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Transport of solutes through cartilage: permeability to large molecules*

A. MAROUDAS
Mechanical Engineering Department, Imperial College, London

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INTRODUCTION

The permeability of cartilage to solutes is relevant to such diverse problems as chondrocyte nutrition, immune reactions and matrix mineralization. In our studies we have attempted to relate physico-chemical structure to cartilage permeability and physiological function.

Since cells occupy only a small fraction of the total volume of human articular cartilage (Stockwell, 1967), it is primarily the extracellular matrix which is relevant to solute and fluid transport. The extracellular matrix consists essentially of collagen fibres embedded in a gel of proteoglycans and water. The gaps between the collagen fibres are relatively large – of the order of 100 nm. These gaps are filled by the glycosaminoglycan–water gel which has an extremely fine porous structure. It is thus the concentration of glycosaminoglycans in the matrix which is the most important factor in determining its permeability (Maroudas, 1970). The glycosaminoglycans contain negatively charged groups – the carboxylate and sulphate ions of the chondroitin and keratan sulphates. Cartilage is thus endowed with a high negative fixed-charge density, which controls ionic equilibria in the matrix (Maroudas, 1968; Maroudas, 1970).

The rate of movement of solutes from an external solution into cartilage and vice versa is governed by three factors:

(*a*) the resistance of a stagnant film at the cartilage–fluid interface (in life this interface is between cartilage and synovial fluid);

(*b*) the equilibrium partition of a solute between cartilage and external solution;

(*c*) the effective mobility, i.e. diffusivity of the solute molecules within cartilage.

It has been shown that, provided the liquid in contact with cartilage is stirred (as it is likely to be during joint motion), liquid film resistance can be neglected in comparison with the resistance to diffusion within cartilage itself (Maroudas, 1968). It is thus possible to express solute permeability in terms of the two factors (*b*) and (*c*) above.

The equilibrium distribution of a given solute between the cartilage and external solution depends on the room available within the matrix network for accommodating the solute molecules, as well as on the affinity of the latter for the solute. Thus we found previously (Maroudas, 1970) that small non-ionic solutes such as urea or glucose distribute themselves equally between the extracellular water in cartilage

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and an external solution (molal partition coefficients around unity). Small cationic solutes are attracted by the negatively charged groups of the glycosaminoglycans and thus have partition coefficients higher than unity. The opposite applies to anionic solutes. Large solutes are partially excluded from the cartilage matrix because their size is comparable with that of the 'pores' in the cartilage gel and they are thus not easily accommodated.

The mobility of a solute within cartilage depends on its normal diffusivity in an aqueous medium as well as on the tortuosity of the path through the cartilage matrix. Most small solutes have a diffusivity within cartilage equal to half their value in aqueous solution (Maroudas, 1970), but special interactions with the matrix components could reduce this value.

The present work is an amplification of our previous studies on the distribution and diffusion of various solutes. In particular, new methods and results relating to the transport of large globular proteins will be described.

MATERIALS AND METHODS

Human post-mortem cartilage taken from the femoral head, femoral condyles and patella was used. The permeability studies on small solutes were carried out in a diffusion cell, using radioactive tracers (Maroudas, 1970). The total glycosaminoglycan content was estimated as fixed charge density by the tracer cation method (Maroudas & Thomas, 1970).

Distribution and diffusion of large globular proteins

The following proteins were examined with regard to partition coefficients: myoglobin (mol. wt. 17000; Stokes radius 19·8 Å); ovalbumin (mol. wt. 45000; Stokes radius 28 Å); serum albumin (mol. wt. 69000; Stokes radius 35·5 Å); transferrin (mol. wt. 76000; Stokes radius 37 Å); IGG (mol. wt. 160000; Stokes radius 56 Å). Most of the work, however, was carried out on serum albumin, particularly as regards diffusion studies.

In order to be able to analyse quantitatively trace quantities of the proteins, we obtained them in an iodinated form, containing the radioactive tracer ^{125}I. (Except for serum albumin which had been supplied by the Radichemical Centre, Amersham, all the above proteins were prepared for us by Dr Alan Barrett, Strangeways Research Laboratory, Cambridge.)

There is, however, a difficulty involved in the use of iodinated compounds because, even with extensive dialysis, it is not possible to get rid of all traces of free iodine from the iodinated protein, and even amounts as small as 0·5 % can cause serious errors when the penetration of the large protein molecules into the tissue is very limited as compared with that of the free iodine. The following example will illustrate the difficulty.

Preliminary results showed that partition coefficients for large solutes are in the range 0·01–0·001 (Maroudas, 1970); this compares with about 0·5 for small anionic solutes. Thus the partition coefficient of iodinated serum albumin should be 50 to 500 smaller than that of free iodine. This means that at equilibrium the ratio of free

iodine to iodinated serum albumin in cartilage will be in the range 0·25:1 to 2·5:1 although the external solution contained only 0·5 % of free iodine and 99·5 % of albumin. A very careful separation of the two components is therefore necessary and so the following procedure was adopted (Snowden & Maroudas, 1976).

A full depth specimen of cartilage, usually about 0·5 cm^2 in cross sectional area, was allowed to equilibrate for 48 hours in Ringer's solution containing 2 % of protein with added (^{125}I) tracer. The count rate of the equilibrating solution was usually of the order of 50×10^6 c.p.m./ml, of which at most 5×10^5 was due to free iodine. The specimen was then given a superficial rinse before being cut into 200–400 μm slices on a freezing microtome. Each slice was placed in 2 ml of Ringer's solution containing 2 % of unlabelled protein and allowed to equilibrate again. Since the slices were thinner, much shorter periods of equilibration were required during desorption.

The desorbates, containing both the (^{125}I) protein and (^{125}I) free iodine were then subjected to a double TCA precipitation. The combined supernatants were 'counted' to determine the free iodine content, whilst the precipitates were dissolved in formic acid and 'counted' to determine the actual amount of iodinated protein. The 'counting' was carried out in the gamma head of a combined gamma and beta Scintillation Counter (Nuclear Enterprises, Cambridge). Column chromatography (Sephadex G-100) was used to check the efficacy of the TCA precipitation and also to verify that there were no breakdown products of the protein under consideration. This proved particularly necessary in the case of IGG, which contained traces of smaller units; these tended to concentrate in cartilage, and their fraction had to be quantitatively determined.

The slices, after desorption, were weighed and their fixed charge density determined by the tracer cation method (Maroudas & Thomas, 1970; Maroudas, Evans & Almeida, 1973). The fixed charge density represents quantitatively the total glycosaminoglycan content (Maroudas, Muir & Wingham, 1969; Maroudas & Thomas, 1970; Venn & Maroudas, in preparation). The partition coefficient of the protein could thus be correlated directly with the glycosaminoglycan content of each slice.

The slice was finally dried in an oven at 67 % to constant weight and its water content thus determined.

The molal partition coefficient of the protein, K, was calculated by means of the formula

$$K = \frac{\overline{m}}{m} = \frac{N_1}{N_0 \times W_1 \times H_0}, \tag{1}$$

where $\overline{m}$ and m = the molal concentrations of the protein in the cartilage and in solution respectively, N_1 = counts due to (^{125}I) protein in the desorbate (i.e. in the precipitate), N_0 = counts per ml due to (^{125}I) protein in the initial equilibrating protein solution, W_1 = weight of cartilage slice, H_0 = water content of cartilage.

As far as the determination of diffusion coefficients by the permeability method is concerned, the difficulties caused by free iodine are much greater than in the case of partition coefficients, for the following reason.

The permeability method consists in the measurement of the flux or the permeability of a given solute across the cartilage membrane in a diffusion cell (Maroudas,

1968). Now preliminary experiments indicate that permeability coefficients for large solutes lie in the range 10^{-10} to 10^{-9} cm^2/sec. This compares with 10^{-6} cm^2/sec for small solutes. Thus the flux of iodinated serum albumin across a cartilage slice will be 1000–10000 times smaller than that of free iodine. This implies that the ratio of free iodine to iodinated serum albumin in the diffusate will be in the range 5:1 to 50:1 although the original solution contained only 0·5 % of free iodine. It is clear that with so high a ratio of free iodine to serum albumin even TCA precipitation cannot give a satisfactory separation, particularly as the count rate due to the protein ^{125}I is extremely low in the diffusate.

A different method was therefore developed for estimating the diffusion coefficients of large solutes. The principle of this method consists in equilibrating a slice of cartilage in a solution containing the labelled protein, as in partition studies, and then desorbing it in several fractions and determining the rate at which the protein is diffusing out. The diffusion coefficient can readily be calculated from the information thus obtained by the method outlined below.

A full depth specimen of cartilage is left to equilibrate for at least 48 hours at 4 °C in a Ringer's solution containing 2 % protein with added (^{125}I) serum albumin. The specimen is then trimmed on a freezing microtome to remove surface slices and the middle slices, about 0·5–1·0 mm in thickness, are retained for the diffusion experiment. The reason for using the middle slice is as follows. In this method of determining diffusion coefficients the slice must be reasonably thick, whilst at the same time it must have a uniform concentration of the solute throughout its depth. Since the partition coefficient of the large solutes depends on the glycosaminoglycan content of the cartilage, the slice must have a constant glycosaminoglycan concentration. This requirement is best fulfilled by the middle slices.

The slice under consideration is then desorbed for accurately timed periods in several successive 4 ml portions of Ringer's solution plus 2 % serum albumin. The total desorption time is approximately 12 hours and the intervals range from 2 minutes at the beginning to 1 hour towards the end. It is essential to blot and transfer the slice from one solution to the next as rapidly as possible. Desorption runs were carried out either at 4 °C or at 37 °C.

Each solution is then treated with TCA to separate the iodinated protein from free iodine, and each precipitate and supernatant is counted.

The cumulative 'counts' for the protein on the one hand and the free iodine on the other are then plotted versus the square root of time, as shown in Figure 1. The diffusion coefficient is calculated from the formula (Crank & Park, 1968)

$$\frac{Dt_{\frac{1}{2}}}{l^2} = 0{\cdot}0492, \tag{2}$$

where D = diffusion coefficient, $t_{\frac{1}{2}}$ = time at which the concentration of the solute in the slice has been reduced to $\frac{1}{2}$ of its maximum value, i.e. at which $N/N_0 = \frac{1}{2}$ where N_0 = number of counts in the slice after the original equilibration, l = thickness of the slice.

It should be noted that the desorption of iodine occurs much more rapidly than that of serum albumin. Thus the slope of the graph, which represents the number of counts in the desorbate during any given time interval, is initially much higher in the

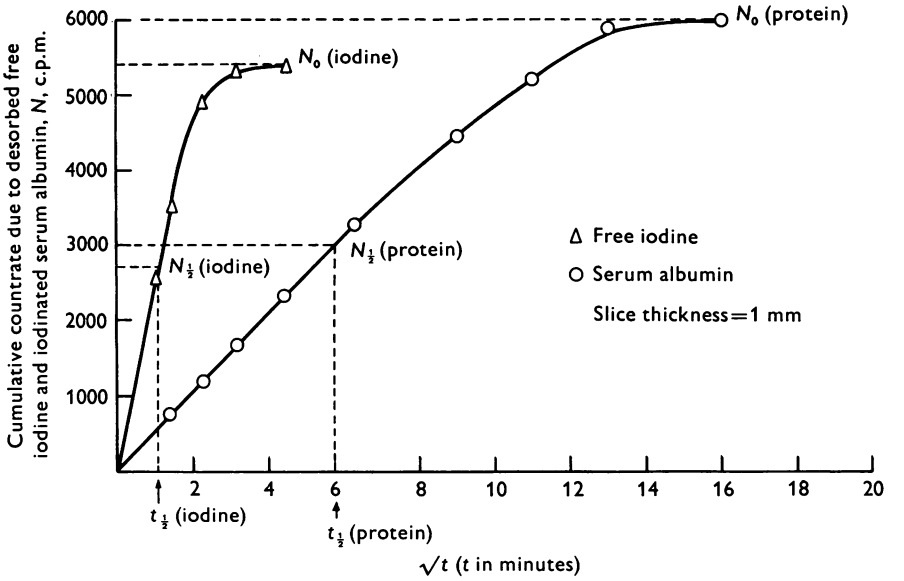

Fig. 1. Desorption curves for iodinated (^{125}I) serum albumin and free iodine (^{125}I) versus square root of time at 37 °C.

case of free iodine than in the case of serum albumin; however, after about 6 minutes the number of counts due to free iodine becomes negligible as compared to those due to serum albumin. This means that whilst the TCA separation between free iodine and protein may not always yield accurate results at the beginning of the desorption, subsequent values for the protein are very reliable. Since the curves remain linear up to more than $N_{\frac{1}{2}}$, it is possible to check the initial points in this manner.

In the example shown in Figure 1, $t_{\frac{1}{2}}$ (iodine) = 0·9 minute and $t_{\frac{1}{2}}$ (serum albumin) = 33·5 min. Hence, by equation (2), with l = 0·1 cm,

$$D_{\text{iodine}} = \frac{0{\cdot}0492 \times (0{\cdot}1)^2}{0{\cdot}9 \times 60} = 9{\cdot}0 \times 10^{-6}\ \text{cm}^2/\text{sec}$$

and

$$D_{\text{serum albumin}} = \frac{0{\cdot}0492 \times (0{\cdot}1)^2}{33{\cdot}5 \times 60}$$

$= 2{\cdot}4 \times 10^{-7}$ cm^2/sec.

RESULTS

Partition coefficients of large solutes

Figure 2 shows a plot of the partition coefficient K versus molecular size (Stokes radius) for a range of solutes, the smallest being urea, the largest IGG. The results in the figure are for cartilage specimens of a given fixed charge density. It can be seen that the partition coefficient decreases very steeply with increase in size, up to serum albumin. For larger solutes there does not appear to be any further decrease; thus the results for serum albumin and IGG practically coincide, which can be seen very clearly in Figure 2 below.

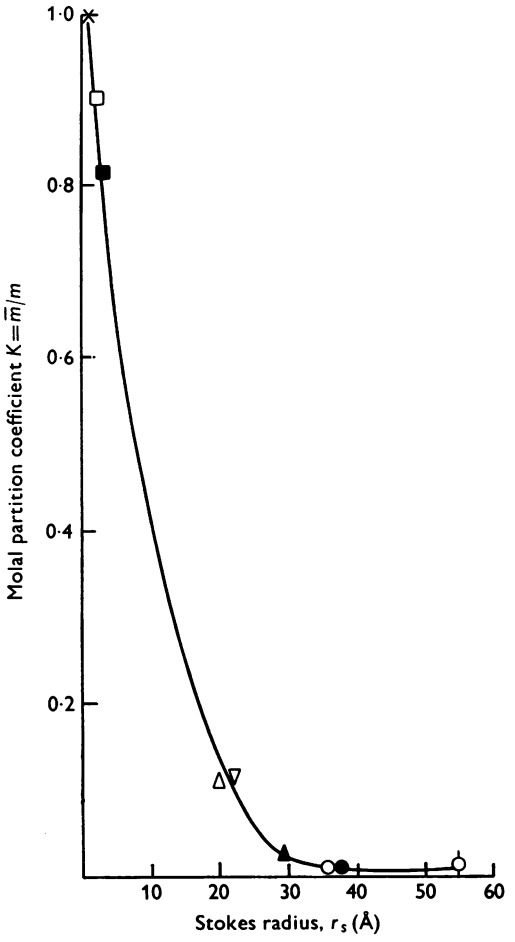

Fig. 2

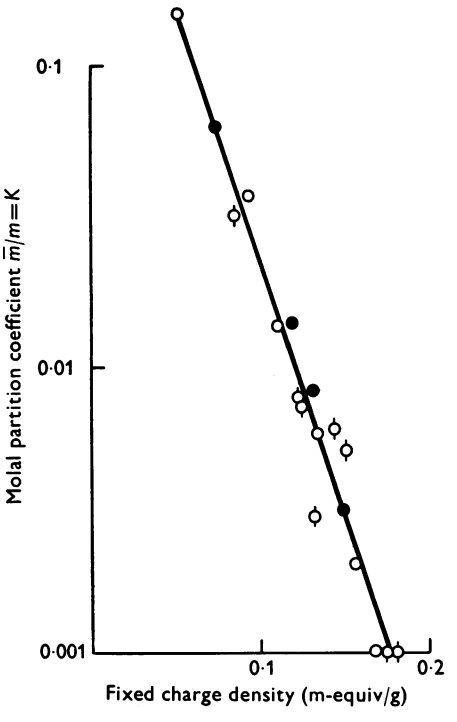

Fig. 3

Fig. 2. Variation of the partition coefficient with molecular size. ×, urea, proline; □, glucose; ■, sucrose; △, myoglobin; ▽, chymotrypsinogen; ▲, ovalbumin; ○, serum albumin; ●, transferrin; ϕ, IGG. Some of the points in this figure have been taken from the results of Snowden, Maroudas & Barrett (in preparation).

Fig. 3. Variation of the partition coefficients of large solutes with fixed charge density. ○, serum albumin; ●, transferrin; ϕ, IGG.

Figure 3 shows the variation of the partition coefficient of serum albumin, transferrin and IGG with the total glycosaminoglycan content, as expressed by fixed charge density. For a threefold increase in fixed charge density (from 0·05 to 0·15) there is a hundredfold decrease in the partition coefficient. For cartilage of fixed charge density greater than about 0·17 m-equiv/g the partition coefficients are lower than 10^{-3} and in this region measurement becomes subject to considerable error. It can be stated, however, that for cartilage of fixed density around 0·19 there is no penetration of globular proteins of size equal to or larger than serum albumin.

Figure 4 shows differences in the penetration of serum albumin into two different

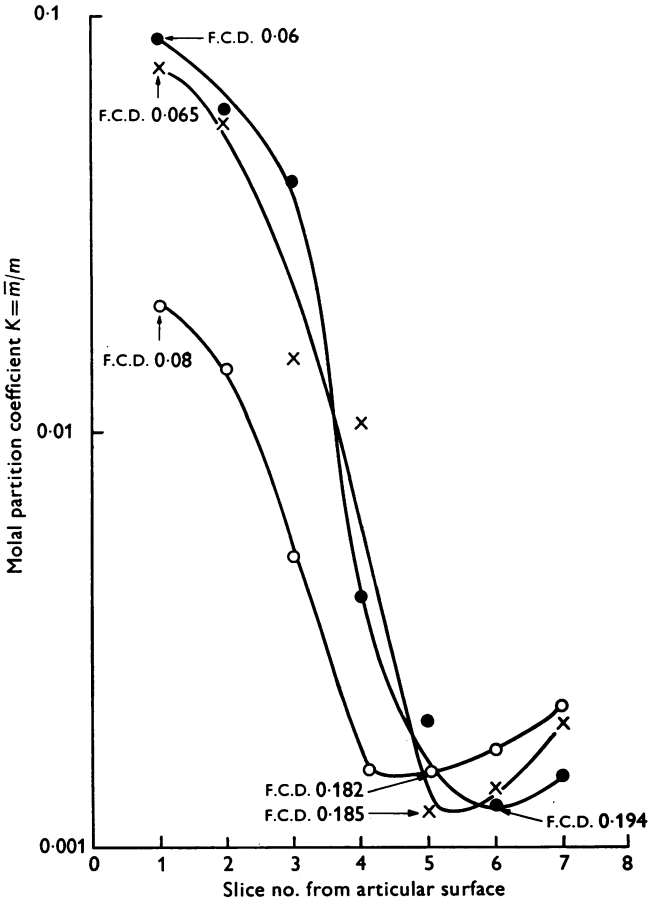

Fig. 4. Comparison between the penetration of serum albumin into different joints and into different zones of articular cartilage. ○, normal femoral head; ×, normal femoral condyle; ●, fibrillated area of femoral head.

joints and into different zones of cartilage from the same joint; normal and surface fibrillated cartilage are also compared. It can be clearly seen that whilst extremely little serum albumin (and the same is true of IGG) penetrates into the middle and deep zones of cartilage, the partition coefficients near the surface, particularly in the case of the femoral condyle and the superficially fibrillated femoral head, are quite high (nearly 0·1). These results are consistent with the variation with depth of the glycosaminoglycan content in different joints (Maroudas, Evans & Almeida, 1973; Maroudas, 1975*a*).

Diffusion coefficients

The mean diffusion coefficient of serum albumin is given in Table 1. It can be seen that it is $2{\cdot}0 \times 10^{-7}$ cm²/sec at 20 °C, i.e. approximately one quarter of its value in aqueous solution. Within the present limited series of experiments no systematic variation with the glycosaminoglycan content was found.

Table 1. *Diffusion coefficients of serum albumin at* 37 °C

Number of specimens	FCD	D (cm²/sec)
15	0·05—0·13	$2{\cdot}0\times10^{-7}\pm0{\cdot}025$

Table 2. *Permeability of cartilage to solutes*

Type of solute	Example	Molal distribution coefficient, m/m		Ratio of diffusion coefficient to that in water, D/D_{aq}
		FCD = 0·08	FCD = 0·16	
Small uncharged	Urea, proline	1·0	1·0	0·45
	Glucose	0·9	0·9	0·40
Small cations	Na^+	1·5	2·2	0·35
	Ca^{2+}	3·0	9·0	—
Small anions	Cl^-	0·75	0·53	0·45
	$H_2PO_4^-$	0·80	0·50	—
	SO_4^{2-}	0·60	0·25	0·45
Large globular proteins	Serum albumin	0·01	0·001	0·25
	Transferrin	0·01	0·001	—
	IGG	0·01	0·001	—

Summary of partition and diffusion data

Table 2 summarizes both our present results on the permeability of cartilage to globular proteins and our previous data on the permeability to small ionic and non-ionic solutes. Partition and diffusion coefficients are given for two levels of glycosaminoglycan content. The two levels (F.C.D. of 0·08 m-equiv/g and 0·16 m-equiv/g) are characteristic, for instance, of cartilage from the femoral condyle and femoral head respectively at a distance of about 400 μm from the articular surface.

DISCUSSION

Effect of solute size on penetration into cartilage

It is of interest to examine qualitatively to what extent the results obtained in the present study on the partition of globular proteins between cartilage and external medium obey Ogston's equation developed for the exclusion of globular solutes by linear rod-like macromolecules in solution. Ogston (1958) derived an equation of the following form for the partition coefficient K of a globular solute:

$$K = \exp[-AC_x(r_x+r_s)^2], \tag{3}$$

where C_x = concentration of the linear rod-like macromolecules, r_x = radius of the rod-like macromolecule, r_s = radius of globular solute, A = constant.

According to equation (3) the logarithm of the partition coefficient should vary linearly with (i) the glycosaminoglycan concentration C_x for a given solute and (ii) the expression $(r_x+r_s)^2$ for a range of solutes of different size, but at a given glycosaminoglycan content.

When plotted on logarithmic paper the graph of K versus C_x is indeed a straight

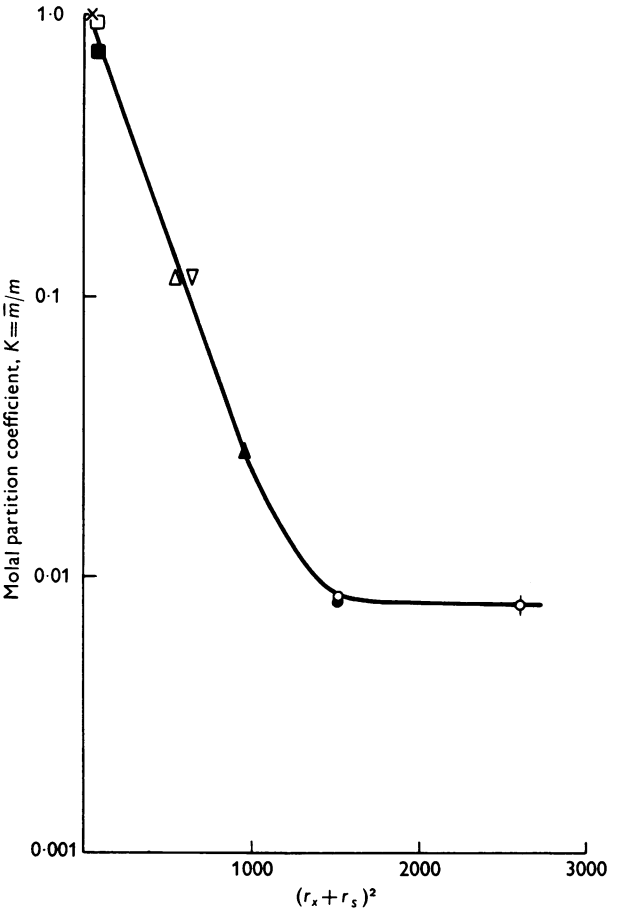

Fig. 5. Variation of the partition coefficient with the expression $(r_x+r_s)^2$ for a range of solutes of different size. Symbols as in Fig. 2.

line (as shown in the results of Figure 2). The same is true if the partition coefficients given in Figure 1 are replotted on a log scale versus $(r_x+r_s)^2$ (Fig. 5), but here the relationship breaks down for the largest solutes. Thus, the concept of excluded volume appears to apply to the partition of solutes between cartilage and external fluid, but only up to serum albumin (Snowden & Maroudas, 1976).

The reason for the fact that the partition coefficients of IGG are of the same order of magnitude as those of serum albumin, although the former solute is much larger, is not clear at the moment. It is possible that a small number of the 'pores' in cartilage are relatively larger than the rest and considerably larger than either serum albumin or IGG, and that the transport of both the latter species occurs predominantly through these large pores. Such a view seems to be consistent with the relatively high diffusion coefficient of serum albumin in cartilage (see Tables 2 and 3). If serum albumin molecules were moving through spaces only slightly larger than themselves,

one would expect a frictional retardation and hence a considerable lowering of their diffusivity.

It is hoped to test the above explanation by carrying out diffusion experiments on IGG and on solutes of even larger molecular weights.

The existence of a small proportion of channels which allow the passage of solutes whose molecular weights lie well above 100000 would explain the fact that proteoglycan fragments of relatively high molecular weight are able to make their way out of the cartilage, though admittedly very slowly, in the course of normal matrix turnover.

Some physiological implications of permeability studies

The following points of physiological interest arise from a consideration of Table 2:

Firstly, the partition coefficients of small metabolites such as amino acids or glucose are close to unity and their diffusion coefficients are approximately equal to half their value in water. Using this information, as well as the data available in the literature on the rate of consumption of these metabolites by cells (e.g. Bywaters, 1937; Rosenthal, Bowie & Wagoner, 1941), it is possible to estimate the maximum thickness of cartilage which can be adequately supplied from the synovial cavity by diffusion alone. Results of such estimates show that even the thickest cartilage (e.g. in the patella, where it can reach 5 mm) should be able to get a sufficient supply of small nutrients from the synovial cavity.

Monovalent cations such as Na^+ have a partition coefficient in the range 1·5–3·0. This coefficient increases with the glycosaminoglycan concentration, in accordance with the Donnan equilibrium (Helfferich, 1962; Maroudas, 1970). It should be noted that the activity coefficients of sodium ion in cartilage are only slightly below their values in an external solution (Maroudas, 1973, 1975*b*), which implies that, although the glycosaminoglycans are responsible for the high sodium concentration in cartilage, they do not show strong interactions with this cation. The relatively high ratio of D/D for the sodium ion (it is only 20 % lower than that of Cl^-) supports the above conclusion. On the basis of the activity coefficients of sodium and chloride ions in cartilage it is possible to calculate their osmotic coefficients and hence the ionic contribution to the osmotic pressure of cartilage (Maroudas, 1973). The osmotic pressure thus calculated is of the order of 1·7 atm, which agrees with our experimental values (Maroudas, 1975*b*).

The divalent calcium ion shows a partition coefficient much higher than that of monovalent sodium, particularly in cartilage of high glycosaminoglycan content. This is only partly explicable on the grounds of higher valency and must be partly due to some additional affinity of the cartilage matrix for calcium. We are at present investigating the possible effect of the high partition coefficient of calcium on the process of deposition of calcium apatite and calcium pyrophosphate crystals in cartilage.

Anions are partly excluded from the cartilage matrix by virtue of the Donnan equilibrium, divalent anions being more affected than monovalent ones (Helfferich, 1962). This is illustrated in Table 2 by the partition coefficients of chloride and sulphate ions.

The partition coefficient of the sulphate ion is particularly relevant in studies of glycosaminoglycan turnover. Using it, one is able to compare the sulphate uptake into the matrix from *in vitro* and *in vivo* ^{35}S tracer studies and to calculate in each case the actual rate of synthesis of sulphated glycosaminoglycans (Maroudas & Evans, 1974; Maroudas, 1975*a*). It has thus been possible to demonstrate a close agreement between glycosaminoglycan turnover rates as obtained from *in vivo* and *in vitro* experiments on animal cartilage and hence to validate the results obtained on human cartilage *in vitro*. The latter show that the glycosaminoglycan turnover in adult human cartilage is very slow, the half-life being of the order of several hundred days (Maroudas, 1975*a*). The slow turnover makes physiological sense if we remember that large proteoglycan fragments make their way through cartilage with great difficulty.

The knowledge of the diffusion coefficient of inorganic sulphate is also relevant to studies of ^{35}S uptake *in vitro*: one needs to know the time required for a cartilage specimen of a given thickness to reach equilibrium with respect to ^{35}S in the medium in order to be able to make this initial time negligible compared to the total incubation period. On the basis of the value of diffusion coefficient given in Table 2 we have been able to calculate equilibration times for different cartilage thicknesses (Maroudas & Evans, 1974). Typically, for a cartilage in contact with medium from both sides, the equilibration time will be 45 minutes for a 2 mm slice and 3 hours for a 4 mm slice.

We do not know at present the effect of the concentration of various ions in the matrix on cellular processes. However, it is likely that these do play a part. Thus, there are indications that the uptake of sulphate by the cells is dependent, amongst other factors, on the sulphate concentration in the matrix (Maroudas & Evans, 1974), which in turn is controlled by its glycosaminoglycan content. This would provide a feedback mechanism whereby glycosaminoglycan depletion in the matrix could lead to increased glycosaminoglycan synthesis.

Whilst all the above solutes are able to diffuse freely in and out of cartilage under all physiological conditions, the passage of larger molecules is very restricted and strongly dependent on the local concentration of glycosaminoglycans. This may have a number of physiological implications.

Thus, unlike small solutes, there could be conditions under which the utilization of large solutes by the chondrocytes might be limited by their rate of diffusion through the matrix.

The variations in glycosaminoglycan content between different joints and between different zones of the same joint lead to considerable differences in the penetration of IGG. If auto-immune destruction of cartilage is involved in rheumatoid arthritis, then the fact, for instance, that the femoral head is relatively less prone to this disease than the more peripheral joints might be explicable in terms of its higher glycosaminoglycan content.

It is also of interest, as pointed out by Dingle (1973), that most of the matrix degrading enzymes have molecular weights below 50000 and can therefore move fairly readily through the cartilage matrix whilst their inhibitors such as α_2-macroglobulin, which are normally present in serum, are too large to be able to penetrate into normal cartilage. Thus, one could envisage a limited action by enzymes such as

the various cathepsins involved in normal turnover of the proteoglycans; however, should the matrix begin for some reason to be seriously depleted of its glycosaminoglycans, the enzyme inhibitors could penetrate and arrest further degradation until the normal levels of proteoglycans were re-established. Thus the 'filtering' action of the matrix proteoglycans could play a part in the regulation of proteoglycan turnover.

SUMMARY

A review of the transport of solutes through articular cartilage is given, with special reference to the effect of variations in matrix composition. Some physiological implications of our findings are discussed.

Also, results of an experimental study of the permeability of articular cartilage to large globular proteins are presented. Because of the very low partition coefficients of large solutes between cartilage and an external solution new experimental techniques had to be devised, particularly for the study of diffusion.

The partition coefficients of solutes were found to decrease very steeply with increase in size, up to serum albumin. There was, however, no further decrease for IGG. The diffusion coefficient of serum albumin in cartilage was relatively high (one quarter of the value in aqueous solution). These two facts taken together suggest that there may be a very small fraction of relatively large pores in cartilage through which the transport of large molecules is taking place.

The permeability of cartilage to large molecules is extremely sensitive to variations in the glycosaminoglycan content: for a threefold increase in the latter there is a hundredfold decrease in the partition coefficient. For cartilage of fixed charge density around 0·19 m-equiv/g, there is no penetration at all of globular proteins of size equal to or larger than serum albumin.

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