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History of a theory

Torvard C. Laurent

Department of Medical and Physiological Chemistry, University of Uppsala, Biomedical Center, Box 575, S-751 23 Uppsala (Sweden)

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ABSTRACT

The article "A theory of gel filtration and its experimental verification" was published in *Journal of Chromatography* in 1964. One of its authors gives an account of the origin of the work.

INTRODUCTION

The Editor asked me to describe the background of the paper "A theory of gel filtration and its experimental verification" which was published almost three decades ago by Johan Killander and myself in the *Journal of Chromatography* [1]. The title and the summary of the paper are shown in Fig. 1. The Editor also asked if I could give an explanation for the apparent impact of the paper as measured by its citation frequency. On this matter I can only speculate.

The research that led to that paper was not at all primarily aimed at the clarification of a chromatographic process. As so many times before, the results arose from the interaction between scientists from different fields at the right time and the right place, Uppsala.

When our paper was written, I had been working in the Department of Medical Chemistry at the University of Uppsala for 2 years. I had earlier received basic training in medicine in Stockholm, but I never went into practice after medical school. Instead, I was recruited to research which led to a doctorate in chemistry at the Karolinska Institute and postdoctoral work at the Retina Foundation in Boston, MA, USA. Research positions were scarce at Swedish Universities but fortunately I received an appointment as investigator in ophthalmic biochemistry at the Medical Research Council and moved to Uppsala in 1961 at the age of 30.

HYALURONAN —A HIGH-MOLECULAR MASS POLYSACCHARIDE IN CONNECTIVE TISSUE

Dr. Endre A. Balazs, my first research teacher, raised my interest in hyaluronan, a carbohydrate component of connective tissue, and this compound has since been a life-long subject of my curiosity [2]. The space between the cells in the connective tissue is filled by a matrix, which is composed of different types of macromolecules, among them a family of polysaccharides. These have linear structures and contain, with one exception, alternating residues of uronic acid and hexosamine. One of them is hyaluronan (previously called hyaluronic acid). It has a high molecular mass (10^6–10^7) and is found as a major component of soft tissues such as skin, umbilical cord, the vitreous body of the eye and joint fluid.

During the 1950s, work in different laboratories, especially by Dr. Alexander G. Ogston (Fig. 2) in

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A THEORY OF GEL FILTRATION AND ITS EXPERIMENTAL VERIFICATION

TORVARD C. LAURENT AND JOHAN KILLANDER

Department of Medical Chemistry and Department of Clinical Chemistry at the University Hospital, University of Uppsala, Uppsala (Sweden)

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SUMMARY

The separation by gel filtration of molecules varying in size is explained as a steric exclusion of solutes from the gel phase.

The volume available for a solute in the gel phase can be determined from the elution volume, the void volume and the total volume of the gel column. It has been calculated for a number of proteins and dextran fractions and for various dextran gels from data given in the literature as well as from some new data. The values were used to test the hypothesis that the exclusion takes place from a three-dimensional random network of straight polymer fibers distributed in the gel. The experimental data were found to verify the hypothesis.

The experimentally determined available volumes in the gel phase for three proteins are approximately the same as the available volumes in dextran solutions, having the same polymer concentrations as the gels. Therefore there seems to be no essential difference between the exclusion phenomenon in a polymer gel and in a polymer solution.

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Fig. 1. Title and summary of the paper in *Journal of Chromatography*, 14 (1964) 317–330.

Oxford, Dr. Endre Balazs in Boston and myself in Stockholm, clarified that hyaluronan behaves as a large extended and hydrated coil structure in solution [3]. It can be calculated but also shown experimentally that entanglement of the individual molecules must occur already at concentrations of the order of 1 mg/ml. At higher concentrations the solution can be regarded as a continuous mesh of polymer chains. It became a challenge in the late 1950s and early 1960s to describe the physiological functions of such networks in physical chemical terms, *e.g.*, in lubrication, water homeostasis, regulation of transport and distribution of plama proteins (for a review see, *e.g.*, ref. 4).

Much of this work was carried out in Ogston's and our laboratories. In both places we approached the problems with a very similar picture of the hyaluronan network in mind. Ogston was intrigued by the question of how much space could be occupied in the network by other molecules, such as proteins, and deduced mathematically the volume available for a spherical particle with a given diameter in a space occupied by a random network of rods of given length and diameter [5]. Ogston and Phelps [6] then measured the exclusion of proteins from hyaluronan compartments by means of equilibrium dialysis and tried to apply the theoretical relationship to the experimental data, although without immediate success. It was later shown that theory and experiment in fact fitted excellently [7]. I was myself studying the physical resistance to transport of globular particles through hyaluronan networks by measuring diffusion and sedimentation and found evidence that the networks acted as filters

Fig. 2. Photographs of some of the workers mentioned in the text, taken approximately at the time when gel filtration was developed. (Left to right) Top row, Arne Tiselius, who suggested the name "gel filtration", Björn Ingelman, who synthesized the first dextran gels, and Alexander G. Ogston, who derived mathematically the excluded fraction for spherical particles in a system of randomly distributed fibres; middle row, Jerker Porath and Per Flodin, wo described the technique of gel filtration; bottom row, Johan Killander, after having received his doctorate, and the author, who collaborated on the theory. (The photographs were kindly supplied by Drs. B. Ingelman, J. Killander and A. G. Ogsten, Mrs. I. Johansson and Mr. B. Ejdesjö.)

or sieves [8]. Thus, within the connective tissue field, steric interaction between proteins and linear polysaccharide chains was well established.

DEXTRAN —A POLYSACCHARIDE USED AS A PLASMA SUBSTITUTE [9]

At the beginning of this century, Uppsala was a centre of protein research. The Svedberg, Professor of Physical Chemistry, had built the ultracentrifuge and shown that proteins are molecules of defined molecular masses. His pupil, Arne Tiselius, developed electrophoresis into an important tool for separating and characterizing proteins. In 1938 a Chair of Biochemistry was created for Tiselius, and he surrounded himself with a number of enthusiastic collaborators from Sweden and elsewhere.

Two young scientists, Björn Ingelman and Anders Grönwall, worked in the same room of the crowded department. Ingelman, studying for his Ph. D., was asked in 1940 to enter a project, supported by the Swedish Sugar Industry, on the macromolecular composition of sugar beet extracts in the hope that one would find proteins, pectin or other products, which could be of use in food production. In one extract he was able to identify by ultracentrifugation a new component which turned out to be a bacterial polysaccharide produced by *Leuconostoc mesenteroides* infecting some of the batches. It was soon identified as a polyglucose, already known as dextran, of very high molecular mass. Anders Grönwall had a medical degree from the University of Lund. He had studied the electrophoretic properties of proteins in Lund and protein solubility at the Carlsberg laboratories in Copenhagen when he became employed by Tiselius in 1942 to work on the chemical composition of tuberculin and its antibodies. Grönwall was thus well oriented in immune techniques.

In order to develop a simple analytical method for dextran, which could be used to identify infected beet extracts, Ingelman tried to raise antibodies against dextran in two rabbits but was unsuccessful. This was a key experiment. Owing to the scientific interaction between Ingelman and Grönwall it was soon realized that the non-immunogenic dextran probably could be a useful plasma substitute, for which there was a great need during World War II. Already in 1943 the two scientists entered into an agreement with the drug firm Pharmacia in Stockholm to develop the idea. Within a few years dextran became a pharmaceutical product. Pharmacia grew and moved to Uppsala in 1950, no doubt owing to the close ties with the Department of Biochemistry.

An immediate consequence of the success of dextran as a plasma substitute and the presence of Pharmacia in Uppsala was a boost in research in both the physical chemistry and the physiology of polymers. Björn Ingelman became Research Direc-

tor at Pharmacia. Dr. Kirsti Granath from the Physical Chemistry Department was recruited to Pharmacia as head of the Polymer Physical Chemical Unit and her well characterized dextran fractions were used in laboratories all over the world. Anders Grönwall was appointed Professor of Clinical Chemistry (and later Hospital Director) at the University Hospital in Uppsala, where research on haemodynamics and infusion therapy flourished.

GEL FILTRATION—A NEW TECHNIQUE TO SEPARATE MOLECULES ACCORDING TO SIZE

Arne Tiselius received the 1948 Nobel Prize in Chemistry for his work on electrophoresis and chromatography, and this increased the fame of biochemistry in Uppsala. At the beginning of the 1950s Per Flodin and Jerker Porath joined Tiselius's department and worked on the zone electrophoretic separation of proteins on a preparative scale on vertical columns of starch gels [10]. During these experiments they made unpublished observations that chromatography on starch columns could separate molecules according to size [11], but similar observations were also made by others [12,13]. There were, however, practical problems in using starch. A breakthrough for utilizing this observation in a new technique came when Flodin was employed at Pharmacia in 1954 and worked with Björn Ingelman.

Already in the 1940s Ingelman had cross-linked dextran with epichlorohydrin and obtained gels[a]. Flodin supplied Porath with a granulated dextran gel from Phamacia to be used as column material in zone electrophoresis. Porath soon observed that this dextran gel had properties that were superior to those of starch gels in separating molecules according to size. When a mixture of compounds of different molecular sizes was chromatographed on a gel column, the substances emerged according to size with the largest molecules first. A new product and a new technique had been born. The observations were published by Porath and Flodin in *Nature* in 1959 under the title "Gel filtration: a method for desalting and group separation" [14]. The name "gel filtration" was suggested by Arne Tiselius (see ref. 15).

Pharmacia soon manufactured dextran gels of various porosities that could be used for the separation of molecules in different size intervals. They were named Sephadex G-10, G-15, G-50, G-75, G-100, G-150 and G-200, where the number stands for the water regain, *e.g.*, Sephadex G-10 takes up 1 g of water and Sephadex G-200 20 g of water per gram of dextran. Soon other types of gels, such as agar (agarose) [16,17] and polyacrylamide [18] gels, were also employed. The impact that gel filtration had on the development of biochemistry is well known.

Jerker Porath succeeded Arne Tiselius as Professor of Biochemistry in Uppsala in 1968. He is now retired but continues to be a major contributor to biochemical separation techniques, *e.g.*, immobilized metal ion affinity chromatography. Per Flodin worked in industry for a while before he was appointed to a Chair in Polymer Technology in Gothenburg.

IMPACT OF PROTEIN CHEMISTRY ON CLINICAL SCIENCE

It was as late as 1926 that Svedberg and Fåhreus by means of ultracentrifugation described for the first time a protein, haemoglobin, as a homogeneous molecular entity with a defined molecular mass. In the following decades research efforts in many laboratories were aimed at finding techniques to isolate and purify individual proteins and Svedberg's and Tiselius's laboratories made major contributions. The greatest impact of this work was seen in medicine where pure proteins were required for diagnosis and treatment. Purification of plasma proteins, including immunoglobulins, enzymes, peptide hormones and toxins, was carried out in laboratories all over Uppsala.

Johan Killander was engaged in graduate work at Anders Grönwall's Department of Clinical Chemistry at the beginning of the 1960s when the new technique of gel filtration emerged. He was especially concentrating his work on the purification of immunoglobulins. Already in 1962 there was an important paper by Flodin and Killander in which

[a] The former Executive Director of Pharmacia, Elis Göth, told me that Björn Ingelman once came into his office to show him a jelly on a dish. Göth asked what it was and Ingelman replied that it was cross-linked dextran. Göth asked what Ingelman was going to use it for and Ingelman answered, "No idea"!

they demonstrated that plasma proteins can be separated into three major peaks by chromatography on Sephadex G-200 [19]. Subsequently, Killander refined his purifications by combining gel filtration with other techniques such as zone electrophoresis and ion-exchange chromatography and he reported a considerable purification of several plasma proteins.

A THEORY OF GEL FILTRATION

Thus, at the beginning of the 1960s in Uppsala, a powerful new product and technique had emerged in the Biochemistry Department, Pharmacia had great interests in polymer chemistry and manufactured the Sephadex products, scientists in many fields were interested in polymer–protein interactions and their biological implications and the scientific community was rather small and most people involved knew each other personally. It was natural that somebody in this community would search for a theory that described the gel filtration process quantitatively.

One obstacle was psychological. The name "gel filtration" implies that the molecules are passing a filter, *i.e.*, that large molecules should be retarded and small ones should pass through the pores of the filter. Similarly, the term molecular sieving [18], which also was used for this chromatographic process, implies that the molecules pass a sieve. At the time many persons really believed that it was a filtration effect, even if it then was a paradox that large molecules emerged first. Steere and Ackers [20,21] explained the phenomenon in terms of restricted diffusion of large molecules in gel pores.

That the phenomenon was due to an exclusion of molecules from the gel grains, however, was apparent to many investigators [13,22–24]. This was also reflected in alternative names given to the technique such as exclusion chromatography [23] and gel permeation chromatography [24]. Personally, I have in later papers used the term gel chromatography to avoid any misinterpretations [25]. However, in 1964 there was a lack of a simple steric model to quantitatively account for the results in terms of exclusion, although various attempts had been or were being made [22,26].

Being well acquainted with Ogston's [5] calculations of steric exclusion of spheres in a system of randomly distributed rods, it was obvious that we should try to use this model to explain the exclusion from gel grains in the gel chromatographic process. Fig. 3 depicts the principle of the model. As Johan Killander had accumulated a large amount of data on the chromatographic behaviour of proteins on dextran gels, we engaged in a collaboration. He had observed that some proteins, *e.g.*, serum albumin and hemoglobin, with similar molecular mass, separated well by gel filtration and he was looking for a theoretical explanation.

Our modified version of Ogston's eqn. 5 was

$$K_{av} = \exp[-\pi L(r_s + r_r)^2]$$

where K_{av}[a] is the fraction of the total gel volume which is available to a spherical protein, L is the total concentration of fibre in the gel expressed in cm fibre per cm^3 gel, r_s is the radius of the protein (the Stokes radius) and r_r is the radius of the fibres. K_{av} can be calculated from the chromatogram by the simple relationship

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e, V_0 and V_t are the elution volume of the

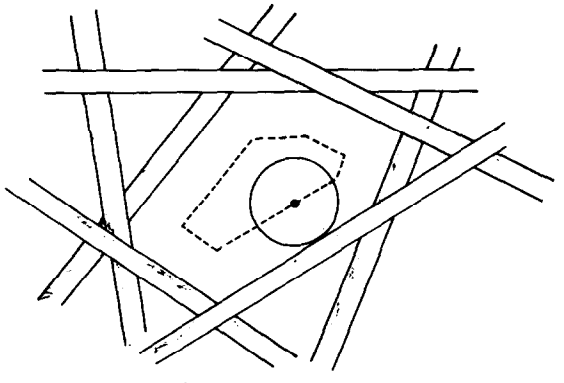

Fig. 3. Demonstration of steric exclusion of a spherical object from a random network of rigid fibres. The centre of the sphere can only move within a volume (available volume; dashed area) which is at a distance from the fibres corresponding to the radius of the sphere. The excluded volume for the centre of the sphere is thus much larger than the volume of the fibres themselves and increases with increasing size of the sphere.

[a] In the designation K_{av} for the partition coefficient, "av" stands for *available*. I regret that we used this designation since it has been a common misunderstanding that "av" stands for *average*.

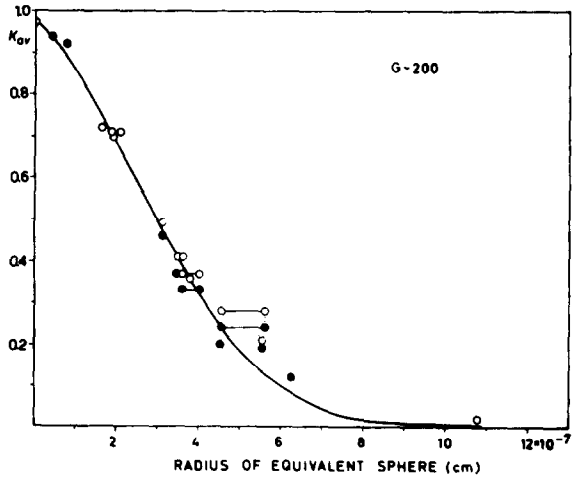

Fig. 4. The available fraction (K_{av}) for a number of proteins chromatographed on Sephadex G-200 plotted as a function of the Stokes radius. The line is the function predicted by Ogston's equation assuming a value of L of $1.6 \cdot 10^{12}$. (Taken from *J. Chromatogr.*, 14 (1964) 317.)

compound and the void volume and total volume of the column, respectively.

Using our own and literature data, we calculated K_{av} for a number of proteins and carbohydrate

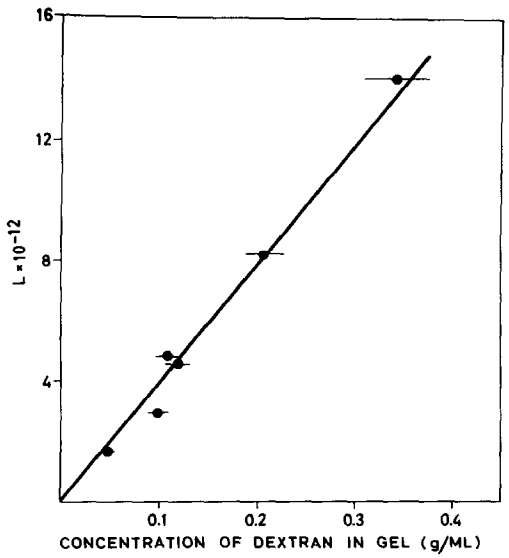

Fig. 5. Experimentally obtained values of L for various Sephadex gels plotted *versus* the dextran concentrations of the gels. As predicted by theory there is a linear relationship. (Taken from *J. Chromatogr.*, 14 (1964) 317.)

fractions chromatographed on Sephadex G-25, G-50, G-75, G-100 and G-200. We chose compounds for which we also could obtain the diffusion coefficients. The diffusion data were used to calculate r_s, the Stokes radius, *i.e.*, the radius of a sphere that has the same diffusion coefficient as the compound. The parameter r_f, the radius of the fibre, had to be estimated but the value was not very critical for the calculations. The dextran chain is coiled and branched and it is therefore shorter and thicker than a stretched linear polysaccharide. We assumed r_f to be 0.7 nm. This value and the calculated L values for the various gels (see below) gave a total volume of dextran per ml that corresponded to the known concentrations of dextran in the various Sephadexes.

When K_{av} was plotted against r_s (an example is shown in Fig. 4), the data followed an S-shaped curve similar to what Ogston's equation predicted. A theoretical curve could be adjusted to the experimental points by choosing the right L value. When the L values obtained for the different gels were plotted against their known concentrations, the relationship was linear, as expected (Fig. 5). For example, in a gel of concentration 0.1 g/ml, L was found to be $4 \cdot 10^{12}$ cm/cm^3. Using the r_f value of $7 \cdot 10^{-8}$ cm one can calculate that the volume occupied by the dextran would be 0.063 ml/ml. As the partial specific volume of dextran is 0.61 g/ml this fits with the concentration 0.1 g/ml. The L value found was 4–5 times shorter than the length of a single stiff linear polysaccharide chain, and this was ascribed to branching and coiling of the dextran molecule.

The success in describing the gel chromatographic data as exclusion of spheres from a simple three-dimensional meshwork of stiff fibres gave a rationale for using chromatography on gels to determine the hydrodynamic size of various compounds, *e.g.*, proteins. The method has subsequently been used for this purpose by numerous workers. It can also be used in the opposite way, however, *i.e.*, to characterize the structure of the gel. When I cross-linked hyaluronan and chromatographed proteins on the gels they had the exclusion properties expected for an unbranched stiff linear polysaccharide network [7]. Results from chromatography on agarose gels corresponded to gel structures that were much more compact [27]. Polyacrylamide gels [28] and elastin networks [29] were also characterized in this way.

To follow up the gel chromatographic studies, my

younger brother and I, with the help of our father, our elder brother and our brother-in-law, built an electrical analogue computer to describe the process [30]. With this machine we could predict the shape of the chromatogram as a function of column length, flow-rate, sample volume and partition coefficient of the compound. This work gave me as much enjoyment as the paper on the theory, but I doubt that it has ever been cited by anyone.

An important observation, which strengthened our conclusion that the gel chromatographic process is based on exclusion from a polysaccharide network and not from structures formed by the cross-linking, came from independent studies with dextran and hyaluronan in solution [7,31,32]. When the exclusion properties of the polysaccharides in solution were determined by independent methods, such as equilibrium dialysis, osmometry and solubility studies, results identical with the chromatographic data were obtained.

Since our paper appeared three decades ago, gel chromatographic techniques have been highly refined, the analytical results have become much more accurate and numerous other papers on the theoretical basis of gel chromatography have been published. By today's standards our approach may look simple, but the basic idea of steric exclusion from the gel matrix at least at slow flow-rates seems still to be valid.

Neither Johan Killander nor I continued to work on chromatographic techniques. Johan Killander defended his thesis "Separation of immunoglobulins and some other plasma proteins by gel filtration" on April 21st, 1964 [33]. Our joint paper was included as part of the thesis. He later became Head Doctor in Clinical Chemistry at one of the major hospitals in Stockholm. I myself continued to work on physiological aspects of connective tissue and later on the metabolism of hyaluronan and I am still working in the same department in Uppsala.

HYPOTHESES ON THE REASON WHY THE THEORY MADE AN IMPACT

Why did our paper become a citation classic? I have not looked up the citations so I really do not know why it was cited. My suggestions are therefore purely hypothetical.

First, the practical concept of separating molecules according to size, which was presented in the paper by Porath and Flodin [14], was of immediate and tremendous importance to biological sciences. There was a need for understanding the process. The term "gel filtration" had created confusion among the users. The presentation of a simple steric model, which could visually and quantitatively explain the phenomenon in terms of exclusion, made the process understandable and could with advantage be used in teaching.

Second, there was a need for simple techniques to determine the molecular size of proteins and the theory gave a rationale for using gel chromatography. It complemented gel electrophoretic techniques, which were developed at about the same time.

Third, the paper promoted the "concept of exclusion", which is important not only for chromatographic techniques but also in understanding physiological and biochemical reactions.

Very often so-called "citation classics" describe the details of a useful technique (the Lowry paper on protein determinations in 1951 had been cited 205 000 times up to 1990). When *Current Contents* identified our paper as among the most cited of those published in the 1960s, its Editor drew the conclusion that the publication was a technique paper. It should be clear from the above description that this is not so, but I must admit that I am slightly worried that the scientific community may have made the same misinterpretation as the Editor of *Current Contents*. The technique was developed by Porath, Flodin and Ingelman. Further, A. G. Ogston must be credited for the concept of exclusion from a three-dimensional network of rods.

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