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The History of Hyaluronic Acid Discovery, Foundational Research and Initial Use

1.1 Discovery

In 1934, Karl Meyer and John Palmer wrote in the *Journal of Biological Chemistry* about an unusual polysaccharide with an extremely high molecular weight isolated from the vitreous of bovine eyes [1]. Being the first to mention it, they gave the new substance the name *hyaluronic acid* (HA, the modern name 'hyaluronan') derived from 'hyaloid' (glassy glass-like in appearance) and 'uronic acid'. While Meyer and Palmer are generally considered to have discovered hyaluronic acid, it is fair to mention that as far back as 1918 Levene and Lopez-Suarez had isolated a new polysaccharide from the vitreous body and cord blood that they called 'mucoitin-sulfuric acid' [2]. It consisted of glucosamine, glucuronic acid and a small amount of sulfate ions. It is now clear this substance was actually hyaluronic acid extracted together with a mixture of sulfated glycosaminoglycans.

At the time of the discovery of hyaluronan, the polysaccharides, which represent the major part of the organic material on our planet, were already quite well known. A number of so-called mucopolysaccharides, currently known as glycosaminoglycans, had already been discovered. Hyaluronic acid is known to belong to this class as well. Mucopolysaccharides were isolated from mucus, to which they give viscous lubricating properties. These properties, in turn, are related to glycosaminoglycan's ability to bind to a significant amount of water.

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1.2 Foundational Research

Soon after the original work was published, unique properties of the new biopolymer were discovered, which proved it different from other similar glycosaminoglycans, According to Meyer and Palmer, the isolated polysaccharide contained uronic acids and amino sugars, as well as pentose, and was not sulfated [1]. They also decided that the molecular mass of the repeatable unit is approximately 450 Da. It was later proved that HA in fact does not contain sulfate groups or pentose. It was also established that the molecular mass of the repeatable disaccharide residue is 397 Da.

Over the next 10 years, Meyer and other authors isolated hyaluronan from various animal organs. For example, the polysaccharide was found in joint fluid, the umbilical cord and recently it has become possible to extract HA from almost all vertebrate tissues. In 1937, F. Kendall isolated hyaluronan from the capsules of *streptococci* groups A and C. This work had great scientific and practical importance, as today *streptococci* groups are the most economical and reliable source for the industrial production of hyaluronic acid [3].

In 1928, F. Duran-Reynals found a certain biologically active compound in rabbit testicles that lead to an extremely important discovery in the chemistry and biology of hyaluronic acid. When the compound was injected with black indian ink subcutaneously, the authors observed extremely fast distribution of the black colour through connective tissue [4]. Similar properties were found for the extracts from semen, leeches, bee sting and snake venom. Further studies confirmed that the observed increasing permeability of connective tissue was mainly caused by the depolymerization of its basic substance, hyaluronic acid. It was thus determined the extract contains a specific enzyme that was given the name ‘hyaluronidase’. The biological material that contains hyaluronidase was recently called the Duran-Reynals *spreading factor.*

The discovery of enzymes that could selectively break down hyaluronan opened the door for the establishment of the polysaccharide molecule’s chemical structure. In those days, a powerful tool for analysing the structure of polysaccharides such as nuclear magnetic resonance spectroscopy NMR was not known. At the present time, NMR makes it possible to determine the monosaccharide biopolymer residue’s composition, centres for substitution reactions, sequence and three-dimensional structure.

In 1943 E.A. Balazs and L. Piller published a paper in which they described a study of role of hyaluronan in dog knee joints. They found that the intercellular substance of connective tissue of the synovium contains sufficient viscous mucin that can replace the mucin removed from the knee [5]. These observations literally opened the door to further studies on the role of hyaluronan in normal and traumatic joints. In 1949, C. Ragan and K. Mayer published a very important paper in which they described the observation of hyaluronan in rheumatoid synovial fluid. This was the first study in which normal and pathological synovial fluids were compared by determination of the concentration and viscosity of hyaluronan [6].

In the short period between 1948 and 1951, several chemists initiated research to elucidate the structure of hyaluronic acid. In 1948 A. Dorfman published the first results of a kinetics of fermentative hydrolysis of hyaluronan [7]. Three years later in 1951, A.G. Ogston and J.E. Stanier published the first significant data about the structure of the HA macromolecule in aqueous solution. They found that the relationship between viscosity

and velocity gradients increased with higher concentrations of the polysaccharide. [8]. It was found that this phenomenon is due to the interlacement of the neighbouring molecules, not individual macromolecule asymmetry. In 1955 an irregular helical configuration of hyaluronan was confirmed by measuring light scattering [9].

Several major research directions on hyaluronic acid were identified in the first half of the twentieth century. Lately, they have developed into independent branches within different fields of science including polymer chemistry, radiochemistry, biochemistry, molecular biology, medicine and glycobiology. The latter term was accepted in 1988 to describe a branch of science that combines a traditional biochemistry of hydrocarbons with a modern understanding of the role of complex sugars in cell and molecular biology.

Causing particular curiosity and scientific wonderment for researchers was the different observed viscosities of the hyaluronan solutions in presence of the different inorganic salts. The largest viscosity was observed for the solution in distilled water. It was proposed that the viscosity could be related to pH values and solution ionic strength. This phenomenon has become common knowledge but was initially described by R. Fuoss only for solutions of the synthetic polyelectrolytes [10].

Fundamental research on the physico-chemical properties of HA is considered to have begun in 1951 with the publication of E.A. Balazs's article [11]. One of the first attempts to sterilize HA by UV light led to a complete loss of the solution viscosity. A similar result was obtained by A. Caputo in 1957 by X-ray exposure of the hyaluronan solution [12]. Later, it was found that when exposed to gamma radiation or electron beams, even at low initial levels of absorbed dose of ionizing radiation, HA degrades completely. The processes of polysaccharide radiolysis, which are associated with polymer degradation and involve free radicals, are now intensively studied in the radiochemistry of biomolecules.

Unlike sulfated polysaccharides, some of the initial proof of HA's ability to interact with living cells came with the observation that hyaluronan accelerates cell growth. It has also been observed that hyaluronan initiates some cell aggregation. This was the first indication of a unique binding of the polysaccharide to the cell surface. Currently, several receptor proteins that bind to the surface of the HA cytoplasmic membrane have been isolated, including high-affinity receptor CD44 and receptor RHAMM (receptor for hyaluronan-mediated motility).

The receptor for HA endocytosis had been found on the membrane of endothelial cells of the liver sinuses and fundamentally differs from other hyaluronan-binding proteins (see [13] and references therein).

These early studies accomplished much in a short period of time, notably the establishment of the structure and monomeric composition of the macromolecule. In 1954, Meyer published an article in *Nature* that presented the result of a study on the decomposition products of HA [14]. The article included the structural formula of the disaccharide, which is the product of HA cleavage by streptococcus hyaluronidase (Figure 1.1).

1.3 Initial Medical Applications

During the second half of the twentieth century, HA was discovered in different tissues and liquids of vertebrae animals as well as humans. It was also found to have clinical applications, mostly for eye surgery, treatment of joint diseases and aesthetic medicine. The first actual use

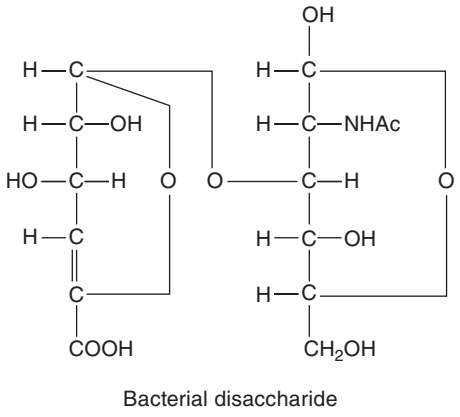

Figure 1.1 *Structure of 4,5-unsaturated disaccharide, obtained by HA cleavage by bacterial hyaluronidase*

of HA in medicinal practise didn't actually occur until 1943 during the Second World War. N.F. Gamaleya (Н.Ф. Гамалея) created complex bandages in order to treat the frostbitten soldiers in the military field hospital no. 1321. The main component of the bandage was an extract from the umbilical cord, which he called a 'factor of regeneration'. The method was later approved by the USSR Ministry of Health and the drug received the name 'Regenerator'. It is apparent that HA was a major contributor towards the positive effect of the treatment, given that the human umbilical cord contains a significant amount of HA. In fact, at this time the umbilical cord was considered to be one of the most important industrial sources of HA alongside other biological materials.

Several practical ventures that explored HA's medical applications followed. In the 1950s, E.A. Balazs initiated experiments with HA to investigate its potential as a prosthesis for the treatment of retinal detachment. In 1969 it was reported that HA was used in order to prevent postoperative soldering. In 1970, hyaluronan was first injected into the joints of racehorses that suffered from arthritis with a clear and positive outcome observed. A few years later, R. Miller started to use HA in implanted intraocular lenses [15]. Since these ground breaking cases, hyaluronan has become one of the most important components in ophthalmology (see the review about hyaluronan application in ophthalmology and the references therein [16]) and has found extremely wide application in aesthetic medicine. Today, HA-contained products are the 'gold standard' for injectable cosmetics.

1.4 Sources of Hyaluronan

Due to its increasing applications, the demand for hyaluronan has grown from the moment of its discovery up until now. As the aforementioned branches of medicine and cosmetology widen, the role of HA is being reconsidered from the passive structural matrix of connective tissue to the understanding of the primary role of that macromolecule in many important physiological processes. These processes include cell communication, migration, differentiation, process regulation in the extracellular matrix and activation of cell structure metabolism.

At the present time, it is known that HA is not an inactive macromolecule of connective tissue, but a metabolically highly active biopolymer. Its half-life in the joints is 1–30 weeks, up to 1–2 days in the epidermis and derma and only 2–5 minutes in the bloodstream. In other words, during one day, approximately 5 g of dry HA can be synthesized and cleaved in the body of an adult 70-kg man, one-third of the whole amount of HA in the body [17].

The HA polysaccharide chain undergoes degradation by endoglucanase (hyaluronidase) and exoglucanase (beta-glucouronidase and beta-N-acetyl hexosaminidase). The testicle hyaluronidases decompose polysaccharides with a hydrolysis of the glycoside bond to tetra-, octa- and other saccharides.

It was pointed out that HA was first discovered as an animal polysaccharide, but soon thereafter it was found that the biopolymer also exists among bacteria. In 1937, Kendall, Heidelberger and Dawson reported about extraction of a polysaccharide from the cultural liquid of the haemolytic streptococcus that was precipitated with acetic acid and ethanol [3]. The authors proposed that the isolated biopolymer is identical to hyaluronic acid, a hypothesis that was later confirmed. It was eventually found that the mammalian glycosaminoglycan exists among several groups of the streptococcus, many of which are pathogenic for humans and animals.

HA was initially produced by extraction from the animal material. Because of the growing demand of HA, however, scientists started looking for new methods for its production, methods that were preferably microbiological. A study of the bacterial synthesis of hyaluronan on the free cells level started when UDP-glucose, UDP-N-acetyl glucosamine and UDPP-glucuronic acid were obtained from streptococcus extracts.

In 1953 Roseman and coworkers published an article in which they describe the precipitation of HA from the cultural liquid (CL) of Group A streptococcus [18]. They reported the yield 200–300 mg from 41 of CL. Later, Warren and Gray found the semi-synthetic media for the cultivation of the HA producers [19].

A significant number of patents have been filed on the production and clinical application of HA. The production of HA by cultivation of the single strain *Streptococcus equi* was described in more than 20 patents from 1985 to 2002. Despite these efforts, however, the problem of HA supply failing to meet worldwide demand remains. The price for highly purified pharmaceutical grade HA has reduced dramatically during last few years, but the current minimum price is still at the level of $10 000/kg. To compare, the price of the polysaccharide xanthan produced from *Xanthomonas campestris* is $11/kg. The enzymes responsible for the metabolism of hyaluronan have been studied since 1959, when the hyaluronate synthase (HAS) from *Streptococcus pyogenes* was first described [20]. Many researchers tried to identify, solubilize and isolate a pure active enzyme that would be able to synthesize HA both in streptococcus and in eukaryotic cells or to clone HAS gene in *E. coli.* Unfortunately, after several decades of persistence, their efforts were not successful.

In 1993 a group of US scientists reported about isolation of HAS, its operon and cloning of hyaluronate synthase gene into *E. coli* [21]. Their findings were recently found to be scientific error. Van de Rijn and Drake isolated three streptococcus membrane proteins with molecular masses of 42, 33 and 27 kDa and proposed that the protein with mass 33 kDa is indeed hyaluronate synthase [22]. Using electrophoresis, other studies have found that streptococcus hyaluronate synthase has a molecular mass 42 kDa, proving this conclusion incorrect.

Soon thereafter, a breakthrough in a study of the mechanism of HA synthesis and regulation occurred. Almost simultaneously, DeAngelis and co-workers reported that the operon of the

HA synthesis was found, isolated, characterized and cloned [23]. It was the first successfully cloned hyaluronate synthase whose expression was confirmed by synthesis of HA in a microorganism that did not synthesize such a polysaccharide before.

1.5 Current Medical Study and Use

At the present time hyaluronan is an object of study in biochemistry, molecular biophysics, bioorganic and radiochemistry and the chemistry of polymer compounds. Medical studies of HA include its role in fertilization, embryogenesis, development of the immune response, the healing of wounds, oncological and infectious diseases, processes of ageing and the problems of aesthetic medicine.

The wide range of practical medical applications of hyaluronan continues to be based upon its anti-inflammatory, disinfectant and wound healing effects. HA promotes epithelial regeneration; prevents the formation of granulation tissue, adhesions and scars; reduces swelling and itching; normalizes blood circulation; promotes scarring of venous ulcers and protects internal eye tissue [24].

Hyaluronan is widely used in applied biochemistry and enzymology as a substrate for the quantitative determination of the enzyme hyaluronidase. Scientific disputes about the possible relationship between HA and hyaluronate lyase and the pathogenicity of some streptococci are to be, as it seems, permanently carried out. Currently, much attention is paid to the study of the secondary and tertiary structures and dynamic conformation of HA in aqueous solutions and biological fluids; the HA interaction with proteins, particularly receptor CD44 and other hyaladherins; and the HA biocatalytic cleavage with different hyaluronidase; the progression toward creating of recombinant strains and chimera products with the desired properties.

The theoretical research on polysaccharide biosynthesis with both bacterial and mammalian enzymes initially focused on gaining an understanding of the reaction mechanisms, but recently attention has been shifted toward solving the application problems, including the creation of recombinant strains and chimera organisms that could produce HA with the initially required properties.

In the last few years a very promising area of medicine and pharmacology has gained significant attention: the targeted delivery of drugs to specific organs or tissues. The development of nanotechnology has brought these studies to a new level because ‘nanocontainers’ have great potential to target delivery biologically active compounds to specific cells in the body. Liposomes, polymeric and lipid particles with a diameter of 100–300 nm, or nanocapsules of the same size, are typically used as nanocontainers. Hyaluronan could be used as an alternative approach in the creation of a macromolecular container. HA macromolecules could bind to the receptors on cytoplasmic membranes of various cells to target deliver biologically active compounds attached to the biopolymer carrier.

Using the technology of solid-state modification of polysaccharides that included the mutual action of super-high pressure and shear deformation, a group of Russian scientists have produced a number of unique products that contain hyaluronic acid. The new products could be considered drug-free (or non-drug) macromolecular therapeutics. These areas of research have made possible a broad range of new products based on the modified hyaluronan and could be used in the various fields of medicine.

1.6 Impact and Future Directions

Seventy-five years have passed since hyaluronan was discovered. In the world of scientific research, at this point past a substance's discovery, research is usually limited to a fairly narrow and specialized group of academics. However, the interest in this compound is far from decreasing; it is actually growing. Publications dedicated to the structure, synthesis, degradation, the biological role of HA and its application in various fields of chemistry, medicine and biology are on the constant rise. Between 1966 and 1975, 790 articles had been published; between 1976 and 1985 the number reached 2200, between 1986 and 1996 – 3300, between 1996 and 2006 – more than 7000. Two fundamental monographs [25,26] and a review [27] have also been published recently.

It is important to mention the most recent fundamental monograph – the series of five volumes under the title *Hyaluronan: From Basic Science to Clinical Application* by Balazs et al. [28]. The monograph covers many aspects of hyaluronan science and application, but what makes this book special is the comprehensive early history overview, followed by the chapters that describe contributions of the most outstanding scientists to hyaluronan chemistry and biology.

HA patents are also on the rise: between 1979 and 1987 USA patent bureau issued five patents regarding HA per year. In 1988 the amount reached a number 35. The same level was till 1995. In 1996 the sharp increase of the patents related to HA was observed, and the amount of the new patents is still increasing every year. As of February 2013, the number of patents involving the keywords 'hylauronic acid' total almost 80 000.

In the beginning of the 1980s it was obvious that hyaluronic acid attracted attention of scientists in chemistry, biology, physics, medicine, and so on from all over the world. It became necessary to gather in order to discuss the latest results and perspectives. The first international meeting dedicated to HA took place in 1985 in Saint-Tropez (France). After that the conferences were held in London (UK) (1988), Stockholm (Sweden) (1996), Padua (Italy) (1999), Wales (UK) (2000), Cleveland (USA) (2003), Charleston (USA) (2007) and Kyoto, Japan (2010). In October 2004 The International Society for Hyaluronan Sciences (ISHAS, www.ishas.org) was created. Initially, it brought together scientists from 16 countries.

In June 2013 Oklahoma City (USA) was the host of the latest International Conference of Hyaluronic Acid. It brought together more than 200 scientists from 22 countries. Since then similar conferences have been carried out every two years, the next one will be in Florence (Italy) in 2015.

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