Biochem. J. (1972) **127**, 249–253
Printed in Great Britain

The Diffusion Coefficient of Caffeine Through Agar Gels Containing a Hyaluronic Acid–Protein Complex

A MODEL SYSTEM FOR THE STUDY OF THE PERMEABILITY OF CONNECTIVE TISSUES

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(*Received* 27 *August* 1971)

A hyaluronic acid–protein complex was embedded into agar gel. This gel complex resembles in some respects the physiological situation in connective tissue, but still permits precise physicochemical measurements to be made. The diffusion coefficient of caffeine into and from such gels has been measured as a function of both agar and hyaluronate concentration. The value for the diffusion coefficient of caffeine was also measured by using a Gouy type diffusiometer. From both types of measurement the value for D (Fick) for caffeine when extrapolated to zero caffeine and agar concentrations agreed at $(6.79 \pm 0.01) \times 10^{-6}\,cm^2 \cdot s^{-1}$ at 25°C. Although agar concentration had only a small effect on caffeine diffusion, hyaluronic acid caused a large decrease in caffeine diffusion coefficient. The presence of the hyaluronic acid–protein complex within the gel tended to oppose gel syneresis, a concentration of 1.7mg/ml abolishing the effect and higher concentrations reversing it. The possible physiological implications of these results are discussed.

Hyaluronic acid is a collective name given to a group of mucopolysaccharides that can be extracted from connective tissues, including Wharton's jelly and the synovial fluid of joints. It is well known that the high viscosity of synovial fluid is largely a consequence of its hyaluronic acid content. It is generally considered that in subcutaneous tissues where the hyaluronic acid has a high concentration of up to 10mg/ml (Rienits, 1960) the functions are, first, to act as barrier to the diffusion of substances, and, secondly, to help stabilize the connective tissue by trapping the water in some way within the connective tissue. A number of studies have been made on the diffusion properties of connective tissues, the earliest being that by Krogh (1919) who showed that the rate of gaseous diffusion across connective tissue was considerably lower than that through muscle; he was unable to explain this result. Since that time several studies have been made on the diffusion coefficients of substances through connective tissues *in vivo* (Wright, 1934; Paulson *et al.*, 1951) and the effects of the simultaneous administration of hyaluronidase on such diffusion or transport (Day, 1950, 1952). In general it has been found that the addition of hyaluronidase to the connective tissues results in a more rapid passage of the substance through the tissue.

Some studies *in vitro* have been made on the effect of preparations of hyaluronic acid on the diffusion coefficient of several substances (Ogston & Sherman, 1961). The results suggest that the large molecular domain of the hyaluronate molecule results in considerable exclusion of other molecular species, which thus decreases the transport of substances across tissues containing appreciable quantities of hyaluronic acid. In general the previously described work relies on the use of membranes to contain the hyaluronic acid within fixed compartments while the diffusing substance is allowed to penetrate the membrane and diffuse into or out of the hyaluronate solution. The reliability of such methods depends on a preliminary calibration of the membrane and the assumption that the permeability characteristics of the membrane are not altered by the subsequent positioning on one side of the membrane of the solution of hyaluronic acid. However, it is not impossible that the hyaluronate molecules will partially penetrate the membrane and thus alter its permeability. A further assumption that transport through such solutions is diffusive is made; however, it is an integral part of all such membrane-limited diffusion measurements that the compartment on the free solution side of the membrane is stirred to prevent the formation of a concentration gradient outside the membrane on that side. Stirring this compartment may well impart vibrations and micro-turbulences to the compartment containing hyaluronate. Such mechanical vibrations have been shown to have very large effects on the transport through fluid systems (Longmuir & Roughton, 1952). There is a third reason why the

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results of such membrane diffusion studies are difficult to interpret, since in connective tissue the hyaluronate fibres are interwoven with collagen fibres (Fessler, 1957), which thus largely immobilize them. A system of 'free' hyaluronate retained only on one side of a membrane does not resemble the physiological situation very closely.

To overcome these limitations the technique of imbedding a biological system in agar gel (McCabe & Longmuir, 1964) has been applied to hyaluronic acid systems. By fixing the hyaluronate in this way its free diffusion is completely prevented and it is thus possible to dispense with the membrane; also, microturbulences are completely prevented and so no special precautions need be taken to prevent vibration. This system of agar–hyaluronate also has the advantage that although it closely resembles the physiological situation it is possible to make precise physicochemical measurements and relate these to controllable variable parameters such as the hyaluronate and agar concentrations.

Caffeine was chosen as the diffusing species since it has a low molecular weight and the transport of such substances through connective tissues is of some interest. Also the pK of caffeine is far from the pH of the synovial fluid extract which was used in the experiments and hence there were no complications due to ionization.

Experimental

Materials

Hyaluronic acid–protein complex. This was prepared by filtration of ox synovial fluid (Ogston & Stanier, 1950, 1952). No attempt was made to remove the accompanying protein, since it was considered important to resemble the situation within connective tissue as far as possible.

Agar–hyaluronate gels. Agar powder (Oxoid Ion Agar no. 2) was added to water to a concentration twice that required in the final gel. This mixture was heated on a water bath until the agar was dispersed and was then allowed to cool to 40°C. Meanwhile a solution of hyaluronic acid containing twice the desired final concentration of hyaluronate in the gel was warmed to 40°C. The two solutions were mixed in equal proportions at 40°C and the resulting mixture was poured into the lower compartment of the diffusion cell, where it was allowed to set.

Caffeine. The concentration of caffeine was determined from the E_{272} of its solution. It was found that during the experiment a material also absorbing at 272nm was leached out from the agar gel. This contamination was minimized by repeatedly washing the agar before using it to prepare the gel. The residual contamination was corrected for by the method of Hufner's Quotients (Dawes, 1965), readings being taken at 245 and 272nm. The error caused by this contaminant could also be decreased by using relatively high concentrations of caffeine with dilution if necessary before readings were taken.

Diffusion apparatus. This was constructed of Perspex and was similar to that described by Ogston & Sherman (1961) with the added refinement of a lid to minimize evaporation from the upper solution of caffeine during an experiment.

Methods

Diffusion coefficients. These were calculated by the method of Ogston & Sherman (1961). In the absence of a membrane, as in this case, the equation simplifies to:

$$C_0 - C_t = \frac{2AC_0}{KV}\left\{\left(\frac{Dt}{\pi}\right)^{\frac{1}{2}} - \frac{ADt}{2KV}\right\}$$

where C_0 is the initial concentration of caffeine in the upper compartment of the cell and C_t is the final concentration after a time t seconds; K is the partition coefficient for caffeine between water and the gel, A is the cross-sectional area of the cell (2.27cm^2), and V is the upper volume of solution, equal to 4.0ml.

The diffusion coefficient of caffeine in water (D) was determined on a Gouy type diffusiometer similar to that described by Gosting *et al.* (1949).

Density of agar gels. This was measured by a modification of the use of a specific-gravity bottle. The agar solution at 40°C was poured into a pre-weighed pre-calibrated bottle almost to fill it; it was then allowed to cool to 25°C and weighed. Finally the remaining space in the bottle was filled with water and again weighed. In this way the residual volume in the bottle not filled by the gel could be calculated and allowed for in the calculation of gel density. The same technique was used for the gels containing hyaluronate. The density thus obtained is that before any syneresis, since there is very little evaporation from the closed specific-gravity bottle. Thus any water squeezed out from the gel remains in the bottle and the total volume of the bottle which still remains to be filled is unchanged. This does not, however, introduce a measurable error into the final calculation since it can readily be shown that even large syneresis effects will have only an extremely small effect on the final gel density. This is due to the fact that the gel density is close to the density of the water that is squeezed out.

Partition coefficients for caffeine between gels and water. These were measured by allowing measured volumes of solutions of known initial concentration of caffeine to attain equilibrium with weighed samples of the gels and the final aqueous concentration of the caffeine was measured.

Hyaluronate concentration. This was determined from the flow time of a capillary viscometer (BSU

type B, water time 65s), which was calibrated by using solutions of known hyaluronate concentration as determined by evaporation to dryness at 105°C.

Results

Free diffusion of caffeine in water

The results of the experiments with the Gouy diffusiometer are shown in Fig. 1. Each point is the mean of three experiments. Over the series of concentrations used there was only a small concentration effect. The extrapolated value at zero concentration and 25°C was $(6.79 \pm 0.01) \times 10^{-6} cm^2 \cdot s^{-1}$.

Density determinations

These are shown in Fig. 2 for a series of gels containing various amounts of hyaluronic acid. Over the range of concentrations used the density increment with both gel and hyaluronate concentration is essentially linear. Distribution coefficients, which are calculated from equilibrium concentration experiments, are shown in Fig. 3. Again the relation between hyaluronate concentration inside the gel and the distribution of caffeine between gel and water is linear over the range of concentrations studied.

Diffusion of caffeine into agar gels

A series of preliminary experiments was conducted to find the effect that agar-gel concentration has on the diffusion coefficient of caffeine. Several agar concentrations were prepared, between 1 and 5% dry weight of agar. It was difficult to obtain consistent results for caffeine diffusion; neither did the extrapolated value for zero agar concentration agree with that measured on the Gouy diffusiometer, the values being lower. It was noticed that the gels exhibited syneresis when they were left, a factor that would tend to oppose the inward diffusion of the caffeine. For this reason each experiment was performed at a fixed time after pouring the gel. The experiment was also conducted in the reverse way, the caffeine being initially incorporated into the gel and then allowed to diffuse out. The experiments were also conducted in triplicate. The calculated values

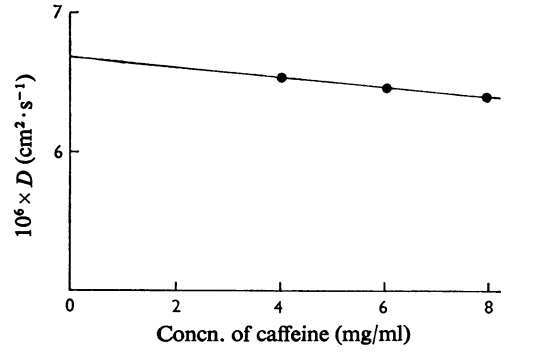

Fig. 1. *Variation of diffusion coefficient of caffeine in water at 25°C as a function of its concentration*

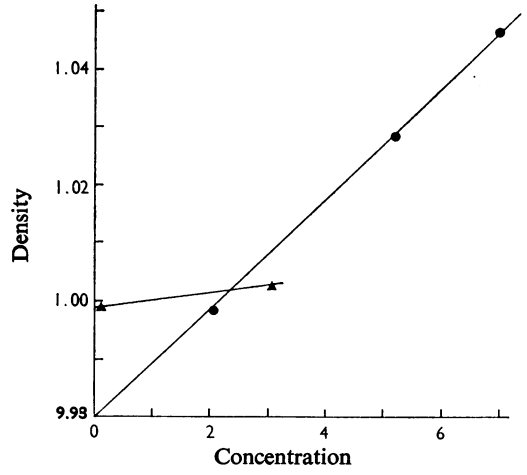

Fig. 2. *Density at 25°C of agar gels and of agar gels containing hyaluronic acid as functions of the concentrations of the components*

●, Agar (concentration in g/100ml); ▲, hyaluronic acid in 2% agar gel (concentration in mg/ml).

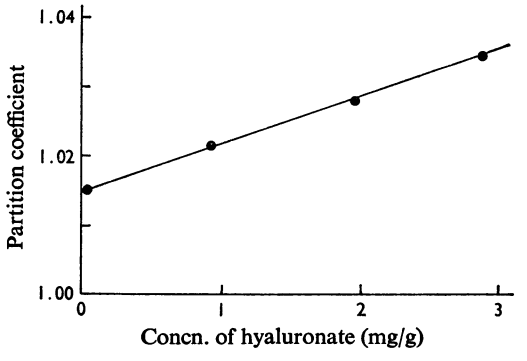

Fig. 3. *Partition coefficient at 25°C of caffeine between water and 2% agar gel containing various concentrations of hyaluronate*

The concentrations of caffeine used were 10–40mg/l; the measured values of the partition coefficients did not depend on the concentration of caffeine in the range used.

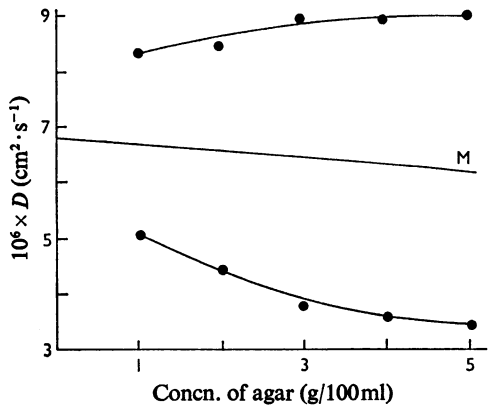

Fig. 4. *Apparent diffusion coefficients for caffeine in agar gels as a function of gel concentration*

Upper curve: apparent diffusion coefficients out from the gel. Lower curve: apparent diffusion coefficients into the gel. M: the mean of these two curves.

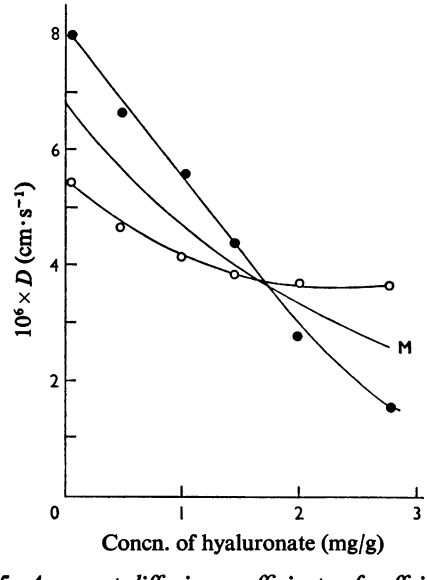

Fig. 5. *Apparent diffusion coefficients of caffeine into and out of 2% agar gels containing the hyaluronate complex as a function of hyaluronate concentration*

●, Apparent diffusion coefficients of caffeine out of the gel complex; ○, apparent diffusion coefficients of caffeine into the gel complex. M: mean curve.

from these reversed experiments gave values that were enhanced by the syneresis. When the mean values of these two sets of experiments were extrapolated to zero agar concentration a value for diffusion coefficient agreeing with that found on the Gouy diffusiometer was obtained. Thus the effect of varying agar concentration on the diffusion coefficient for caffeine is quite small, being approximately proportional to the dry weight of the gel, i.e. a 5% gel decreased the diffusion by approx. 5% of that through water (Fig. 4).

These experiments were then repeated by using a final agar concentration of 2% and incorporating various amounts of hyaluronic acid. The results are shown in Fig. 5. The incorporation of relatively small amounts of hyaluronic acid into the gel resulted in a large decrease in the diffusion coefficient of the caffeine. It was found difficult to increase the concentration of hyaluronate in the gel much above 3mg/ml, but this concentration caused a decrease of the diffusion coefficient of approx. 65%.

Discussion

It is thought that in subcutaneous connective tissue the concentration of hyaluronate reaches approx. 10mg/ml. The extrapolated values from these experiments suggest that this concentration must present a very formidable barrier to the diffusion of even small molecules. It is understandable why Wharton's jelly should have a high hyaluronate content, since this will help to decrease the fall in oxygen partial pressure owing to diffusion losses through the walls of the blood vessels in the umbilical cord.

One unexpected result of investigation was the demonstration that the addition of hyaluronate to an agar gel tends to oppose gel syneresis: a concentration of hyaluronate in the gel of 1.8mg/ml completely prevents syneresis, whereas higher concentrations cause a reversal of the effect, water being drawn into the gel; this may be caused by the osmotic pressure exerted inside the gel by the hyaluronate. Preston *et al.* (1965) have shown that hyaluronic acid–protein complexes have an anomalously high osmotic pressure. One of the functions of the protein that is attached to the hyaluronic acid may be to raise the osmotic pressure of the complex, thus stabilizing connective tissue gels by opposing the external osmotic pressure of excluded proteins that surround the tissue.

I thank Professor A. G. Ogston for his continuing advice and encouragement during the period of this work, and Professor L. W. Nichol for his comments and helpful criticism.

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