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An early look at macromolecular crowding

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Abstract

Interest in the interaction between proteins and polysaccharides in semidilute systems developed in the late 1950's and early 1960's both in the field of matrix biology and in the construction of new separation techniques. The author gives an account of how his work in the 50's on the characterization of a connective tissue polysaccharide, hyaluronan, led him into studies on polysaccharide networks, sieve effects, molecular exclusion, a theory of gel filtration, solubility of proteins and chemical equilibria in polymer solutions, water and protein homeostatis in vivo, and ordered convectional flow in concentrated polymer systems.

Keywords: Polysaccharide; Macromolecular interaction; Semidilute systems; Sieve effects; Exclusion; Ordered convection

1. Introduction

Although the cell and often the extracellular matrix are crowded with macromolecules, something everybody is aware of, there were few studies on the properties of concentrated (semidilute) solutions of biological macromolecules when we started our work on the behavior of polysaccharide networks forty years ago. Most physical chemical techniques, which were then used to characterize molecular parameters, were based on the ideal behavior of the macromolecule and all data were therefore extrapolated to infinite dilution. Except for expressing non-ideality in the form of virial coefficients, there was hardly any theoretical basis for physical chemical studies of molecular crowding. The situation is different now and the present volume is an example of the increasing awareness of the biological importance of concentrated systems.

When the Editor asked me to contribute to this special issue I had to admit with regret that I had not worked in the field for ten years. He then generously asked me to summarize our early efforts. I have tried to recollect the process of thinking and the external influences which effected the progress of our work but I have not attempted to make an historical account of the development of the whole field in this time period.

2. Hyaluronan chains form a continuous network

A number of polysaccharides are found in the vertebrate extra-cellular ground substance. One of them is hyaluronan (hyaluronic acid) which is especially accumulated in soft connective tissue such as skin, synovial fluid, the vitreous body of the eye and the umbilical cord. It is a linear polydisperse polymer, built from alternating units of glucuronic acid and N-acetylglucosamine, and usually has a weight-

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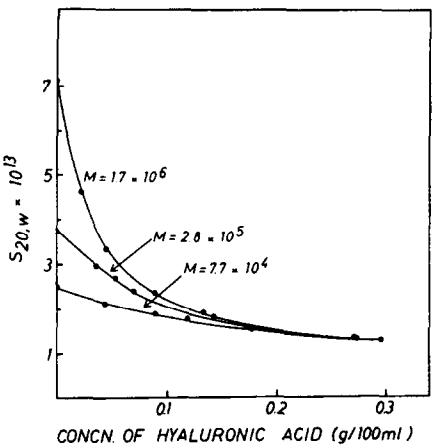

Fig. 1. The sedimentation of three samples of hyaluronan of different molecular weights. Note the sedimentation behavior at high concentrations. Data from Ref. [2]. Reproduced from Ref. [55] by permission.

average molecular weight of several millions. In solution it behaves as an expanded random coil. My teacher, Dr. Endre Balazs, had introduced me to this compound in 1949 and I defended my doctoral thesis on the macromolecular characterization of hyaluronan in 1957 [1]. This polysaccharide has since then been my favorite research object.

When we had developed a technique to fractionate hyaluronan according to molecular weight and had characterized the fractions by ultracentrifugation and light-scattering [2] we found an unexpected pattern for the concentration dependence of the sedimentation rates (Fig. 1). At infinite dilution the sedimentation constant was a function of the molecular weight but at higher concentrations (above 1–2 g/l) there was no molecular weight dependence and the sedimentation rate was low. Searching the literature we found that Ogston and collaborators had found a similar behavior for other polymers and that they explained the behavior by the formation of a continuous polymer network that sedimented like a plug through the cell. The slow sedimentation rate was explained by a high resistance to backflow of water through the plug [3]. This concept was very attractive as we could calculate from the dimensions of the hyaluronan coils [4] that they should be in close contact already at concentrations of 1 g/ml. Later it was shown that viscosity measurements gave a similar entanglement point [5]. Polysaccharide concentrations above this point are not uncommon in vivo and we drew the conclusion that hyaluronan exists in the tissues in the form of a three-dimensional continuous randomly distributed chain network between the cells. This was the starting point for our studies on semidilute systems.

3. Transport through polysaccharide networks

A network of randomly distributed chains should act as a filter for transport of other macromolecules, i.e. small molecules should move relatively freely through the meshwork while large molecules should be hindered. This was tested by determining the sedimentation rate of globular proteins and other particles in the presence of hyaluronan of increasing concentration [6,7]. The results were striking. Serum albumin moved relatively unhindered through hyaluronan solutions while a virus and large latex particles were dramatically retarded. In control experiments it was demonstrated that diffusional transport was retarded to the same extent as sedimentation and the decrease in sedimentation rate was therefore not an artifact due to the technique [7]. The hyaluronan network acted as a true molecular sieve which even could be used for molecular size separations in the ultracentrifuge [8]. Also other polymers acted likewise [9].

An analysis of the data from hyaluronan experiments showed that they could be organized into an empirical equation of the form [7]:

$$S/S_0 = A \exp\left(-k \cdot d \cdot C_{HA}^{1/2}\right)$$

where S/S_0 is the sedimentation rate of a globular probe in the presence of hyaluronan divided by the sedimentation rate in free solution, C_{HA} is the concentration of hyaluronan, d is the diameter of the probe, k is a constant and A is different for each probe but it is not far from unity. A theoretical explanation to this empirical equation was later presented by Ogston et al. [10]. It was based on the probability that there would be an open space of sufficient size in the network next to the probe into which the latter could move.

Many molecules moving through the extracellular space are asymmetric or chain molecules and from the concept which was developing the asymmetric

molecules should have a larger chance to find space in a polymer network if they moved end-on rather than sideways. When the diffusion of asymmetric molecules was studied in the ultracentrifuge [11] their movements were indeed much less retarded in hyaluronan than expected from the diameter of their hydrodynamic units. This was interpreted as if elongated molecules preferentially moved along their long axis like a bow-arrow shot through a bush.

To further check that the retardation of transport of macromolecules in a polysaccharide network was due to a steric hindrance for the probe rather than friction between the moving probe and the stationary polysaccharide we used fluorescence polarization techniques to measure diffusional rotation of globular proteins in the presence of polysaccharides (dextran and hyaluronan) at high concentrations. The rotational diffusion was only marginally retarded in spite of the large number of probe–polysaccharide contacts in these experiments [12–14] indicating similar friction between protein and polysaccharide surfaces as between macromolecules and solvent.

These experiments thus left us with a general idea of how a polymer network exerted its sieve effect. Since then a large amount of work has been carried out on other polymer systems due to the great interest in separating macromolecules by electrophoresis in polyacrylamide and agarose gels.

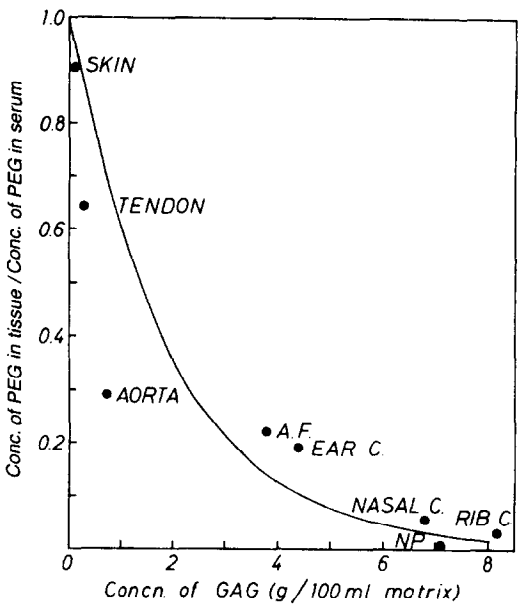

Fig. 2. Radioactively labelled polyethylene glycol was equilibrated in a rabbit in vivo. Its concentration in the extracellular space of various tissues was measured and related to that in serum. The polysaccharide content (glycosaminoglycans) was measured in each tissue. The line is the exclusion estimated from the polysaccharide concentration. C, cartilage; A.F. *annulus fibrosus*; NP, *nucleus pulposus*. Data taken from Hallén [18].

4. The concept of excluded volume in hyaluronan solutions

While our group was making dynamic studies on probes moving through polysaccharide networks, Ogston and Phelps [15] made equilibrium studies on the partition of proteins between hyaluronan solutions and buffer. They found that the proteins partitioned in favor of the buffer side. The conclusion was that hyaluronan excluded proteins and other solutes from part of the space and that this excluded volume increased with increasing hyaluronan concentration and increasing size of the solute. Ogston had previously from first principles calculated the available space for spheres in a network of randomly distributed rods [16] but the experimental results exceeded the theoretically calculated values. However, a reinvestigation of protein distributions between hyaluronan solutions and buffer led to a close fit with Ogston's theory if one assumed that the hyaluronan chains could be regarded as stiff infinitely long fibers with a diameter of 7 Å [17]. Subsequently Anund Hallén in our laboratory showed that the exclusion of polyethylene glycol from polysaccharide containing tissues in vivo is of the expected order of magnitude [18] (Fig. 2) but other components like collagens are of course also effective excluders [19].

5. The principle of gel chromatography

In 1960, two new techniques to separate proteins and other compounds had been developed in the Biochemistry Department in Uppsala. The two techniques, gel chromatography (gel filtration) [20] and counter current distributions in two polymer phase systems [21] utilized concentrated polymers. Of these two techniques the first one has been the most

widely applied. In 1961 the author moved to Uppsala and was confronted with the lively discussions on the molecular mechanisms behind the separations. It was logical to apply the reasoning from the studies on hyaluronan and a theory was developed to explain gel chromatography [22]. At that time the gels were made by cross-linking various concentrations of dextran. We assumed that the dextran chains in these gels formed a three-dimensional network of fibers and assumed that solutes were partitioned to equilibrium between the gel and the mobile phase. Available gel chromatographic data for different solutes and different dextran gels obeyed Ogston's relationship [16] if one assumed that dextran was somewhat coiled, i.e it formed shorter and thicker fibers (diameter 14 Å) than a linear stiff polysaccharide chain. When hyaluronan was cross-linked and used for gel chromatography, it excluded proteins as if it was a stiff chain (diameter 7 Å) [17]. Agarose gels could also be accommodated in the theory but in this case the fibers were much thicker (diameter 50 Å) [23]. An historical account of the development of the theory of gel filtration was recently published [24].

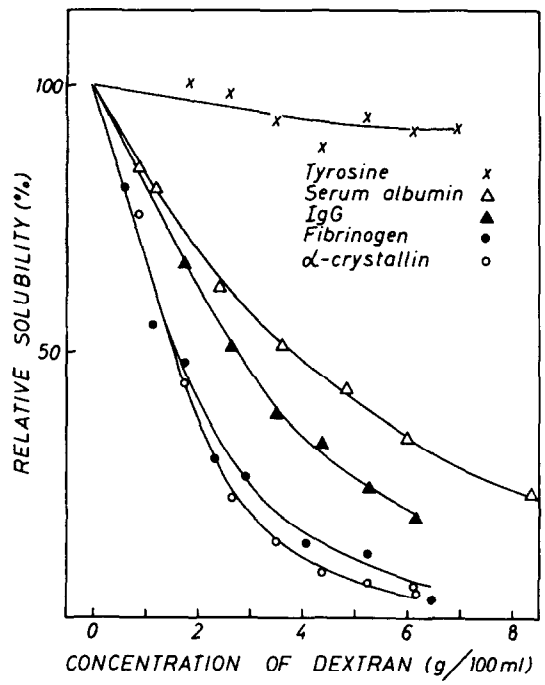

Fig. 3. The relative solubility of tyrosine and various proteins in the presence of dextran. Data from Ref. [27].

6. The solubility of proteins in polymer solutions

The concept of volume exclusion had several biologically interesting consequences. For example, by addition of a polymer to a protein solution, the latter should become 'more concentrated' i.e. its chemical activity should increase. At sufficiently high polymer concentrations the protein could reach its solubility limit and precipitate. As a matter of fact a few reports before 1960 indicated that polymers could cause macromolecular precipitations although the nature of the phenomenon was never clarified. For example, Cohen [25] could crystallize plant viruses and hemocyanin by adding polysaccharides. Polymers were also used to enhance immune reactions (for early references see [26]).

To explore this hypothesis we measured the solubility of various proteins in the presence of dextran and found a correlation between their solubility and the dextran concentration [27,28]. The larger the size of a protein, the more its solubility was depressed in the presence of the polymer (Fig. 3). To our delight the lowering of the solubility closely corresponded to what was expected from data on the exclusion of the proteins from a dextran gel of equal polymer concentration. No specific interaction between the polysaccharide and the protein had thus to be invoked to explain the precipitation.

The observation was expanded by three of my graduate students to three problems of direct biomedical interest. Krister Hellsing studied the influence of polysaccharides on the precipitation of immune complexes formed by serum albumin and antialbumin IgG [26,29]. In the region of antigen excess, where the complexes were soluble, dextran caused a dramatic decrease in solubility. Linear connective tissue polysaccharides had an even larger effect [30]. There was no binding of either of the proteins to the polysaccharides [31]. The large immune complexes precipitated before the small [32,33] and the relative effect of the polymer was independent of the affinity of the antibody for the antigen [34]. All data taken together strongly indicated that it was an excluded volume reaction that Hellsing studied. These observations have been widely utilized to increase the sensitivity in immunological analyses.

Per-Henrik Iverius identified proteins which precipitated when dextran or polyethylene glycol were added in increasing concentrations to blood plasma [35]. Polyethylene glycol was more effective than dextran but in both cases it was the high molecular weight proteins that first appeared in the precipitate: fibrinogen, IgM, β-lipoprotein, α_2-macroglobulin etc. A closer analysis of the solubility of β-lipoproteins in dextran concurred with the exclusion hypothesis [36]. Björn Öbrink studied the interactions of connective tissue polysaccharides with collagen monomers and in the case of hyaluronan he found that the two macromolecules mutually excluded each other [37]. For this reason hyaluronan also accelerated the formation of collagen fibers from the monomers [38]. Although in these investigations the exclusion of proteins by a polysaccharide was studied there are other conditions when the two components attract each other. For example, under certain conditions, sulfated polysaccharides bind to both lipoproteins and collagen.

7. Osmotic pressure in mixed polysaccharide–protein systems

A way of determining the chemical activity of the components in mixed macromolecular solutions is osmometry. For this reason we determined the osmotic pressure of mixtures of hyaluronan and serum albumin [39] and analyzed the data in thermodynamic terms according to Ogston [40]: the analysis gave values of exclusion of albumin in the order of 25 ml/g hyaluronan at low polysaccharide concentrations. When data for the partition of albumin between hyaluronan gel/solution and buffer subsequently were published [17] they were in general agreement with the osmometric results. The osmometry data on albumin–hyaluronan mixtures were soon confirmed by Preston et al. [41]. The osmotic pressures in albumin–dextran systems were also in concordance with data on the exclusion of albumin from dextran gels [42].

The biological consequences of the osmotic contributions due to exclusion were first realized by capillary physiologists because the interstitium contains both proteins and polysaccharides and the difference between the oncotic pressures of the vascular and interstitial compartments is one of the factors that determine the hydration of tissues.

8. The effect of exclusion on chemical equilibria

Chemical equilibria ought to be influenced by exclusion if reactants and products are excluded to different degrees. In order to test this hypothesis three enzymatic reactions were followed in the presence of high concentrations of dextran or polyethylene glycol [43]. When the results were analyzed it was found that the polymers caused an apparent decrease in Michaelis–Menten constants or inhibitor constants in agreement with theoretical predictions. As the substrates and products were of low molecular weight the effects were small in these experiments but they should have been of a much larger magnitude if macromolecules had been involved. In addition to effects on chemical equilibria, reaction rates are also changed by polymers due to the decrease in diffusion and collision rates of the reactants.

One type of reaction, which is common in biological systems is the change in conformation of a macromolecule. If this change in conformation results in a change of effective molecular volume the reaction should be sensitive to exclusion effects. To test this we studied the stability of the DNA double helix in the presence of dextran and polyethylene glycol [44]. As two single chains in random coil configuration occupy a larger volume than a double helix formed from them we expected that the presence of neutral polymers should move the equilibrium from single chains towards the compact double helix. Dextran and polyethylene glycol raised the melting temperature of the DNA dramatically as the hypothesis had predicted.

It has later been described that connective tissue polysaccharides similarly raise the denaturation temperature of collagen in cartilage [45].

9. Ordered convection in concentrated polymer systems

During the 1970's there was a close collaboration on transport processes in semidilute polymer solutions between Barry Preston at Monash University in

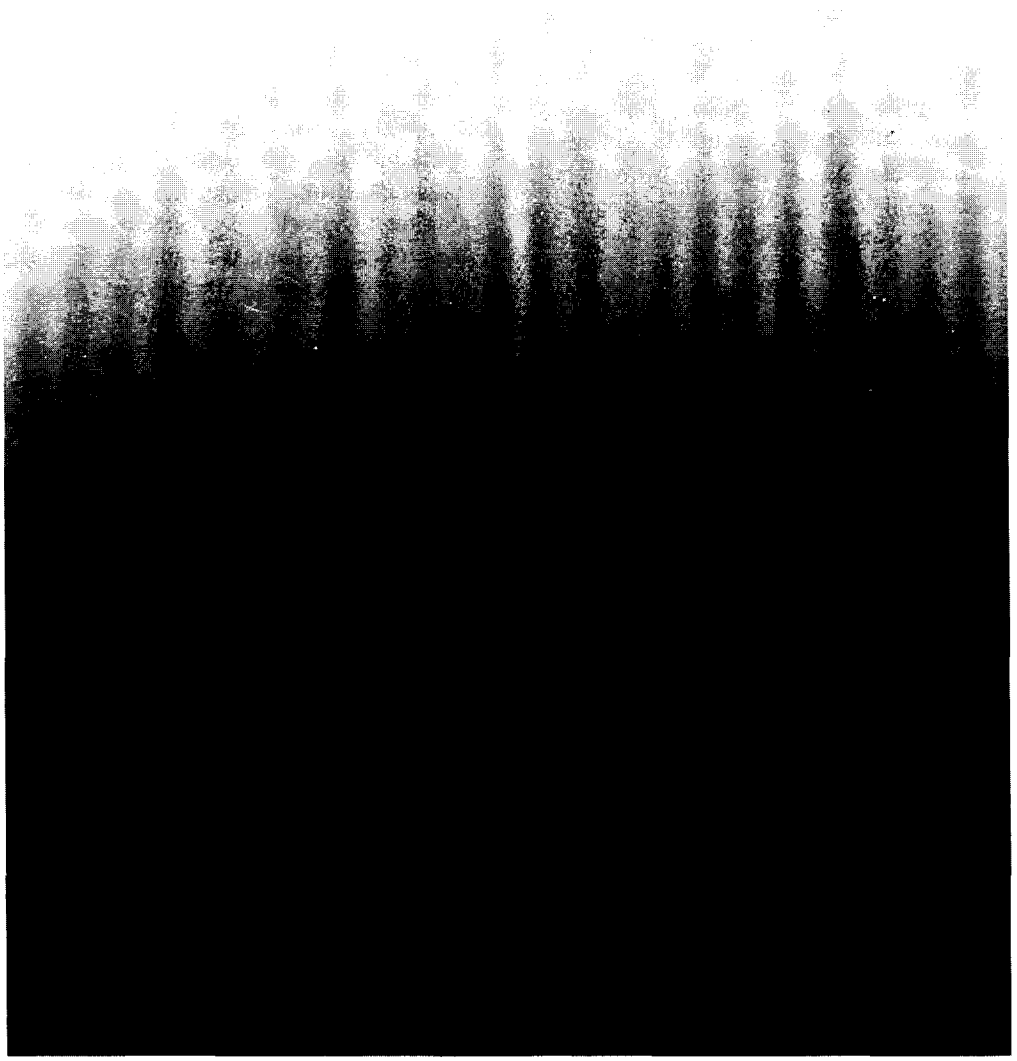

Fig. 4. Ordered convection in a mixed polymer system. 13.5% dextran was layered on top of 13.5% dextran + 0.5% blue polyvinylpyrrolidone and photographed one hour later. Vertical fingers are moving through the solution. Described in Ref. [49]. Reproduced from Ref. [52] by permission.

Australia, Lars-Olof Sundelöf at the Pharmaceutical Faculty in Uppsala and our laboratory. Barry Preston spent a sabbatical in Uppsala in 1978 and he brought data from measurements in Australia on the diffusion rates of polymers in mixed polymer systems. These 'rates' were very high and even higher than the diffusion rates of low molecular weight solutes. We tried to explain the results in terms of increased chemical activity of the components due to exclusion [46]. When I spent a sabbatical in Australia a year later I brought a simple instrument constructed by Lars-Olof Sundelöf [47,48]. With this instrument we could simultaneously in a large number of cells measure the diffusion rate of labelled probes over interphases formed by two polymer solutions. When we layered 13.5% dextran over a mixture of 13.5% dextran and 0.5% polyvinyl pyrrolidone [PVP] and also included ^{3}H-labelled PVP (mol. wt. 360 000) and ^{14}C sorbitol in the lower phase we found that the PVP moved 40 times faster over the boundary than in pure buffer while the diffusion rate of sorbitol decreased by about a third in the presence of dextran [49].

The explanation to this remarkable observation came unexpectedly when we stained the PVP blue. We then found that finger-like ordered structures were formed in the boundary and grew upwards and downwards in the system until they extended through the whole cells (Fig. 4) [49]. PVP was transported in these fingers while sorbitol apparently formed a normal diffusion boundary.

Further analyses of the conditions for the formation of ordered structures in semidilute polymer systems have clarified the process [50,51]. At the interphase there is a solvent flow, driven by an osmotic gradient, from the solution above the boundary to that one below, and this causes a density inversion at the interphase and the formation of the structures which are stabilized by gravity and growing due to a continuous water flux. Large molecules which are immobilized in the polymer network are transported in the finger-like structures which are moving upwards and downwards. Low molecular weight compounds, which can diffuse between the upwards and downwards going structures are transported in a counter current type fashion. When the compound is easily diffusing between the two phases a normal diffusion pattern is obtained at the original boundary.

A large amount of work has subsequently been carried out to characterize and explain various systems in which ordered convective flow occurs in polymer systems. The early contributions were reviewed by Comper and Preston in 1984 [52] and Wayne Comper has since continued to study systems of possible biological interest.

10. Concluding remarks

Our work on 'concentrated' polysaccharide systems is a good example of how the research process functions. It is seldom possible to predict the long term direction. Every new result raises new questions and new hypotheses and furthermore new ideas are continuously fed into the work from the outside. Our main interest has been to solve biological problems. Our original goal was to characterize hyaluronan as a macromolecule. After we had developed a technique to molecular weight fractionate the polysaccharide we found the interesting sedimentation behavior of the polymer fractions. We drew the conclusion that the polymer chains formed a continuous network in the interstitium. Such a network should influence transport through the tissues and we started to characterize the sieve effect of the meshwork. When Ogston showed that the hyaluronan fibers also had exclusion properties it was logical to look also for biological phenomena, which could be explained by this parameter. It led us into capillary physiology, biological precipitations and chemical equilibria. The recently developed gel filtration technique seemed to be an excellent example of molecular exclusion and that led us into the theory of separation techniques. Our studies on the transport in polymer systems led us to the discovery of ordered convective flow.

When I left the studies on crowded macromolecules more than a decade ago it was also due to a new development which carried us into a new field. One of my collaborators Anders Tengblad developed an analytical technique to measure nanogram quantities of hyaluronan [53]. So instead of continuing studies on crowded systems we went into biological systems containing very dilute concentrations of hyaluronan. It lead us into studies of the turnover and catabolism of hyaluronan and its use as a marker in clinical medicine [54].

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