Osmotic flow caused by chondroitin sulfate proteoglycan across well-defined Nuclepore membranes

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Osmotic flows generated by solutions of Swarm rat chondrosarcoma proteoglycan subunit have been analysed using Nuclepore membranes of well-defined straight-through cylindrical pores of known radius r_p. Membranes with r_p in the range of 27–500 nm were studied. For semipermeable membranes, which are impermeable to the proteoglycan, the flows were consistently related to r_p^2 and not to r_p^4 (Poiseuille's Law) which demonstrates that the flow is a diffusion-controlled process as described previously (R.P.W. Williams and W.D. Comper, J. Phys. Chem. 91 (1987) 3443). We have also identified a characteristic distance, approx. 50% of the average interparticle spacing, out from the pore surface of the membrane over which the proteoglycan has to move to generate flow. The proteoglycan generates similar osmotic permeability coefficients with membranes up to 125 nm in pore diameter which is significantly larger than the average intermolecular distance. These results have been interpreted in terms of the membrane pore recognising dynamic transient aggregates in the proteoglycan solution.

1. Introduction

Recent studies by the present authors [1,2] have provided a new interpretation of osmotic flow. Experimentally, we consider osmotic flow across an inert semipermeable membrane caused by the osmotically active solute. The flow is considered to be analogous to gel swelling. Specifically, it is a diffusion-driven process consisting of a series of events involving (1) a rate-limiting solvent-solute exchange governed by the diffusional relaxation of the solute concentration gradient in the semi-dilute solution immediately adjacent to the membrane; (2) the exchange process then draws water flow through the membrane. The new factor in the osmotic flow model is the recognition of the solution-dependent process governing osmotic flow,

namely, the hydrodynamic frictional coefficient of the osmotically active solute.

This study sets out to investigate osmotic flow created by chondroitin sulfate proteoglycans across Nuclepore polycarbonate membranes of well-defined pore size and density and with a range of pore diameters. This study will not only yield quantitative measurements of osmotic flow produced by the proteoglycan but will also provide information on the nature of the thin layer on the solution side of the membrane where solute-solvent exchange, driving osmotic flow, takes place and the overall contribution of the membrane to osmotic flow. The understanding of osmotic flow mechanisms for chondroitin sulfate proteoglycan will also provide information concerning its dynamic organisation in cartilage extracellular matrices. In such matrices the proteoglycan endows osmotic properties which are important for the overall biomechanical properties of the tissue.

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2. Theory

The theoretical and experimental approach employed in this study is one where we examine water flow across inert, porous membranes of well-defined porosity separating a solution of proteoglycan from a compartment containing solvent. For membranes impermeable to the osmotically active solute, the osmotic flow can be described by the following equation [2]:

$$J_v = \Delta\Pi / \left[(1/L_p) + \left(f_{12}l/v_1(1 - \phi_1)^2 M_1 A_{eff} \right) \right]$$

(1)

where J_v is the volume flow, $\Delta\Pi$ the osmotic pressure across the membrane, L_p the hydraulic permeability of water through the membrane when $\Delta\Pi = 0$, f_{12} the hydrodynamic frictional coefficient between proteoglycan (component 1) and solvent (regarded as a combination of water and simple electrolyte NaCl), l the distance in front of the pore over which solute-solvent exchange takes place, v_1 the partial specific volume of the proteoglycan, ϕ_1 the volume fraction, M_1 the molecular weight and A_{eff} the effective area of the membrane. Experimental measurement of J_v with knowledge of $\Delta\Pi$ and A_{eff} will yield values of l for the osmotically active solute involved.

For permeable membranes the solute will partition itself at the membrane pore. The simplest approach is to regard the proteoglycan as an effective sphere of radius r_1 that is sterically excluded from the pore of radius r_p. This gives a partition coefficient (λ)

$$\lambda = (1 - \alpha)^2 = C_1'/C_1$$

(2)

where $\alpha = r_1/r_p$, and C_1' and C_1 are the concentrations of component 1 just inside the membrane pore and in the bulk solution, respectively. This approach regards processes at the membrane solution interface as critical in determining the degree of osmosis. For osmotically active solute initially on one side of the membrane only, it is then the corresponding difference in osmotic pressure across the mouth of the pore, $\Pi(C_1) - \Pi(C_1')$, that will drive osmotic flow. This will be generated by molecules excluded by the pore and undergo-

ing diffusional exchange with solvent, with a frictional coefficient corresponding to the concentration C_1, over the characteristic distance l in front of the pore. It is assumed that all other molecules will enter the pore and do not become osmotically active. The volume flow generated by diffusion of solute in the pore is also assumed to be negligible. With these considerations we can rewrite eq. 1 for permeable membranes as

$$J_v = \left[\Pi(C_1) - \Pi(C_1') \right] / \left[(1/L_p(C_1')) \right.$$
$$\left. + \left(f_{12}(C_1)l/v_1(1 - \phi_1)^2 M_1 A_{eff} \right) \right]$$

(3)

where $L_p(C_1')$ is the hydraulic permeability of the proteoglycan solution assumed to be at a concentration C_1' through the membrane. Values of L_p were calculated using the Poiseuille equation (eq. 4) taking the viscosity function corresponding to water for impermeable membranes and proteoglycan solution at C_1' for permeable membranes.

3. Experimental

3.1. Materials

Carbazole (laboratory grade) was obtained from British Drug Houses, Australia. Track etched polycarbonate membranes with nominal pore diameters of 0.03, 0.05, 0.08, 0.1, 0.2, 0.4, 0.6 and 1.0 μm were obtained from Nuclepore Corp. (Pleasanton, CA). Proteoglycan subunit (PGS) was purified from Swarm rat chondrosarcoma tumors that were grown in female Wistar rats as described previously [3]. Unless otherwise stated, all other reagents were of the highest grade commercially available.

3.2. Osmotic flow measurements

Osmotic flows were measured in a bivalve perspex cell, described in detail elsewhere [2], that incorporated a Nuclepore membrane in the vertical position, of exposed surface area of 3.14 cm^2, to separate a compartment that contained phosphate-buffered saline (0.14 M NaCl, 2.6 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, pH 7.5)
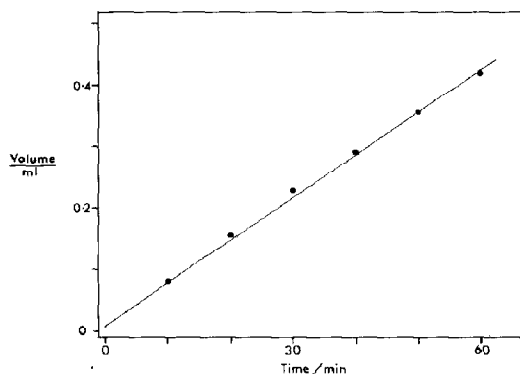
Fig. 1. Kinetics of osmotic flow as determined by capillary volume flow for an experiment with a stirred solution of PGS at an initial concentration of 19.8 mg ml^{-1} on the solution side of a Nuclepore polycarbonate track etched membrane with nominal pore diameter of 400 nm (see table 1).

and another that contained the osmotically active proteoglycan solution. The solutions were stirred at speeds of 200 rpm. The Nuclepore membranes were not pretreated in any way prior to their use in the flow cell. A new membrane was used for each flow measurement. Volume transfer across partially permeable membranes exhibited linear kinetics over a 60 min period (fig. 1). The resultant concentration change in the solution compartment was 14%. The flow was measured by monitoring the movement of the meniscus in capillary bore glass tubing, with an internal diameter of 1.75 mm, connected to each compartment. For routine analysis, volume flows were measured over the initial 1 h period. In control experiments we observed no detectable volume flow across the membranes used when $\Delta \Pi = 0$ and with the stirrers operating at maximum speed.

3.3. Osmotic pressure

Osmotic pressure of the proteoglycan solution was determined from the sedimentation-diffusion data of Comper and Williams [2,3].

3.4. Membrane characterization

The polycarbonate track etched Nuclepore membranes contain pores that are essentially uni-

form circular cylinders [4]. The membrane thickness (L) was determined from the membrane weight per unit area by using a value of 1.19 g cm^{-3} for the density of polycarbonate, knowledge of the porosity, and a slight correction to account for the small number of pores which are not aligned exactly perpendicular to the membrane surface [5]. Further, no correction was made for the small proportion of overlapping pores at the surface as it is likely that only a fraction of these pores continue to overlap entirely through the membrane, since they are not all aligned normal to the membrane surface [5]. Pore density for each membrane was determined by counting the number of pores (number range on photograph 60–180) of at least 10 scanning electron microscope (SEM) photographs (Hitachi S-570, Hitachi, Tokyo, Japan) obtained at 3000–14000 × magnification. No differences in pore densities were noted between different membranes taken from the same lot. The membranes were prepared for microscopy by lightly coating with platinum and the magnification was determined by an internal calibration system within the SEM. For membranes with nominal pore diameters greater than 100 nm the pore radius was determined directly from SEM photographs by measurement of at least 40 pores. Accurate resolution of the pore diameter for smaller pores was not possible. For these membranes, pore radii were determined from measurements of the hydraulic permeability. In this case, the membranes were mounted in the osmotic flow cell and subjected to applied pressures of 20–40 cmH$_2$O. Flow rates were determined by continuous weight measurement of the membrane effluent and pore radii (r_p) were calculated using the Poiseuille equation

$$r_p = \left[\frac{8\eta LQ}{n\pi A\Delta P} \right]^{0.25} \tag{4}$$

where η is the solvent viscosity, Q the flow rate, n the number of pores per unit area, A the area of the exposed membrane and ΔP the applied pressure gradient across the membrane. Values of r_p obtained for membranes with $2r_p < 125$ nm were larger than those suggested by the manufacturer. This has also been found in other characterizations of these membranes [5].

3.5. Viscosity

The viscosity of PGS in phosphate-buffered saline was measured in a capillary viscometer with a flow time of 30 s.

3.6. Preparation of solutions

The proteoglycan solutions were dialysed against phosphate-buffered saline to ensure thermodynamic equilibrium of simple electrolyte. All dilutions of the proteoglycan solutions warranted gentle mixing at 4°C for at least 24 h prior to use.

3.7. Analytical procedures

Uronic acid was determined by an automated carbazole method [6]. The physical constants and concentration conversion factors associated with the proteoglycan preparation have been described previously [3].

4. Results

The osmotic flows generated by 19.8 mg ml^{-1} Swarm rat chondrosarcoma PGS as a function of membrane pore diameter are shown in fig. 2. A concentration of approx. 20 mg ml^{-1} PGS was chosen as it is well above the critical concentration for molecular overlap [3] ($\sim$ 5 mg ml^{-1}). When the data are expressed as osmotic volume flow per unit effective area of the membrane (table 1), it is seen that the flow is quantitatively similar for pore diameters from 54 to 125 nm, suggesting proteoglycan impermeability within this range. At higher pore diameters the osmotic flow decreases. At $2r_p$ of 200 and 400 nm, the flows were approx. 70 and 30% of that obtained for $2r_p < 125$ nm. Significant flows were also recorded at $2r_p = 1000$ nm.

Since the influence of L_p in eq. 1 for the different membranes on J_v/A_{eff} is not readily apparent (as impermeable membranes may have varying J_v/A_{eff} depending on the value of L_p), the measure of impermeability is much better appreciated by estimating the parameter l in eq. 1. This is shown in the inset of fig. 2 where it is
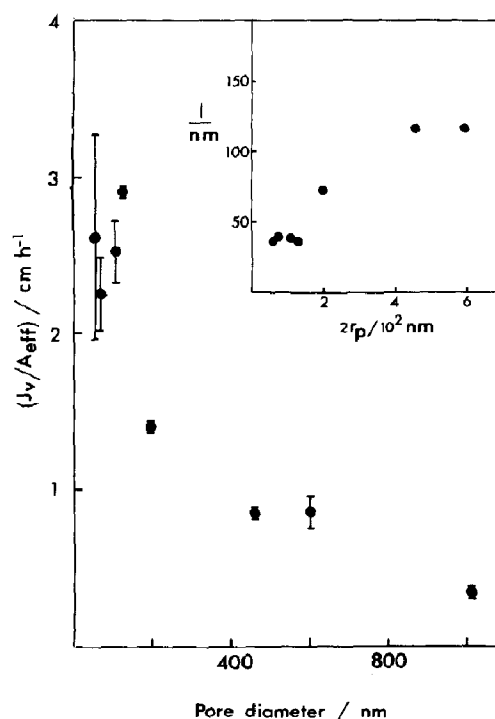

Fig. 2. The osmotic volume flow J_v/effective membrane area (A_{eff}) obtained over a 1 h period generated by 19.8 mg ml^{-1} PGS across Nuclepore capillary membranes with various pore diameters (table 1). Stirred at 200 rpm (●). Standard deviations are shown as error bars ($n = 3$). (Inset) The corresponding distance l in front of the pore, calculated from eq. 1.

Table 1

Nuclepore polycarbonate membrane characterisation

Nominal pore diameter (μm)	Membrane length (L) (μm)	Pore density [a] (n)($\times 10^8$) .cm^{-2})	Pore diameter ($2r_p$) (nm)	Effective area ($n\pi r_p^2$)
1.0	11.12	0.18	1019 [b]	0.151
0.6	9.96	0.40	600 [b]	0.113
0.4	11.47	0.94	457 [b]	0.154
0.2	8.97	2.97	194 [b]	0.0878
			207 [c]	
0.1	5.97	2.96	125 [c]	0.0365
0.08	6.00	5.28	106 [c]	0.0467
0.05	6.25	6.50	68 [c]	0.0310
0.03	6.26	5.92	54 [c]	0.0136

[a] S.D. varied from ±6 to ±9%.
[b] S.D. varied from ±5 to ±10% as determined by direct measurement of SEM photographs.
[c] Estimates from hydraulic flow.

demonstrated to have a constant value of approx. 37 nm for pore diameters in the range 54–125 nm and then its value increases substantially for larger pore membranes. These results indicate that membranes with $2r_p \leqslant 125$ nm are impermeable to the proteoglycan and that the effective diameter of the molecule is between 125 and 194 nm at 19.8 mg ml^{-1}.

Further significance of these results can be realised in terms of the average intermolecular distance in the PGS solution at 19.8 mg ml^{-1}. The hydrodynamic volume of the proteoglycan can be approximated in terms of a sphere [7]. Therefore, if we assume that the structure of the PGS solution can be regarded as a hexagonal close packing of spheres, with the molecular weight of the PGS taken to be 2.5×10^6, this will give an average intermolecular distance of 74 nm. The hexagonal packing model will tend to maximise the inter-

Pore diameter / nm

Fig. 4. Predicted osmotic flow for PGS at 19.8 mg ml^{-1} from eq. 3 with $l = 37$ nm, A_{eff} from table 1, and L_p (C_1') from eqs 2 and 4 and the viscosity data in the inset. The values have been calculated for $r_1 = 37$ nm (one half the average intermolecular distance between PGS molecules at 19.8 mg ml^{-1}) (curve 1) and $r_1 = 60$ nm (curve 2).
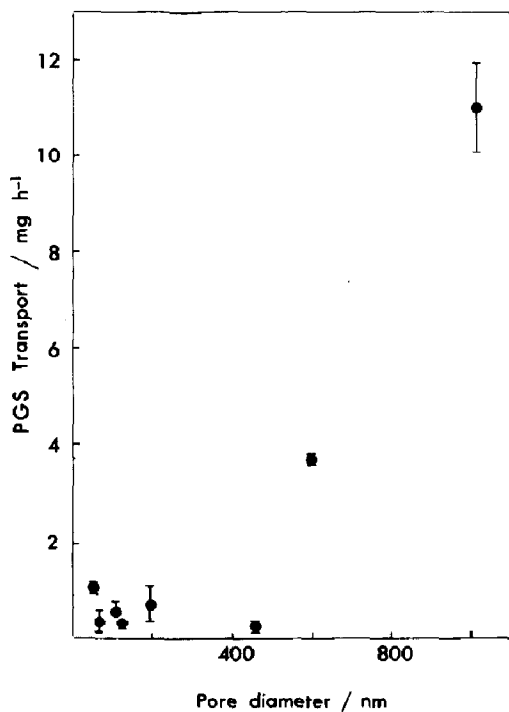

Pore diameter / nm

Fig. 3. The total quantity of PGS/A_{eff}, as determined by uronic acid assay, that has been transported across Nuclepore membranes in 1 h for the experiments described in fig. 2. Standard deviations are shown as error bars ($n = 3$).
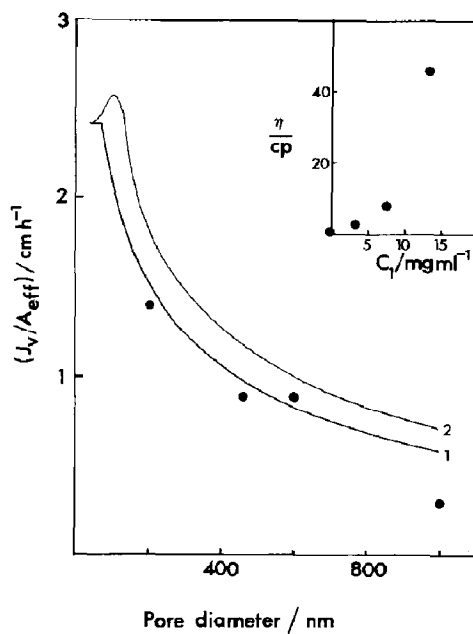
molecular distance. On this basis, for pore diameters less than 68 nm we would expect impermeability. The fact that these membranes gave identical l values up to $2r_p = 125$ nm provides further evidence that impermeability existed up to membranes of this diameter. The constant value of l of approx. 37 nm suggests that the proteoglycan at 19.8 mg ml^{-1} has to undergo an exchange with water over a distance corresponding to approx 50% of the average diameter of the proteoglycan.

The amount of PGS transported through the membrane, as determined by uronic acid assay, also confirms the relatively high effective diameter of PGS as estimated above (fig. 3). For membranes with $2r_p < 450$ nm there is a relatively low level of uronic acid containing material being transported, namely, less than 0.5% of the material on the solution side of the membrane. The small quantities that leak across the membranes probably reflect the polydispersity of the preparation.

We also note that for $2r_p \leqslant 125$ nm that the ratio of volume flow associated with osmotic flow of water across the membrane to the volume flow of proteoglycan transport across the membrane is greater than 4000 : 1. For $2r_p > 450$ nm the amount of PGS transported increases markedly indicating a transition in the nature of the size selectivity of permeability.

Efforts aimed at predicting proteoglycan-generated osmotic flow through permeable membranes using eq. 3 are shown in fig. 4. While we do not place emphasis on this interpretation it is of interest to examine whether the data give meaningful values of proteoglycan size and how flow is predicted to vary with r_p. It is evident that reasonable agreement is apparent for molecular radii in the range of 37 nm up to 60 nm although the analysis is not accurate enough, and the system too complex, to nominate a particular effective size of the material. It is apparent, however, that the viscosity component, embodied in the L_p term is insignificant in relation to the frictional coefficient term in eq. 3. Further, there is a strong dependence predicted of the J_v/A_{eff} term in relation to the variation of r_p, especially in the range of r_p 125–194 nm. This is essentially derived from the marked nonideality of the osmotic pressure term [2].

5. Discussion

Experiments involving equilibrium partitioning between phases based on molecular size have demonstrated that the PGS does occupy reduced volumes based on hard-sphere approximations at finite concentrations in high salt [7]. A different situation occurs in an osmotic flow experiment as the actual flow generated is the result of the effective kinetic unit on the solution side of the membrane. This unit could be a transient structure associated with the semi-dilute solution of the osmotically active solute which the membrane pore has to recognise so that flow is generated. Equilibrium partitioning experiments or experiments involving partitioning such as membrane transport at constant volume may not detect these transient structures. Therefore, the membrane pore may recognize a reduced macromolecular volume char-

acteristic of the individual molecule or it may recognize transient networks that form in semi-dilute solution by purely entropic entanglement effects [8]. Electrostatic effects would be minimal as electrolyte dissipation for the proteoglycan in PGS is negligible [9]. Transient aggregates may make individual PGS molecules appear larger to the membrane pore and create a more efficient osmotically active solute.

The present results suggest that the membrane pore excludes not only just individual PGS molecules but also transient network structures. These dynamic structures have diameters of the order of 100–120 nm, at least, and represent a unit which is significantly larger than the average intermolecular distance. For a simple hexagonal close packing of molecules with diameters $2r_1 = 74$ nm, then the transient structure would represent a pentamer of PGS molecules. These 'loose lattices' are not aggregates in the conventional sense with close proximity binding. Such aggregation processes are often characterised by significant negative virial coefficients and low osmotic pressure. The opposite is found as demonstrated by measurement of PGS osmotic pressure by the sedimentation-diffusion techniques which gave a strongly increasing function of Π with C_1 [2]. The transient aggregate structure is probably a loose aggregation of molecules brought about by physical entanglements.

The errors in the analysis leading to the hypothesis of transient networks of PGS are most likely to be associated with three factors, namely, the accuracy associated with pore size dimensions, the average intermolecular distance and the identification of membrane impermeability. We have established good agreement between the pore size identified by hydraulic flow and that estimated by direct SEM for the 200 nm membranes (table 1). It is also unlikely, if the estimate of 125 nm for the diameter of the nominal 100 nm membrane is incorrect, that this membrane would have a pore diameter less than 100 nm which would still make the proteoglycan appear to participate in transient aggregates. With regard to average intermolecular distance its value of 74 nm at 19.8 mg ml^{-1} PGS is likely to be on the high side considering the polydispersity of the proteoglycan preparation. An average molecular weight of 2.0×10^6 would give

an intermolecular distance of 68 nm. Again, in spite of possible errors in molecular weight estimation the differences in intermolecular distance and membrane pore diameter appear significant enough to demonstrate transient networks of PGS. The relationship of the partial permeability to molecular radius could be a source of error particularly for estimating the molecular radii for values near the transition of partial permeability to impermeability. Previous studies employing the Kedem-Katchalsky equations have demonstrated that permeability as represented by the standard reflection coefficient may take on a sigmoidal form [10]. However, it is clear that in working with highly osmotically active materials, like proteoglycan, and the new derivation of osmotic flow as embodied in eq. 3, that this transition, expressed in terms of volume flow (fig. 4) or the distance, l (fig. 2) is marked for this material. This would give us confidence to suggest that the effective

diameter of the proteoglycan is indeed greater than 125 nm. A further source of error that would lead to an underestimate of the effective size of the osmotically active proteoglycan may arise through the influence of stirring on the magnitude of the flows measured. While we cannot exclude the presence of 'unstirred layers' [1,2] it seems unlikely that they would significantly affect flow results in the stirred system for cylindrical pore membranes for a number of reasons: (1) the use of relatively high stirrer speed and the stirrer-membrane design which has the stirrer placed to within 0.5 mm of the membrane to maximise convection at the solution-membrane interface; (2) the consistent results obtained for osmotic flow obtained for stirred systems in this apparatus and similar osmotic flow cells when measured over a wide variety of osmotically active solutes including proteoglycan with tortuous pore membranes [1,2]; and (3) the relatively constant l values obtained for membranes with $2r_p \leqslant 125$ nm. The latter finding would also suggest that proteoglycan-membrane interaction is insignificant, since as the effective area of the pore varies by a factor of 3 for $2r_p \leqslant 125$ nm we could also expect, but do not observe, the influence of a similar variation in the proteoglycan membrane interaction area around the pore. Further evidence against this interaction is the linearity of flows over the first hour (fig. 1).

We consider that the results with the Nuclepore membranes offer significant confirmation of our new model of osmotic flow. For membranes impermeable to the proteoglycan the variation of flux is to r_p^2 which indicates that the process is diffusion-controlled. If we take the common assumption that the coefficient in eq. 1 is directly related to Poiseuille's law (eq. 4) then the flows would be related to r_p^4. This is clearly not the case as shown in fig. 5.

6. Conclusions

The major aim of this paper is an experimental study of the measurement of osmotic flows generated by a nonideal osmotically active solute, namely, the chondroitin sulfate proteoglycan, across well-characterized membranes of known
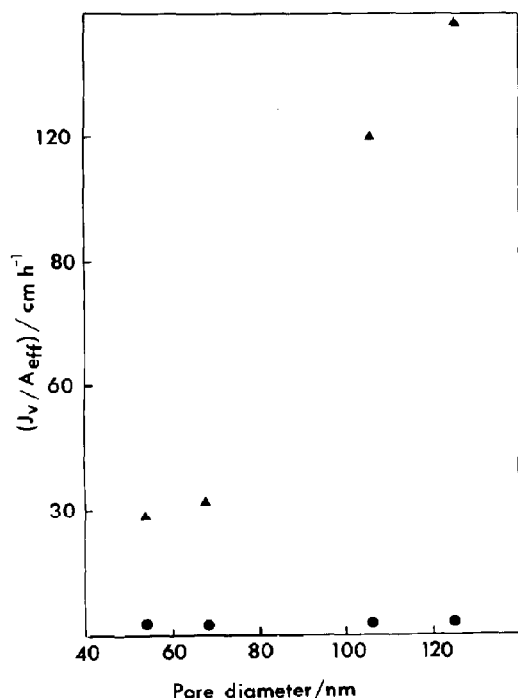

Fig. 5. Osmotic flow, J_v, represented as J_v/A_{eff} (●) and its predicted value (▲) calculated from Poiseuille's law (eq. 4) as a function of membrane pore diameter.

pore density and geometry. The conclusions are drawn essentially from experiments where the membrane is impermeable to the solute; the Nuclepore cylindrical pore membrane-proteoglycan system is one of the few systems that enable this type of study. The results demonstrate that dynamic aggregates may exist in the proteoglycan solution. These aggregates participate in a diffusion-controlled exchange process that drives water flow across the membrane. The process occurs over a small distance, of the order of a molecular dimension, in the solution immediately adjacent to the membrane.

Acknowledgements

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