Permeability of Composite Chondrocyte-Culture–Millipore Membranes to Solutes of Varying Size and Shape

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(Received 7 August 1978)

A model connective-tissue system was developed that is amenable to the determination of permeability coefficients of diffusing solutes. The system involves the culture of 13-day chick-embryo chondrocytes on a Millipore filter (HA:0.45 μm pore size) to form what is, in effect, a confluent, extremely thin cartilage slice of uniform thickness. These cultured chondrocyte membranes were used to measure the steady-state flux of radioactively labelled low-molecular-weight solutes and micro-ions. Similarly, the permeability coefficients of either radioactively labelled or enzymically active proteins across the membranes were determined. The membrane was found to have no marked effects on the diffusional behaviour of low-molecular-weight non-electrolytes (water, proline, ribose, glucose, sorbitol, raffinose). For micro-ions (Na^+ , SO_4^{2-} , Cl^- , glutamate, glucuronate), the diffusive behaviour was found to be markedly affected by the ionic strength of the solvent used in a manner which was consistent with a Donnan distribution resulting from the immobilized proteoglycans. Globular proteins permeated the membrane at rates which decreased as the molecular size of the diffusing solute increased. The apparent diffusion rates of fibrinogen and of collagen through the membranes were greater than would be expected on the basis of their diffusion coefficients in free solution. Reasons for this behaviour are discussed.

The permeability of cell and tissue compartments to solutes plays an important role in the regulatory function of many biological systems such as cell membranes, blood-capillary walls and connective tissues. In this regard, we have been particularly interested in the permeability of connective tissues such as articular cartilage. This system is characterized by a comparatively small number of cells embedded in an avascular extracellular matrix. The major macromolecular components of the matrix are collagen and proteoglycans. We are primarily concerned with the effects of this matrix on the transport of nutrients and metabolites to and from the cells, with particular reference to macromolecular transport involved in tissue turnover and deposition.

It is generally considered that the extracellular space can be compared with an aqueous gel consisting of a network of highly entangled proteoglycans which immobilize water and are resistant to water flow (Day, 1952; Fessler, 1960). It has been suggested that the major transport mechanism of solutes within connective tissues is diffusive flow (Laurent, 1966; Renkin, 1969).

Various studies have been made into the diffusive permeability of certain connective tissues such as intervertebral disc (Paulson *et al.*, 1951), articular cartilage (Kantor & Schubert, 1957; Brower *et al.*, 1962; Gerber & Schubert, 1964; Maroudas, 1970; Lotke & Granda, 1972) and cornea (Hedbys, 1963). The difficulties that present themselves in studying

transport across connective-tissue membranes are many. Biological tissues may be unstable and components may be leached out into the surrounding medium. There is often variability between tissues and within samples from a single tissue. Also it is difficult to obtain suitably thin slices from these tissues which retain their molecular integrity and a uniform distribution of extracellular macromolecules.

Another approach to the understanding of the permeability properties of connective tissues which enables the permeabilities of diffusing solutes to be related to the composition of the tissue is the use of model systems (Preston & Snowden, 1972, 1973a; Comper & Preston, 1975).

In the present study we have used a sophisticated model system which involves the use of mechanically strong matrices produced by cultured chondrocytes. The chondrocyte layers have morphological and physical properties comparable with those of ultra-thin slices of embryonic cartilage. The permeability of these culture layers to both small and macromolecular solutes has been determined.

Experimental

Materials

Commercially supplied. Edestin (hemp seed), thyroglobulin (bovine), peroxidase (horseradish) and glucose oxidase (*Aspergillus niger*) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Catalase (bovine liver) was obtained from Boehringer, Mannheim, Germany. ^{125}I -labelled human serum albumin, Na^{36}Cl (3 mCi/g of Cl), D-[U- ^{14}C]glucose (230 mCi/mmol), D-[U- ^{14}C]glucuronic acid, potassium salt (50 mCi/mmol), L-[U- ^{14}C]glutamic acid (250 mCi/mmol), [2- ^3H]glycine (4 Ci/mmol), [^{125}I]iodine, carrier-free (100 mCi/ml), L-[2,3,4,5- ^3H]proline (677 mCi/mmol), D-[1- ^{14}C]ribose (50 mCi/mmol), $^{22}\text{NaCl}$ (100 mCi/mg of Na), NaB^3H_4 (10 Ci/mmol), $\text{Na}_2^{35}\text{SO}_4$ (100 mCi/mmol, D-[U- ^{14}C]sorbitol (150 mCi/mmol) and [^3H]water (5 Ci/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [G- ^3H]Raffinose (15 mCi/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A.

All other chemicals were commercially available analytical reagents unless otherwise stated.

Preparation of radioactively labelled macromolecules. (i) Iodinated proteins. ^{125}I -labelled edestin and ^{125}I -labelled thyroglobulin were prepared by the method of Greenwood *et al.* (1963). Iodinated proteins were separated from free iodide on a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The proteins were then exhaustively dialysed against phosphate-buffered saline, pH 7.4 (Ca^{2+} - and Mg^{2+} -free) (Dulbecco & Vogt, 1954). By this method ^{125}I -labelled edestin and ^{125}I -labelled thyroglobulin with specific radioactivities of 120 mCi/mmol and 360 mCi/mmol respectively were prepared.

(ii) [^3H]Collagen. Calvaria were removed from 17-day chick embryos and were cleaned of extraneous tissue. The cleaned calvaria were then placed in Eagle's basal medium (CSL, Melbourne, Victoria, Australia) containing 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, 5 mM- Na_2SO_4 , ascorbate (50 $\mu\text{g}/\text{ml}$) and β -amino-propionitrile (64 $\mu\text{g}/\text{ml}$). The calvaria were incubated in a shaker water bath at 37°C for 25 min, with ten calvaria/incubation flask. Then 0.5 mCi of [2- ^3H]glycine and 1 mCi of L-[G- ^3H]proline were added to the incubation flasks and the tissue was allowed to incubate for 7 h. The tissue was then removed from the incubation medium, rinsed in cold water and homogenized in a small volume of 1.0 M-NaCl in 0.5 M-Tris/HCl, pH 7.45, by using an Omnimix homogenizer (Sorvall, Norwalk, CT, U.S.A.). The homogenized tissue was stirred overnight in the cold, in 1.0 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.45. The solution was then clarified by centrifugation at 35000g for 30 min at 4°C, the supernatant decanted, made up to 25% (w/v) with respect to NaCl and stirred overnight in the cold. The precipitate was collected by centrifugation at 35000g for 1 h at 4°C and the supernatant decanted. The precipitate was suspended in a small volume of 0.1 M-acetic acid. The solution was stirred gently overnight at 4°C, then clarified by centrifugation at 35000g for 30 min at 4°C and exhaustively dialysed against 0.1 M-acetic

acid. The integrity of the labelled collagen was verified by applying a sample to a sodium dodecyl sulphate/polyacrylamide gel and subjecting it to electrophoresis (Furthmayr & Timpl, 1971). The gel was then cut into 1 mm slices and the radioactivity released by digestion for 2 h at 55°C with 0.5 ml of Nuclear-Chicago Solubilizer (Amersham/Searle, Arlington Heights, IL, U.S.A.). Scintillant (4 ml) (Bruno & Christian, 1961) was added and the radioactivity measured in a Packard Tri-Carb model 3003 liquid-scintillation spectrophotometer (Packard Instruments, Downers Grove, IL, U.S.A.). The resulting radioactive profile of the gel is shown in Fig. 1.

Methods

Culture of embryonic cartilage cells on Millipore filters. Chondrocytes were isolated from the epiphyses of the long bones of 13-day embryonic White Leghorn chicks as described by Handley *et al.* (1975). Primary cultures of the cells were grown at an initial

Fig. 1. Radioactivity profile of [^3H]collagen subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The positions of dansylated (Dns) α - and β -collagen chains and of the dye front are indicated. The dansylated markers were prepared from acid-soluble foetal calf skin. The dansylated α -chains were detected as a single band, probably corresponding to the α_1 species. The radioactive sample contained 34000 c.p.m. and the recovery was 97%. Identical profiles were obtained from collagen that had passed through the chondrocyte culture membrane.

density of 3×10^6 cells in 2ml of the growth medium in a high-sided Petri dish (diameter 31mm). In the bottom of the culture dish there was a Millipore membrane (0.45 μm pore size : HA) of diameter 29mm which had been sterilized by autoclaving and was held in place by a glass ring of diameter 28mm. The growth medium was changed every 24h and consisted of Dulbecco's modified Eagle's medium (CSL, Melbourne, Victoria, Australia) supplemented with 0.1mM each of ascorbic acid, L-alanine, L-asparagine, L-aspartate, L-glutamate, L-proline, L-serine and glycine, and buffered at pH7.2 with 15mM-Hepes, 10mM-Bes {2-[bis-(2-hydroxyethyl)-amino]ethanesulphonic acid}, 10mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid, 2mM- NaH_2PO_4 and 3.7mM- NaHCO_3 and contained 20% (v/v) foetal calf serum (GIBCO, Grand Island, NY, U.S.A.) (Handley & Lowther, 1977).

Primary cultures of embryonic-chick chondrocytes grown in this manner grow rapidly on the Millipore membrane to give a cell culture five to eight cell layers thick and each synthesizes a uniformly thick (50–80 μm), dense cartilage matrix (Oakes *et al.*, 1977) (Plate 1). The chemical and physical properties of the extracellular matrix and of the two main components of the matrix, namely collagen and proteoglycan subunit, are similar to those found in embryonic-chick epiphyseal cartilage (Handley *et al.*, 1975; Oakes *et al.*, 1977). The purpose of culturing the chondrocytes on a Millipore membrane was to achieve a mechanically strong system amenable to use in the permeability apparatus.

Permeability measurements. Permeability studies were carried out in a cylindrical Perspex cell (Fig. 2) with an upper chamber 'A' of diameter 1.7 and depth 4.0cm. The lower chamber 'B', with a diameter of 1.7cm and a depth of 0.5cm, was connected to a constant-flow pump (model 352: Sage Instruments, White Plains, NY, U.S.A.) on one side and eluate fractions were collected on the opposite side by using a fraction collector (model 1200; ISCO, Lincoln, NE, U.S.A.). The chambers were separated by a cultured membrane with the culture facing the lower chamber 'B'.

Both chambers were stirred with magnetic stirrers. Pump rates used were 40ml/h for low-molecular-weight solutes and 10ml/h for macromolecular solutes.

Cultured membranes were always soaked for approx. 1h in phosphate-buffered saline to remove all traces of the culture medium. Similar permeability data were obtained for water, Na^+ , ribose and glucose whether the cultures were soaked in buffer alone or in buffer containing azide, which effectively poisoned the chondrocytes.

The cell was made operational for each permeability experiment as follows: the lower chamber of the

Fig. 2. Schematic diagram of the diffusion cell. A, upper chamber, which contains a solution of the species under study; B, lower chamber; C, connective-tissue culture supported by Millipore filter; D, magnetic stirring bars; E, Neoprene 'O' ring for sealing the chambers; and F, inlet and outlet lines allowing for flow through lower chamber.

assembled cell was filled with the buffered saline, pH 7.4, ensuring that no air bubbles remained. Buffer containing the solute was then pipetted into the upper chamber of the cell. For radioactively labelled proteins the final concentration in the upper chamber did not exceed 3mg/ml. At concentrations up to this, osmotic imbalance caused negligible differences in permeation rates. The level to which the upper chamber was filled was that at which there was no hydrostatic-pressure difference between the two chambers. A sample of the top solution was taken, weighed and analysed to determine the solute concentration. The experiment was continued until a steady state was achieved as measured by the collection of three consecutive fractions that were of the same solute concentration. Such a steady state was reached in about 20min for the low-molecular-weight solutes and in 0.75–1 h for the macromolecular species. A second sample of the top solution was taken, weighed and the solute concentration determined. The upper chamber was then emptied of solution and rinsed several times with buffer. The pump was allowed to continue until the solute concentration in the effluent was not detectable. The procedure was then repeated for a series of other solutes by using the same cultured membrane. It was possible to use one membrane to carry out a number of experiments within a period of 12h.

Experiments were carried out either at room temperature (22°C) or at 4°C, depending on the nature of the solutes to be permeated through the membrane. Experiments involving collagen were always performed at 4°C because of the tendency of collagen to gel at room temperature. Other proteins were permeated at both room temperature and at 4°C. Permeabilities were always corrected to 37°C as shown by eqn. (21–29) of Tanford (1961), by using

viscosity values of 0.15M-NaCl from Svedberg & Pedersen (1940). For solutes that were permeated at both 4°C and room temperature, similar values of the corrected permeability coefficients were obtained, thus suggesting that the transport was by a simple passive diffusion mechanism. Since the flux of macromolecular species through the membrane was expected to be very low, it was necessary to choose molecules for which there were sensitive assays that would enable the concentrations in the diffusate to be measured accurately. In practice, this was achieved by using either radioactive proteins or enzymically active proteins.

The permeability coefficient for the unidirectional flux of solute across the membrane may be determined as described by Comper & Preston (1975) by their eqn. (4):

$$P_{AB} = \frac{dv}{dt} \cdot \frac{C_B}{C_A - C_B} \cdot K^{-1}$$

P_{AB} is the permeability coefficient in $\text{cm}^2 \cdot \text{s}^{-1}$ for solute moving from A to B (Fig. 2), dv/dt is the pump rate in ml/s, C_A is the concentration (molar) of solute in the upper chamber, C_B that in the lower and K is the membrane constant (cm), which is a measure of the effective cross-sectional area/diffusional path length of the membrane. In practice, the membrane constant, K , was determined by permeating $[^3\text{H}]$ -water through the membrane. It is assumed that water is able to diffuse through the aqueous phase of the membrane at the same rate as in free solution and any apparent decrease in the rate of diffusion of water may be attributed to a decrease in the area available for diffusion, i.e. $P_{AB} = \lambda D_0$, where D_0 is the diffusion coefficient in free solution and λ is the

distribution coefficient of the solute. For small solutes $\lambda = 1$.

The membrane constant, K , was re-determined by measurement of $[^3\text{H}]$ water flux after each macromolecular species had been passed through the membrane.

Measurement of radioactively labelled solutes. For radioactive solutes labelled with ^{14}C or ^3H the radioactivity was measured by standard counting techniques in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The radioactivity of a 1.0ml aqueous sample was determined by using 4.0ml of a scintillation mixture described by Fox (1968). The γ -radiation emitted from ^{22}Na and ^{125}I samples was measured on a scintillation counter (E.S.I. Nuclear type 5350; Reigate, Surrey, U.K.) by using a thallium-activated NaI crystal as scintillator.

Preparations of iodinated proteins were used and, as has been found by other workers, these preparations contained significant amounts of free iodide even after extensive dialysis (Maroudas, 1976). It was therefore necessary to compensate for the rapidly diffusing iodide in the determination of the permeability coefficients of the iodinated proteins. Since it appears that the release of free iodide from the iodinated solutes is time-dependent, a preliminary dialysis step was carried out to remove most of the free label from the sample. However, in the analysis of the permeability data, the solution from the top chamber and the effluent from the permeability cell were then analysed by gel chromatography on Sephadex G-25 (see Fig. 3). It was possible to determine the percentage of radioactivity on each side of the composite membrane that could be attributed to the macromolecular species and to the free iodide

Fig. 3. Gel-chromatography profile of ^{125}I -labelled human serum albumin (a) and of ^3H -labelled collagen (b). Profiles obtained on a column ($15\text{cm} \times 1.6\text{cm}$) of Sephadex G-25 (Pharmacia, Uppsala, Sweden): —, solute from solution in top chamber of diffusion cell; ----, solute that had passed through the composite membrane into the lower chamber of the diffusion cell. The void volume (V_0) and total volume (V_t) of the column were evaluated by use of Blue Dextran and $[^3\text{H}]$ water respectively. Note the increase in the percentage of free ^{125}I in the diffusate of the albumin. The ^3H -labelled collagen showed no free label in either diffusand or diffusate.

EXPLANATION OF PLATE I

Light micrograph of a 32-day-old chondrocyte culture (C) grown on a Millipore filter (M)

The thickness of the culture is formed largely by a metachromatic matrix which contains round chondrocytes. A few superficial elongated cells containing vacuoles are seen on the surface of the cultures. Note the absence of fibroblast cells. The section was embedded in Epon and stained with Toluidine Blue (magnification $\times 400$). (Courtesy of Dr. B. W. Oakes.)

respectively. To ensure that the material eluted in the void volume was macromolecular, it was rechromatographed immediately on Sephadex G-25 and no free iodide peak was detected. The columns gave approx. 100% recovery and good resolution between the two peaks.

Gel chromatography of proteins. The macromolecular integrity and behaviour of many of the globular permeant species were checked before and after passage through the cultured membranes. A column (20cm×1.6cm) of Sepharose 4B-CL (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with phosphate-buffered saline, pH 7.4. The column was operated at flow rates of approx. 7 ml/h per cm² and at room temperature. Samples (1 ml) were applied and fractions (1 ml) were collected. The void volume (V_0) and total volume (V_t) of the column were evaluated by use of Blue Dextran and [³H]-water respectively. The column was calibrated with the following proteins: ribonuclease, ovalbumin, γ -globulin and glutamate dehydrogenase.

The gel chromatography was used to show that the macromolecular species on either side of the membrane were chromatographically identical and that the values of $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is elution volume of the protein, were consistent with their molecular dimensions (Table 3).

Assay of enzymically active proteins

The activity of catalase, glucose oxidase and peroxidase were measured by using the assay procedures given by the suppliers of the enzymes. The enzyme solutions from the upper chamber of the diffusion cell were diluted before being assayed for enzyme activity. The enzyme concentration in the effluent was such that no dilution was required.

Analytical methods. The glycosaminoglycan content of the membranes was measured by digesting them with papain and applying the digests to DEAE-cellulose columns (6cm×1.5cm) as described by Handley & Lowther (1976). The hexuronic acid content of the purified glycosaminoglycans was assayed as described by Bitter & Muir (1962), with glucuronolactone as a standard.

The collagen content of the membranes was measured by subjecting them to an overnight papain digestion followed by hydrolysis in 6M-HCl for 16h at 110°C in sealed tubes. The samples were then dried *in vacuo* and assayed for hydroxyproline by the method of Stegemann & Stalder (1967).

Results

The hexuronate and hydroxyproline contents of the cultured chondrocyte membranes were determined as a function of age (Fig. 4). In a 40-day-old culture, collagen comprises approx. 1.1% of the

wet weight and proteoglycan 0.9% of the wet weight, which is typical of embryonic articular cartilage (Oohira *et al.*, 1974). The increase in these macromolecular components parallels the increase in the number of cells (Oakes *et al.*, 1977). The variation of the membrane constant, K , with age was also determined (Fig. 5).

The stability of the membranes under experimental conditions was examined by analysing the effluent from the lower chamber for uronic acid and protein. The effluent was collected over a 12h period, dialysed against distilled water and concentrated by freeze-drying. No uronic acid-containing material and a negligible amount of protein material (less than 20 μ g) were present when cultures older than 30

Fig. 4. Uronic acid and hydroxyproline content of the chondrocyte culture layers as a function of age

The analysis of uronic acid and of hydroxyproline content was carried out as outlined in the text. The two components were used to indicate the composition of the chondrocyte layer in terms of proteoglycan and of collagen respectively. ○, Uronic acid (μ g/membrane); Δ, hydroxyproline (μ g/membrane).

Fig. 5. Variation of the membrane constant, K , with age of the chondrocyte culture

Membrane constant, K , was determined by measurement of [³H]-water through the culture membrane.

days were used. Furthermore, the values obtained for K remained constant for cultures grown in excess of 30 days. Membranes younger than 30 days were found to give values of K which increased during a 12h experiment.

In the present studies, over 200 separate cultured membranes of varying ages have been used. In the presentation of data we have chosen to group the experimental results in terms of membranes that had been in culture for approximately the same time.

The validity of using $[^3\text{H}]$ water flux to determine the membrane constant was verified by permeating a second solute such as any of those listed in Table 1. The permeability coefficients calculated were similar to their diffusion coefficients in free solution ($\pm 4\%$).

If the ionic strength of the buffer was lowered, the effect on the permeability coefficients of small charged species was quite marked (Table 2). These results may be interpreted by considering the polyanionic nature of the proteoglycans constrained within the matrix. The permeability of the cultures to a series of compact proteins is shown in Table 3. The proteins in the top group are generally regarded as globular, since their frictional ratios (f/f_0) are less than 1.4. For this group there is a progressive decrease in the ratio P_{AB}/D_0 with increasing molecular weight of the permeant species. The results reported

in the lower section of Table 3 emphasize the effect of molecular shape on the permeability of proteins of similar molecular weight through the chondrocyte membranes. The permeability coefficient of the spherical protein edestin is decreased to 0.42 of its diffusion coefficient in free solution, whereas the compact asymmetric protein fibrinogen exhibits an increased permeability compared with its behaviour in free solution. A more marked effect is seen with the behaviour of collagen, where a permeability coefficient three times its diffusion coefficient in free solution is observed.

Collagen solutions were chromatographed on Sepharose 4B/CL columns and the protein was eluted in the void volume. To ascertain the macromolecular integrity of the collagen that had passed through the cultured membranes, the diffusate was also chromatographed on Sepharose 4B/CL. Radioactivity was found only in the void volume of the column and recovery of the radioactivity was approx. 100%. Furthermore, samples of the diffusate were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and profiles identical with that shown in Fig. 1 were obtained.

To confirm the difference in behaviour of collagen from that of the spherical macromolecular solutes studied, experiments were carried out involving the

Table 1. *Permeability of cultured chondrocyte membranes to low-molecular-weight solutes*

The measurements were carried out in phosphate-buffered saline, pH 7.4, at room temperature. The results have been corrected to 37°C and the values are averages $\pm$ s.d. for the numbers of membranes used shown in parentheses.

Solute	$10^7 \times P_{AB} \text{ (cm}^2 \cdot \text{s}^{-1}\text{)}$	$10^7 \times D_0 \text{ (cm}^2 \cdot \text{s}^{-1}\text{)}$	P_{AB}/D_0
$[^3\text{H}]$ Water	311.3 ± 3.8 (20)	311.3*	1.00
Proline	108.0 ± 1.4 (3)	109.7§	0.98
Ribose	99.6 ± 1.9 (4)	100.2§	0.99
Glucose	95.5 ± 3.5 (20)	91.4†	1.04
Sorbitol	95.4 ± 3.8 (20)	91.4†	1.04
Raffinose	56.1 ± 1.2 (3)	58.2†	0.96
Na^+	180.0 ± 2.6 (20)	178.8‡	1.01

* From Devell (1962).

† From Weast (1966).

‡ From Mills (1955).

§ G. J. Checkley (personal communication).

Table 2. *Permeability of cultured chondrocyte membranes to ionic species*

The measurements of permeability coefficients were carried out in phosphate-buffered saline, pH 7.4 (P_{AB}), and in phosphate-buffered saline, pH 7.4, which had been diluted 1:10 with water (P_{AB}^*). All experiments were performed at room temperature and the results corrected at 37°C as described in text. The values of the permeability coefficients are averages $\pm$ s.d. for the numbers of membranes used shown in parentheses.

Ion	$10^7 \times P_{AB} \text{ (cm}^2 \cdot \text{s}^{-1}\text{)}$	$10^7 \times P_{AB}^* \text{ (cm}^2 \cdot \text{s}^{-1}\text{)}$	P_{AB}^*/P_{AB}
Sodium	180.2 ± 2.6 (10)	231.8 ± 3.5 (10)	1.28
Chloride	270.6 ± 4.8 (3)	241.4 ± 2.9 (3)	0.89
Sulphate	127.5 ± 1.8 (3)	103.3 ± 3.2 (3)	0.81
Glutamate	85.9 ± 1.4 (3)	65.8 ± 2.6 (3)	0.75
Glucuronate	85.2 ± 1.5 (3)	69.0 ± 2.9 (3)	0.81

Table 3. *Permeability of cultured chondrocyte membranes to macromolecular solutes*

The permeability measurements were carried out in phosphate-buffered saline, pH 7.4, and, unless otherwise indicated, at 22°C. All experiments involving collagen and some using edestin and fibrinogen as permeant species were carried out at 4°C. All results have been corrected to 37°C as described in the text. The values of P_{AB}/D_0 are averages $\pm$ s.d. for the numbers of membranes used shown in parentheses. Gel chromatography was carried out on Sepharose 4B/CL as described in the text.

Solute	$10^{-4} \times \text{Mol.wt.}$	Frictional ratio (f/f_0)	$10^7 \times D_0$ ($\text{cm}^2 \cdot \text{s}^{-1}$)	$K_{av.}$	P_{AB}/D_0
Horseradish peroxidase	4.40*	1.36*	10.5*	0.82	0.92 ± 0.05 (6)
Human serum albumin	6.50*	1.29*	9.40*	0.68	0.75 ± 0.13 (20)
Glucose oxidase	18.6*	1.19*	7.73*	0.61	0.67 ± 0.10 (5)
Catalase	25.0*	1.25*	6.31*	0.59	0.45 ± 0.01 (3)
Edestin	30.0*	1.30†	6.05*	0.58	0.42 ± 0.03 (4)
Thyroglobulin	67.0*	—	4.02*	0.48	0.26 ± 0.04 (6)
Fibrinogen	33.0†	2.34†	3.11†	—	1.32 ± 0.05 (10)
Collagen	34.5†	6.8†	1.06†	0.0	3.00 ± 0.37 (14)

* From Sober (1968).

† From Tanford (1961).

‡ From Svedberg & Pedersen (1940).

Table 4. *Values of P_{AB}/D_0 obtained from experiments involving the simultaneous migration of two solutes*

Measurements were carried out in phosphate-buffered saline, pH 7.4, at 4°C. Values of P_{AB}/D_0 are averages $\pm$ s.d. for the numbers of membranes used shown in parentheses.

Solute	Expt. no.	In presence of	P_{AB}/D_0
$[^3\text{H}]\text{Collagen}$	—	—	3.00 ± 0.37 (14)
	A	Catalase	3.40 ± 0.27 (3)
Catalase	B	$[^{14}\text{C}]\text{Glucose}$	3.00 ± 0.23 (3)
	—	—	0.45 ± 0.01 (3)
$[^{14}\text{C}]\text{Glucose}$	A	$[^3\text{H}]\text{Collagen}$	0.47 ± 0.01 (3)
	C	$[^3\text{H}]\text{Water}$	0.40 ± 0.03 (3)
$[^3\text{H}]\text{Water}$	—	—	1.04 ± 0.04 (10)
	B	$[^3\text{H}]\text{Collagen}$	0.97 ± 0.12 (3)
	—	—	1.00
	C	Catalase	0.97 ± 0.15 (3)

simultaneous migration of (A) $[^3\text{H}]\text{collagen}$ and catalase, (B) $[^3\text{H}]\text{collagen}$ and $[^{14}\text{C}]\text{glucose}$ and (C) $[^3\text{H}]\text{water}$ and catalase. The results are presented in Table 4: in all cases, permeability values were obtained similar to those found in experiments involving only single solutes.

Discussion

The membrane system developed for these studies offers many advantages for the study of transport through connective-tissue matrices. The major advantage of this system is that the chondrocyte cultures produce a confluent matrix of uniform thickness which is extremely thin (approx. $80\mu\text{m}$). The resultant large surface-area/thickness ratio makes the system suitable for the measurement of the flux of macromolecular solutes across these membranes. Also the glycosaminoglycan content of the matrix confers a high charge density to the membrane that would be difficult to attain in a model gel system.

The membranes used in the permeability studies had been cultured for more than 30 days. By this age, they appeared to be stable with respect to their ability to constrain their macromolecular components. To confirm that any 'leaching out' of the soluble components of the matrix had a minimal effect on the physical properties of the cultures, the membrane constant, K , was re-determined after the permeation of each solute. Fig. 4 shows that the numerical value of K becomes constant for cultures older than 30 days.

It is evident from Table 1 that the diffusion of low-molecular-weight solutes is unaffected by the aqueous phase of the culture membrane, as defined by the $[^3\text{H}]\text{water}$ flux. The results obtained with small molecules are comparable with those obtained from other investigations using different model systems (Maroudas, 1968; Preston & Snowden, 1972; Comper & Preston, 1975).

Because of the charged nature of the immobilized glycosaminoglycans, the extracellular phase may be viewed as having properties similar to an ion-exchange

resin, and its effect on the migration of small ionic species can be interpreted in this fashion. Owing to the relatively higher concentration of the counter-ion in the membrane, differences in ion flux become quite marked at low ionic strengths. The uni-directional flux of the counter-ion (Na^+) tends to be higher and that of the co-ion lower when the ionic strength of the buffer is lowered (Table 2). It should be noted that at physiological ionic strength (Table 1) the charge effect of the cultured membrane on the migration of Na^+ ions was not statistically significant. An estimate of the fixed charge density of the membrane may be obtained from a consideration of the uronic acid content and dimensions of the structure. On the basis of a uronic acid content of $100\mu\text{g}/\text{membrane of volume } 0.033\text{cm}^3$, and assuming the proteoglycan comprises equimolar amounts of sodium glucuronate and sodium *N*-acetylgalactosamine sulphate, the fixed charge density of the cultured membrane is found to be $30.5\text{ mequiv./litre}$. In membranes older than 30 days, a range in fixed charge density of between 30 and 40 mequiv./litre was found. It has been predicted by Comper & Preston (1975) that marked effects on ion transport by matrix-mobile-ion interactions should only be expected at physiological ionic strengths in the most densely charged tissues such as intervertebral disc and the nasal septum.

It is evident from the present studies that the permeability of compact globular proteins through the cultured chondrocyte membranes decreases with increasing molecular size of the permeant (Table 3). Similar behaviour has been shown in sedimentation studies of globular particles through polysaccharide solutions (Laurent & Pietruszkiewicz, 1961; Laurent *et al.*, 1963; Laurent & Persson, 1964) and by diffusion studies in the ultracentrifuge (Laurent *et al.*, 1963; Preston & Snowden, 1973b). It has been proposed (Laurent *et al.*, 1963; Ogston *et al.*, 1973) that such decrease in translational migration in concentrated polymer solutions is due to a sieving mechanism whereby the particles act as if they are trapped in the network and their movement is hindered owing to steric obstruction.

We have been unable to demonstrate a permeability 'cut-off' with the culture layer, since the largest particle studied, thyroglobulin (mol.wt. 6.7×10^5), is able to pass through the culture. This behaviour may resemble the situation found in soft connective tissues. It would appear that it is very different from that observed in adult articular cartilage, where it was found that no penetration occurred with globular proteins of size equal to or larger than serum albumin (Maroudas, 1976).

The results of the permeability studies of asymmetric particles support suggestions made earlier (Lieb & Stein, 1971; Laurent *et al.*, 1975) that molecular shape may play an important role in the

determination of the permeability of molecules through a network. In this regard, the results of the simultaneous transport of catalase and collagen (Expt. A in Table 4) are of particular importance. Whereas for catalase, a globular protein, of axial ratio approx. 4 (Tanford, 1961), the permeability is decreased to 0.47 of its value in free solution, the transport rate of collagen, with an axial ratio of 300 (Tanford, 1961), is enhanced by a factor of 3.

Any suggestion that the transport of collagen or of fibrinogen is mediated by an 'active' process arising from the chondrocyte cells can be discounted in view of the observation that cultures poisoned with azide gave similar results to cultures soaked in buffer.

In consideration of the behaviour of the asymmetric particles, it is of interest to note the early work of Perrin (1936), which discussed the anisotropy of translational diffusion of ellipsoidal particles. For such structures, the resistance to motion is characterized by three different frictional coefficients, each representative of the resistance to motion of the ellipsoid parallel to one of its principal axes. The free diffusion of a system of such molecules does not of course follow a direction parallel to any of the molecular axes, since the particles are oriented at random. However, it is interesting to note that, for a system where the Brownian movement is considered to be parallel to the major axis of the particle, the diffusion coefficient describing this 'end-on' movement is dependent on the degree of asymmetry of the particle and reaches a limiting value of three times that based on a random orientation. Such behaviour reflects the fact that a rod-like particle would encounter less resistance moving lengthwise than when moving broadside.

For fibrinogen and collagen, having axial ratios of 31 and 300 respectively (Tanford, 1961), it can be calculated that the ratio of the value of the 'end-on' diffusion coefficient to its values based on a random orientation is approx. 1.6 and 2.8 respectively. It is tempting to suggest that the observed permeability of these proteins to the culture membranes (P_{AB}/D_0 having values of 1.3 and 3.0 respectively; Table 3) reflect an orientation effect and can be partially understood in terms that the matrix does severely restrict broadside movements of the particles while offering little resistance to 'end-on' movement. Such behaviour may result in the permeability of particles to a network being correlated predominantly with their minor axis. It should be emphasized that we are not suggesting that the particles are oriented relative to the concentration gradient, that is relative to the overall macroscopic direction of solute flow.

If we adopt a simplified view of the 'pore' theory of membrane transport (Pappenheimer *et al.*, 1951) and consider that the diffusive flux of solute molecules is affected only by (a) steric hindrance at the entrance

of the pores and (b) frictional forces within the pores, then the observed permeability of fibrinogen and collagen suggests that hindrance of entry to the culture membrane may be of minimal significance.

The present studies suggest that asymmetrical particles should penetrate the ground substance of connective tissues with greater ease than globular structures of similar hydrodynamic volume. This suggestion is in support of earlier findings (Laurent *et al.*, 1975; Dudman, 1966) and of preliminary permeability studies of flexible polymeric solutes such as dextran, hyaluronate and sulphated proteoglycan (Cumming *et al.*, 1977). In tissues such as cartilage where the chondrocytes are surrounded by an extracellular matrix, this behaviour could facilitate the transport of various macromolecules to and from the cells. It is of interest that the major biosynthetic products of chondrocytes, namely collagen and sulphated proteoglycan, are essentially asymmetric or flexible molecules.

In the present investigation, we have chosen to describe the transport properties in terms of the overall compound structure of the membrane. No attempt has been made to analyse its behaviour in relation to its component parts, that is of the culture layer and of the Millipore base. However, preliminary studies on Millipore membranes clearly indicate that these porous structures also display permeability properties dependent on the shape of the permeant macromolecular species (G. T. Cumming & B. N. Preston, unpublished work). Although the transport of the globular proteins is not retarded to any marked degree through the Millipore membrane (0.45 μm pore size : HA), the permeability of the collagen is increased compared with its behaviour in free solution.

We approached this work with the intention of applying the results to diffusion processes occurring within the extracellular matrix of connective tissues. However, the biochemical and physiological significance of our findings may be more far-reaching. For example, in most biological systems, inter- and intracellular transport probably takes place through polymeric or fibrous networks.

Note Added in Proof (Received 3 April 1979)

As pointed out by the referee, the evidence given in this paper does not rule out the possibility that the Millipore-membrane behaviour might be the determinant factor with regard to the movement of the asymmetric proteins. However, an estimate of the permeability coefficient of collagen in the chondrocyte culture layer can be made, by using eqn. 4.12 of Crank (1956). If we assume the thicknesses of the Millipore and of the chondrocyte layer as 150 μm and 80 μm respectively, and given that the permeability coefficient of collagen through the Millipore

membrane (0.45 μm pore size) has been determined as $3.88 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, then the estimate of the permeability coefficient of the collagen to the culture layer is $2.37 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$.

The value of P_{AB}/D_0 is 2.24, suggesting that the connective-tissue layer is like other membranous structures in increasing the apparent diffusion rate of asymmetric particles (Cumming & Preston, 1978).

We thank Dr. T. C. Laurent and Dr. W. D. Comper for discussions during the preparation of the manuscript, Dr. B. W. Oakes for the light micrograph of the chondrocyte membrane, the expertise of Dr. J. F. Bateman in the preparation of labelled collagen and Mrs. M. T. Scott for her valuable technical assistance. This work was supported by the Australian Research Grants Committee (grant no. D68/16898 and D2 73/14127), G. J. C. was in receipt of a postgraduate award from the Australian Government.

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