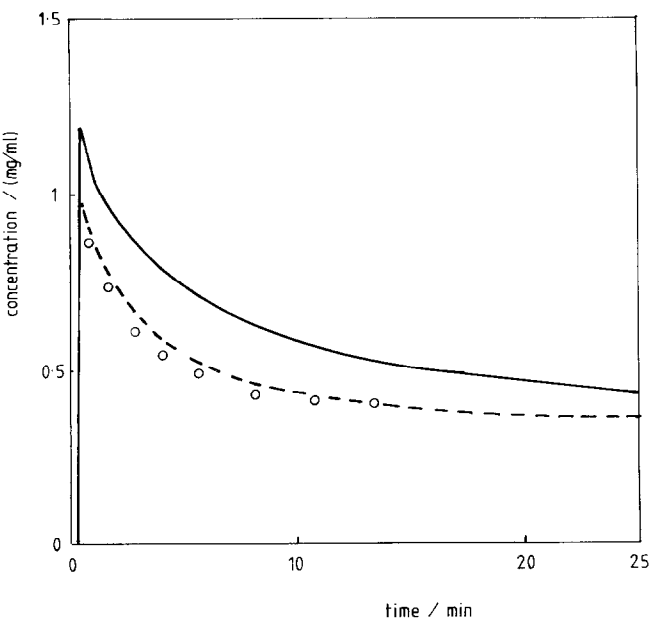

Fig. 4. Concentration as monitored by spectrophotometer of γ-globulin, *versus* time, in stirred suspension of Sepharose–protein A following addition of γ-globulin solution. ———, Large beads; – – –, small beads. ○, Theoretical points for small beads based on ratio of squares of the diameters (see Results).

beads, the concentration fell more rapidly, indicating a faster rate of uptake of γ-globulins.

It has been suggested that the dominant mechanism governing the rate of uptake onto beaded affinity supports is diffusion from the outer surface of the beads to the interior [4]. Provided that the spatial distribution of protein A throughout the interior of the beads is similar in the case of the two different size beads, this would mean that the rate of uptake would be proportional to the square of the bead diameter. The mean effective diameter is taken as that giving an equivalent surface area per volume [12]. This mean may be calculated as the sum of the cubes of the diameters of the beads divided by the sum of their squares. For the beads used here, the mean effective diameters were, from the microscopic examination of 100 of each type, calculated as 72 μm and 126 μm. (The arithmetic means and standard deviations were 65 ± 15 μm for the smaller, and 111 ± 32 μm for the larger beads.) If the rate of uptake is controlled by the diameter, then this rate should be $(126/72)^2 = 3.1$ times faster in the case of the smaller beads. Accordingly, the theoretical points in Fig. 4 show the predicted decrease of the reservoir concentration with time for the smaller beads, obtained by dividing the time-points for the larger beads by a factor of 3.1. The rate of uptake onto the smaller beads is close to that predicted. This gives a clear indication of the importance of bulk diffusion from the outer surface of the beads to the interior.

Increasing the stirrer speed from 900 to 2900 rpm increased the rate of reaction by about 13%, which suggested that diffusional resistance in the fluid film surrounding the particles was small but not insignificant.

Diffusion coefficient of γ-globulin in the membrane

For the purpose of the theoretical modelling, a measure of the diffusion coefficient governing movement of γ-globulin molecules through the membrane was needed.

The theory behind the measurement is simple [13], but the practice is very often fraught with potential error. In order to minimise mechanical convection and gravitational effects, the diffusion apparatus was designed so that the volume of fluid in the donor chamber remained constant since it was not open to the air, and so that the γ-globulin molecules diffused upwards.

As a quantitative description of the transport process, we have adopted Fick's assumption that the flux J, defined as a number of moles of substance crossing a unit area in a unit time, is proportional to the concentration gradient, *i.e.*

$$J = -D\frac{\mathrm{d}n}{\mathrm{d}x} \tag{2}$$

where n is the molar concentration of the solute, x a measure of the path of diffusion (in this case measured as thickness through the membrane). Using the above definition of flux we arrive at:

$$D = \frac{V\Delta x\, c_t}{tA\, \bar{c}_0} \tag{3}$$

where $\bar{c}_0$ is the mean concentration (mg ml^{-1}) of the solute in the donor chamber, based on measurements before and after the experiment, c_t is the concentration in the upper chamber, which contains volume V of fluid, and is exchanged after time t. The surface area of the membrane is represented by A.

Mean values of coefficient of diffusion across the membrane are shown in Table II. We note that the concentration in the donor chamber did not fall significantly during the course of the experiments.

TABLE II

MEASURED DIFFUSION COEFFICIENT OF γ-GLOBULIN IN THE MEMBRANE

$A = 3.14$ cm^2; $\Delta x = 161$ μm. Diffusion coefficient represents a mean of ten measurements.

Concentration of γ-globulin in the donor chamber ($\bar{c}_0$) (mg ml^{-1})	Diffusion coefficient (D) (10^{-7} cm^{2}s^{-1})	Variation of concentration of γ-globulin in the donor chamber (%)
10.0	4.1 ± 0.8	−7.1
7.5	4.2 ± 1.2	+1.2
5.0	4.4 ± 0.9	−1.3

The diffusion coefficient tends to increase slightly toward a limiting value as the solution is diluted. Accordingly, D refers to a diffusion coefficient rather than a diffusion constant and was estimated as $4.2 \pm 0.1 \cdot 10^{-7}$ cm^{2}sec^{-1}. As compared with the quoted value of human γ-globulin in free solution $4.0 \cdot 10^{-7}$ cm^{2}sec^{-1} [14] we therefore assume that the rate of the diffusion of γ-globulin molecules in the membrane is close to that in free solution, which is not unreasonable given that the void fraction of the membrane is quite large (0.65). It is impossible to eliminate entirely convection from the experiment, which tends to cause an artificially high value of diffusivity to be recorded.

Theoretical modelling of reaction rates, based on diffusion coefficients

An attempt has been made to model the rates of reaction on the basis of the rate of diffusion of the γ-globulin from the surface to the interior of the membrane or beads. It is assumed that once γ-globulin molecules have penetrated to a depth inside the material where vacant binding sites are situated, binding occurs virtually instantaneously. The model does not take into account convection, and so, in the case of the membrane, can only apply to those experiments where the membrane was simply immersed in the γ-globulin, not to those where the γ-globulin solution was forced to flow through the pores. The model used is that of the "unreacted core" [15], and is based on the following four simplifying assumptions:

(1) The interior of each bead or membrane is treated as a continuum. The model does not attempt to describe the pore structure, which in both instances is fine relative to the gross dimensions (*i.e.* bead diameter or membrane thickness) of the material. However, a partition coefficient, K_{av}, representing the fraction of the internal volume

of the beads or membrane accessible to the γ-globulin molecules, is included to allow for the fact that the protein is partially excluded.

(2) The protein A is bound uniformly across the diameter of the beads, and through the thickness of the membrane. Staining and sectioning the beads and the membrane, with gold particles and fluorescent dyes, showed that there was protein A present in all parts of the beads and of the membrane. Although it was difficult to assess the distribution quantitatively by this method, the assumption of an uniform distribution appeared reasonable. Furthermore during the preparation of the beads and membrane, an excess of protein A was applied, which suggests that all parts of the beads and membrane were saturated with protein A.

(3) It is assumed that resistance to mass transfer through the fluid adjacent to the surface of the beads or membrane is small. At least in the case of the beads, this could be justified [16] by the application of Pfeffer's analysis [17].

(4) The reaction is regarded as being irreversible during adsorption at pH 7.2. This is only approximately true. For human IgG, the dissociation constant K_d for the reaction with protein A is reported to be in the range 3 to 8 μg ml^{-1} [18], compared to the concentrations of 10 to 40 μg ml^{-1} used here. An experiment was carried out to determine the dissociation constant for the interaction of porcine γ-globulin with Sepharose–protein A. A non-linear Scatchard plot was obtained (data not shown), suggesting a hetereogeneous reaction [19]. An average K_d of 0.2 mg ml^{-1} was estimated, compared to the concentrations of 0.5 to 8 mg ml^{-1} used in the present studies.

The "unreacted core" model divides the beads, or membrane, into an outer region, where the protein A is fully saturated with bound γ-globulin molecules, and an inner core, into which γ-globulin molecules have not yet penetrated. As γ-globulin diffuses from the outer surface to this core, the core diminishes in size and disappears completely once the reaction is complete. The mathematical treatment is straightforward provided that the concentration c of γ-globulin surrounding the beads, or membrane, is substantially smaller than the concentration q_m in which it finally binds, a condition which is generally met in these experiments. For the beads, which are spherical, the appropriate solution is [15]:

$$\frac{t}{T} = 1 - 3\left(1 - \frac{q}{q_m}\right)^{\frac{2}{3}} + 2\left(1 - \frac{q}{q_m}\right) \tag{4}$$

where T, the time taken to complete the reaction, is given by:

$$T = \frac{d^2 q'_m}{24 D K_{av} c} \tag{5}$$

where d is the mean effective diameter (giving equivalent surface area/volume). Here, $d = 98$ μm.

In the case of the membrane, the expression used is:

$$\frac{q}{q_m} = \sqrt{\frac{8ctK_{av}D}{q_m(\Delta x)^2}} \tag{6}$$

where t is time measured from when the reaction begins. The concentration q'_m in eqn. 5 must be referred to the volume of the beads themselves, not the whole bed including the fluid surrounding the beads. In separate experiments, in which DNA was used to measure the exclusion from the columns of Sepharose packed down under comparable pressures (a method similar to that used by Laurent [20]), it was found that the beads typically occupied two-thirds the total column volume. A similar factor was assumed here, giving q'_m as $1\frac{1}{2}$ q_m. For the beads, the values of D and K_{av} were taken from ref. 16 as $1.12 \cdot 10^{-7}$ cm^2 sec^{-1} and 0.62, respectively. The value of q_m was taken as the maximum reached, *i.e.* 19.8 mg/ml, using the measure of uptake based on the total of the γ-globulin eluted using the pH 3.0 and pH 7.2 buffers. For the membrane, q_m was taken from Table I, row 4, the free solution diffusivity was assumed for D, and K_{av} was taken as the void fraction, which was determined as 0.65 by measuring the density of the material, and comparing it with that of solid nylon. The curves of q against t resulting form these equations are shown in Fig. 3 (broken lines).

The model tends to predict faster rates of reaction than observed, and this is attributed to the reversible nature of some of the interactions taking place during the process of adsorption, *i.e.* condition 4 above is not fully met.

It has been reported [21] that weak interactions occur between the Fab part of the γ-globulin molecules and protein A. In addition, electrostatic interactions, for example between bound and unbound γ-globulin [22], or involving the support matrix, could be important. By competing with the stronger interactions on which affinity chromatography using protein A relies, these weak interactions may effectively slow down the adsorption.

In the case of the membrane, the possibility of a significant fluid film resistance (not taken into account by the model) cannot be discounted.

It is clear that the "unreacted core" model is only partially applicable to the systems studied. It is likely to give an upper limit to the rate at which uptake occurs. On the other hand, more complicated models would probably require parameters which would be difficult to determine accurately, and the mathematical solution would soon become impossible except by numerical means.

DISCUSSION

Our comparative studies have established that the rate of association of γ-globulin with protein A supported on a microporous nylon membrane, when the γ-globulin solution was forced through the pores of the membrane, was 200–300 times faster than in the conventional agarose bead affinity system. However, when the membrane was simply immersed in γ-globulin solution, so that the fluid inside the pores remained static, this increase was not obtained. These findings are in general agreement with those of Brandt *et al.* [3], although quantified for the first time. In order to explore the mechanisms underlying these differences, the experimental data have been compared with the predictions of theoretical models based on various assumptions.

It has already been suggested [4] that diffusion from the surface of the bead to the interior may limit the reaction rate in agarose-based affinity media, and the finding that the rate of reaction increased in inverse proportion to the square of the bead diameter supports this. By extension, it is thought a similar diffusive mechanism limits

the rate of reaction which followed immersion of the membrane without imposed flow, *i.e.* that the rate is governed by the membrane thickness in this instance. Therefore we have attempted to model the rate of reaction on the basis of such diffusive processes. Independently determined values of the diffusion coefficients have been used. By the use of an analytical model based on a shrinking, unreacted core, we are at least able to estimate an upper limit of the reaction rate in affinity media in which there is no flow through the pores.

In the presence of flow through the pores, convection dominates over diffusion in transporting molecules from the surface to the interior. Nevertheless, a diffusive mechanism of rate control may still apply, within a smaller region around the sites of binding. We discount diffusion from the centre of the pores to their walls as a rate-limiting process, as increasing the pore size from 0.45 μm to 3.0 μm did not slow down the reaction. It is thought, however, that above a certain threshold of pore-size, a decrease in reaction rate must occur.

The importance of molecular dimensions for diffusion-controlled reactions may be modelled by the Smoluchowski formula [27]:

$$\text{forward reaction rate constant (per molecule)} = 4\pi DR \tag{7}$$

where R is the sum of the radii of the two reacting molecules, estimated as 8 nm. To account for the motion of the fluid, the use of a formula due to Levich [24] is suggested [25]. However, in this instance Levich's formula only produces a relatively small correction to the Smoluchowski result. It may also be considered appropriate to divide the prediction of Smoluchowski's formula by two, since the formula was derived on the basis that the molecules may approach each other from all directions, whereas here the protein A molecules may only be approached from one side because they are attached to a solid surface. Whereas there is some difficulty in applying this formula to a heterogeneous biochemical reaction, it might nevertheless be expected to give an order-of-magnitude indication.

In any case, the formula gives a reaction constant of about 10^6 mg^{-1} ml min^{-1}, which exceeds by a factor of about 10^5 the experimental value. Whereas Smoluchowski's model assumes that reaction occurs as a result of all collisons between the molecules, in reality the reaction only takes place if they come together in the correct mutual orientation. For this, and other, reasons, Smoluchowski's model often gives predictions which are in considerable error. However, it is difficult to modify the model in such a way as to make it more realistic [23].

Finally, it is also possible that the reaction rate in the flow-through membrane system is controlled by a barrier of chemical activation energy. This would correspond to a situation in which the molecules frequently encounter each other with the correct mutual orientation, but only occasionally have sufficient kinetic energy to overcome the repulsive forces which tend to prevent them from reacting.

It has to be concluded that it is difficult to elucidate the mechanism of reaction rate limitation in the membrane with flow through the pores. It is possible that more than one mechanism is of importance.

Microporous membrane affinity systems may be compared to the use of affinity adsorbents in high-performance liquid chromatography (HPLC), in that both achieve a higher reaction rate by minimising the diffusion path, whilst their capacities are

maintained at about one quarter of those obtained in agarose bead systems. Rapid adsorption rates have been reported by Eveleigh and Levy [26] using non-porous, glass particles. The flow through the interstices between particles of this kind, when used in an HPLC column, may be compared to the flow through the pores of the membrane.

In both systems, the gain in reaction rate is paid for in terms increased operating pressures. However, a possible advantage of the membrane system is the easier manipulation of the geometry of the device. For example, by passing the fluid through a flat disc of the membrane, substantial flow-rates can be maintained at quite low pressures (<1 bar). For particulate adsorbents, the corresponding design would be a column of very small length to diameter ratio; whereas in practice it may be difficult to maintain an even flow through such a column.

With regard to permeability, it may be important to optimise the membrane pore size. We have found that a 3.0 μm pore size membrane has a permeability ten times greater than the 0.45 μm pore size membrane studied here, whilst its capacity is similar.

It seems to us that the rapid reaction kinetics of affinity membranes, together with the fairly low operating pressures which they require, should enable them to be used economically in large-scale operations.

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SYMBOLS

A	surface area of the membrane
c	concentration of the solute (mg ml^{-1})
$\bar{c}_0$	mean concentration of the solute in the donor chamber
c_t	concentration of the solute in the upper chamber after time t
d	mean effective diameter of the beads giving equivalent surface area/volume
D	diffusion coefficient
J	number of moles of substance crossing a unit area in a unit time
k_{eff}	effective forward reaction rate constant
K_{av}	partition coefficient
K_{d}	dissociation constant
n	molar concentration of the solute
q	uptake of the solute after time t
q_{m}	effective capacity to adsorb the solute
q'_{m}	capacity to adsorb the solute, per cm^3 of the beads themselves excluding the surrounding fluid
R	sum of the radii of the two reacting molecules
t	time
T	time required to complete the reaction
u	mean velocity of the liquid in the pore
V	volume of liquid in the donor chamber
Δx	thickness of the membrane

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