Aquaporin Water Channels

All living matter is made up of cells. A single human being has as many as the stars in a galaxy, about one hundred thousand million. The various cells - e.g. muscle cells, kidney cells and nerve cells - act together in an intricate system in each one of us. Through pioneering discoveries concerning the water and ion channels of cells, this year’s Nobel Laureates Peter Agre and Roderick MacKinnon, have contributed to fundamental chemical knowledge on how cells function. They have opened our eyes to a fantastic family of molecular machines: channels, gates and valves all of which are needed for the cell to function.

**Molecular channels through the cell wall**

To maintain even pressure in the cells it is important that water can pass through the cell wall. This has been known for a long time. The appearance and function of these pores, remained for a long time as one of the classical unsolved problems of biochemistry. It was not until around 1990 that Peter Agre discovered the first water channel. Like so much else in the living cell, it was all about a protein.

Water molecules are not the only entities that pass into and out of the cell. For thousands of millions of cells to be able to function as something other than one large lump, coordination is required. Thus communication between the cells is necessary. The signals sent in and between cells consist of ions or small molecules. These start cascades of chemical reactions that cause our muscles to tense, our eyes to water - indeed, that control all our bodily functions. The signals in our brains also involve such chemical reactions. When we stub a toe this starts a signal moving up towards the brain. Along a chain of nerve cells, through interaction...
between chemical signals and ion currents, information is conveyed from cell to cell like a baton in a relay race.

It was in 1998 that Roderick MacKinnon succeeded for the first time in showing what ion channels look like at atomic level - an achievement which, together with Agre’s discovery of water channels, opened up entirely new research areas in biochemistry and biology.

The medical consequences of Agre’s and MacKinnon’s discoveries are also important. A number of diseases can be attributed to poor functioning in the water and ion channels of the human body. With the help of fundamental knowledge of what they look like and how they work, there are now new possibilities for developing new and more effective pharmaceuticals.

Fig 1. The dividing wall between the cell and the outside world - including other cells - is far from being an impervious shell. On the contrary, it is perforated by various channels. Many of these are specially adapted to one specific ion or molecule and do not permit any other type to pass. Here to the left we see a water channel and to the right an ion channel.

**Water channels**

The hunt for the water channels
As early as the middle of the nineteenth century it was understood that there must be openings in the cell membrane to permit a flow of water and salts. In the
middle of the 1950s it was discovered that water can be rapidly transported into and out of cells through pores that admit water molecules only. During the next 30 years this was studied in detail and the conclusion was that there must be some type of selective filter that prevents ions from passing through the membrane while water molecules, which are uncharged, flow freely. Thousands of millions of water molecules per second pass through one single channel!

Although this was known, it was not until 1992 that anybody was able to identify what this molecular machinery really looked like; that is, to identify what protein or proteins formed the actual channel. In the mid-1980s Peter Agre studied various membrane proteins from the red blood cells. He also found one of these in the kidney. Having determined both its peptide sequence and the corresponding DNA sequence, he realized that this must be the protein that so many had sought before him: the cellular water channel.

Agre tested his hypothesis in a simple experiment (fig. 2) where he compared cells which contained the protein in question with cells which did not have it. When the cells were placed in a water solution, those that had the protein in their membranes absorbed water by osmosis and swelled up while those that lacked the protein were not affected at all. Agre also ran trials with artificial cells, termed liposomes, which are a type of soap bubble surrounded on the outside and the inside by water. He found that the liposomes became permeable to water if the protein was planted in their membranes.

What is osmosis?
The liquid pressure in plant and animal cells is maintained through osmosis. In osmosis, small molecules (such as water) pass through a semi-permeable membrane. If the membrane does not admit macromolecules or salts that are in higher concentrations on one side of the membrane, the small molecules (water) will cross to this side, attempting to “dilute” the substance that cannot pass through the membrane. The osmotic pressure thus arising is the reason why cells are often swollen and stiff, in a flower stalk, for example.
Fig 2. Peter Agre's experiment with cells containing or lacking aquaporin. The aquaporin is necessary for making the cell absorb water and swell.

Peter Agre also knew that mercury ions prevent cells from taking up and releasing water, and he showed that water transport through his new protein was prevented in the same way by mercury. This made him even more sure of that he had discovered what was actually the water channel. Agre named the protein aquaporin, "water pore".

How does the water channel work? A question of form and function
In 2000, together with other research teams, Agre reported the first high-resolution images of the three-dimensional structure of the aquaporin. With these data, it was possible to map in detail how a water channel functions. How is it that it only admits water molecules and not other molecules or ions? The membrane is, for instance, not allowed to leak protons. This is crucial because the difference in proton concentration between the inside and the outside of the cell is the basis of the cellular energy-storage system.

Selectivity is a central property of the channel. Water molecules worm their way through the narrow channel by orienting themselves in the local electrical field formed by the atoms of the channel wall. Protons (or rather oxonium ions, H₃O⁺) are stopped on the way and rejected because of their positive charges.
Animations »

Fig 3. Passage of water molecules through the aquaporin AQP1. Because of the positive charge at the center of the channel, positively charged ions such as $\text{H}_3\text{O}^+$, are deflected. This prevents proton leakage through the channel.

The medical significance of the water channels

During the past ten years, water channels have developed into a highly topical research field. The aquaporins have proved to be a large protein family. They exist in bacteria, plants and animals. In the human body alone, at least eleven different variants have been found.

The function of these proteins has now been mapped in bacteria and in plants and animals, with focus on their physiological role. In humans, the water channels play an important role in, among other organs, the kidneys.

The kidney is an ingenious apparatus for removing substances the body wishes to dispose of. In its windings (termed glomeruli), which function as a sieve, water, ions and other small molecules leave the blood as ‘primary’ urine. Over 24 hours, about 170 liters of primary urine is produced. Most of this is reabsorbed with a series of cunning mechanisms so that finally about one liter of urine a day leaves the body.
From the glomeruli, primary urine is passed on through a winding tube where about 70% of the water is reabsorbed to the blood by the aquaporin AQP1. At the end of the tube, another 10% of water is reabsorbed with a similar aquaporin, AQP2. Apart from this, sodium, potassium and chloride ions are also reabsorbed into the blood. Antidiuretic hormone (vasopressin) stimulates the transport of AQP2 to cell membranes in the tube walls and hence increases the water resorption from the urine. People with a deficiency of this hormone might be affected by the disease diabetes insipidus with a daily urine output of 10-15 liters.

**Ion channels**

The cells signal with salt!
The first physical chemist, the German Wilhelm Ostwald (Nobel Prize in Chemistry 1909) proposed in 1890 that the electrical signals measured in living tissue could be caused by ions moving in and out through cell membranes. This electro-chemical idea rapidly achieved acceptance. The notion of the existence of some type of narrow ion channel arose in the 1920s. The two British scientists Alan Hodgkin and Andrew Huxley made a major breakthrough at the beginning of the 1950s and for this were awarded the Nobel Prize in Physiology or Medicine in 1963. They showed how ion transport through nerve cell membranes produces a signal that is conveyed from nerve cell to nerve cell like a relay race baton. It is primarily sodium and potassium ions, $\text{Na}^+$ and $\text{K}^+$, that are active in these reactions.

Thus as much as fifty years ago there was well-developed knowledge of the central functions of the ion channels. They had to be able to admit one ion type selectively, but not another. Likewise, it had to be possible for the channels to open and shut and sometimes to conduct ions in one direction only. But how this molecular machinery really worked was long to remain a mystery.

**Ion-selective channel**

During the 1970s it was shown that the ion channels were able to admit only certain ions because they were equipped with some kind of “ion filter”. Of particular interest was the finding of channels that admit potassium ions but not sodium ions - even though the sodium ion is smaller than the potassium ion. It was suspected that the oxygen atoms in the protein played an important role as “substitutes” for the water molecules with which the potassium ion surrounds itself in the water solution and from which it must free itself during entry to the channel.
But further progress with this hypothesis was difficult – what was now needed was simply high-resolution pictures of the type only X-ray crystallography can provide. The problem was that it is extremely difficult to determine the structure of membrane proteins with this method, and the ion channels were no exception. Membrane proteins from plants and animals are more complicated and difficult to work with than those from bacteria. Using bacterial channel proteins that resemble human ion channels as closely as possible might perhaps offer a way forwards.

Many researchers tried in vain. The breakthrough came from an unexpected direction. Roderick MacKinnon, after studying biochemistry, turned to medicine and qualified as medical doctor. After working as a physician for some years, he grew so interested in ion channels that he started to do research in the field: "My scientific career in effect began at the age of 30", he has admitted. But his career took off quickly. Realizing that better and higher-resolution structures were needed for understanding how ion channels function, he decided to learn the fundamentals of X-ray crystallography. It was then only a few years before he astonished the whole research community by presenting a structure of an ion channel. This was in April 1998.

First ion channel mapped – atom by atom

In 1998, then, MacKinnon determined the first high-resolution structure of an ion channel, called KcsA, from the bacterium *Streptomyces lividans*. MacKinnon revealed for the first time how an ion channel functions at atomic level. The ion filter, which admits potassium ions and stops sodium ions, could now be studied in detail. Not only was it possible to unravel how the ions passed through the channel, they could also be seen in the crystal structure – surrounded by water molecules just before they enter the ion filter; right in the filter, and when they meet the water on the other side of the filter (fig. 4). MacKinnon could explain why potassium ions but not sodium ions are admitted through the filter: namely, because the distance between the potassium ion and the oxygen atoms in the filter is the same as that between the potassium ion and the oxygen atoms in the water molecules surrounding the potassium ion when it is outside the filter. Thus it can slide through the filter unopposed. However, the sodium ion, which is smaller than the potassium ion, can not pass through the channel. This is because it does not fit between the oxygen atoms in the filter and therefore remains in the water solution. The ability of the channel to strip the potassium ion of its water and allow it to pass at no cost in energy is a kind of selective catalyzed ion transport.
The cell must also be able to control the opening and closing of ion channels. MacKinnon has shown that this is achieved by a gate at the bottom of the channel which opened and closed a molecular “sensor”. This sensor is situated close to the gate. Certain sensors react to certain signals, e.g. an increase in the concentration of calcium ions, an electric voltage over the cell membrane or binding of a signal molecule of some kind. By connecting different sensors to ion channels, nature has created channels that respond to a large number of different signals.

Fig 4. The ion channel permits passage of potassium ions but not sodium ions. The oxygen atoms of the ion filter form an environment very similar to the water environment outside the filter. The cell may also control opening and closing of the channel.

High resolution image (jpeg 137 kB) »
OUTSIDE THE ION FILTER (A)
Outside the cell membrane the ions are bound to water molecules with certain distances to the oxygen atoms of the water.
INSIDE THE ION FILTER
(B)
For the potassium ions the distance to the oxygen atoms in the ion filter is the same as in water.

The sodium ions, which are smaller, do not fit in between the oxygen atoms in the filter. This prevents them from entering the channel.

Understanding diseases

Membrane channels are a precondition for all living matter. For this reason, increased understanding of their function constitutes an important basis for understanding many disease states. Dehydration of various types, and sensitivity to heat, are connected with the efficacy of the aquaporins. The European heat waves of recent years, for example, resulted in many deaths where the cause has sometimes been connected to problems in maintaining the body-fluid balance. In these processes the aquaporins are of crucial importance.
Disturbances in ion channel function can lead to serious diseases of the nervous system as well as the muscles, e.g. the heart. This makes the ion channels important drug targets for the pharmaceutical industry.

Illustrations: Typoform

Source:

The Laureates

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The Nobel Prize in Physiology or Medicine for 2005

jointly to

Barry J. Marshall and J. Robin Warren

for their discovery of

"the bacterium Helicobacter pylori and its role in gastritis and peptic ulcer disease"

Introduction

This year’s Nobel Laureates in Physiology or Medicine made the remarkable and unexpected discovery that inflammation in the stomach (gastritis) as well as ulceration of the stomach or duodenum (peptic ulcer disease) is the result of an infection of the stomach caused by the bacterium Helicobacter pylori.

Robin Warren (born 1937), a pathologist from Perth, Australia, observed small curved bacteria colonizing the lower part of the stomach (antrum) in about 50% of patients from which biopsies had been taken. He made the crucial observation that signs of inflammation were always present in the gastric mucosa close to where the bacteria were seen.

Barry Marshall (born 1951), a young clinical fellow, became interested in Warren’s findings and together they initiated a study of biopsies from 100 patients. After several attempts, Marshall succeeded in cultivating a hitherto unknown bacterial species (later denoted Helicobacter pylori) from several of these biopsies. Together they found that the organism was present in almost all patients with gastric inflammation, duodenal ulcer or gastric ulcer. Based on these results, they proposed that Helicobacter pylori is involved in the aetiology of these diseases.

Even though peptic ulcers could be healed by inhibiting gastric acid production, they frequently relapsed, since bacteria and chronic inflammation of the stomach remained. In treatment studies, Marshall and Warren as well as others showed that patients could be cured from their peptic ulcer disease only when the bacteria were eradicated from the stomach. Thanks to the pioneering discovery by Marshall and Warren, peptic ulcer disease is no longer a chronic, frequently disabling
condition, but a disease that can be cured by a short regimen of antibiotics and acid secretion inhibitors.

**Peptic ulcer – an infectious disease!**

This year’s Nobel Prize in Physiology or Medicine goes to Barry Marshall and Robin Warren, who with tenacity and a prepared mind challenged prevailing dogmas. By using technologies generally available (fibre endoscopy, silver staining of histological sections and culture techniques for microaerophilic bacteria), they made an irrefutable case that the bacterium *Helicobacter pylori* is causing disease. By culturing the bacteria they made them amenable to scientific study.

In 1982, when this bacterium was discovered by Marshall and Warren, stress and lifestyle were considered the major causes of peptic ulcer disease. It is now firmly established that *Helicobacter pylori* causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers. The link between *Helicobacter pylori* infection and subsequent gastritis and peptic ulcer disease has been established through studies of human volunteers, antibiotic treatment studies and epidemiological studies.

*Helicobacter pylori* causes life-long infection

*Helicobacter pylori* is a spiral-shaped Gram-negative bacterium that colonizes the stomach in about 50% of all humans. In countries with high socio-economic standards infection is considerably less common than in developing countries where virtually everyone may be infected.

Infection is typically contracted in early childhood, frequently by transmission from mother to child, and the bacteria may remain in the stomach for the rest of the person's life. This chronic infection is initiated in the lower part of the stomach (antrum). As first reported by Robin Warren, the presence of *Helicobacter pylori* is always associated with an inflammation of the underlying gastric mucosa as evidenced by an infiltration of inflammatory cells.

**The infection is usually asymptomatic but can cause peptic ulcer!**

The severity of this inflammation and its location in the stomach is of crucial importance for the diseases that can result from *Helicobacter pylori* infection. In most individuals *Helicobacter pylori* infection is asymptomatic. However, about 10-15% of infected individuals will some time experience peptic ulcer disease. Such
ulcers are more common in the duodenum than in the stomach itself. Severe complications include bleeding and perforation.

The current view is that the chronic inflammation in the distal part of the stomach caused by *Helicobacter pylori* infection results in an increased acid production from the non-infected upper corpus region of the stomach. This will predispose for ulcer development in the more vulnerable duodenum.

**Malignancies associated with *Helicobacter pylori* infection**

In some individuals *Helicobacter pylori* also infects the corpus region of the stomach. This results in a more widespread inflammation that predisposes not only to ulcer in the corpus region, but also to stomach cancer. This cancer has decreased in incidence in many countries during the last half-century but still ranks as number two in the world in terms of cancer deaths.

Inflammation in the stomach mucosa is also a risk factor for a special type of lymphatic neoplasm in the stomach, MALT (mucosa associated lymphoid tissue) lymphoma. Since such lymphomas may regress when *Helicobacter pylori* is eradicated by antibiotics, the bacterium plays an important role in perpetuating this tumour.

**Disease or not – interaction between the bacterium and the human host**

*Helicobacter pylori* is present only in humans and has adapted to the stomach environment. Only a minority of infected individuals develop stomach disease. After Marshall’s and Warren’s discovery, research has been intense. Details underlying the exact pathogenetic mechanisms are continuously being unravelled.

The bacterium itself is extremely variable, and strains differ markedly in many aspects, such as adherence to the gastric mucosa and ability to provoke inflammation. Even in a single infected individual all bacteria are not identical, and during the course of chronic infection bacteria adapt to the changing conditions in the stomach with time.

Likewise, genetic variations among humans may affect their susceptibility to *Helicobacter pylori*. Not until recently has an animal model been established, the
Mongolian gerbil. In this animal, studies of peptic ulcer disease and malignant transformation promise to give more detailed information on disease mechanisms.

**Antibiotics cure but can lead to resistance**

*Helicobacter pylori* infection can be diagnosed by antibody tests, by identifying the organism in biopsies taken during endoscopy, or by the non-invasive breath test that identifies bacterial production of an enzyme in the stomach.

An indiscriminate use of antibiotics to eradicate *Helicobacter pylori* also from healthy carriers would lead to severe problems with bacterial resistance against these important drugs. Therefore, treatment against *Helicobacter pylori* should be used restrictively in patients without documented gastric or duodenal ulcer disease.

**Microbial origin of other chronic inflammatory conditions?**

Many diseases in humans such as Crohn's disease, ulcerative colitis, rheumatoid arthritis and atherosclerosis are due to chronic inflammation. The discovery that one of the most common diseases of mankind, peptic ulcer disease, has a microbial cause, has stimulated the search for microbes as possible causes of other chronic inflammatory conditions.

Even though no definite answers are at hand, recent data clearly suggest that a dysfunction in the recognition of microbial products by the human immune system can result in disease development. The discovery of *Helicobacter pylori* has led to an increased understanding of the connection between chronic infection, inflammation and cancer.
Helicobacter pylori
the bacterium causing peptic ulcer disease

Infection
*Helicobacter pylori* infects the lower part of the stomach, antrum.

Inflammation
*Helicobacter pylori* causes inflammation of the gastric mucosa (gastritis). This is often asymptomatic.

Ulcer
Gastric inflammation may lead to duodenal or gastric ulcer. Severe complications include bleeding ulcer and perforated ulcer.

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The Nobel Prize in Physiology or Medicine for 2004 awarded

jointly to

Richard Axel and Linda B. Buck

for their discoveries of

"odorant receptors and the organization of the olfactory system"

Introduction

The sense of smell long remained the most enigmatic of our senses. The basic principles for recognizing and remembering about 10,000 different odors were not understood. This year’s Nobel Laureates in Physiology or Medicine have solved this problem and in a series of pioneering studies clarified how our olfactory system works. They discovered a large gene family, comprised of some 1,000 different genes (three per cent of our genes) that give rise to an equivalent number of olfactory receptor types. These receptors are located on the olfactory receptor cells, which occupy a small area in the upper part of the nasal epithelium and detect the inhaled odorant molecules.

Each olfactory receptor cell possesses only one type of odorant receptor, and each receptor can detect a limited number of odorant substances. Our olfactory receptor cells are therefore highly specialized for a few odors. The cells send thin nerve processes directly to distinct micro domains, glomeruli, in the olfactory bulb,
the primary olfactory area of the brain. Receptor cells carrying the same type of receptor send their nerve processes to the same glomerulus. From these microdomains in the olfactory bulb the information is relayed further to other parts of the brain, where the information from several olfactory receptors is combined, forming a pattern. Therefore, we can consciously experience the smell of a lilac flower in the spring and recall this olfactory memory at other times.

Richard Axel, New York, USA, and Linda Buck, Seattle, USA, published the fundamental paper jointly in 1991, in which they described the very large family of about one thousand genes for odorant receptors. Axel and Buck have since worked independent of each other, and they have in several elegant, often parallel, studies clarified the olfactory system, from the molecular level to the organization of the cells.

**The olfactory system is important for life quality**

When something tastes really good it is primarily activation of the olfactory system which helps us detect the qualities we regard as positive. A good wine or a sun ripe wild strawberry activates a whole array of odorant receptors, helping us to perceive the different odorant molecules.

A unique odor can trigger distinct memories from our childhood or from emotional moments - positive or negative - later in life. A single clam that is not fresh and will cause malaise can leave a memory that stays with us for years, and prevent us from ingesting any dish, however delicious, with clams in it. To lose the sense of smell is a serious handicap - we no longer perceive the different qualities of food and we cannot detect warning signals, for example smoke from a fire.

**Olfaction is of central importance for most species**

All living organisms can detect and identify chemical substances in their environment. It is obviously of great survival value to be able to identify suitable food and to avoid putrid or unfit foodstuff. Whereas fish has a relatively small number of odorant receptors, about one hundred, mice - the species Axel and Buck studied - have about one thousand. Humans have a somewhat smaller number than mice; some of the genes have been lost during evolution.

Smell is absolutely essential for a newborn mammalian pup to find the teats of its mother and obtain milk - without olfaction the pup does not survive unaided.
Olfaction is also of paramount importance for many adult animals, since they observe and interpret their environment largely by sensing smell. For example, the area of the olfactory epithelium in dogs is some forty times larger than in humans.

**A large family of odorant receptors**

The olfactory system is the first of our sensory systems that has been deciphered primarily using molecular techniques. Axel and Buck showed that three per cent of our genes are used to code for the different odorant receptors on the membrane of the olfactory receptor cells. When an odorant receptor is activated by an odorous substance, an electric signal is triggered in the olfactory receptor cell and sent to the brain via nerve processes. Each odorant receptor first activates a G protein, to which it is coupled. The G protein in turn stimulates the formation of cAMP (cyclic AMP). This messenger molecule activates ion channels, which are opened and the cell is activated. Axel and Buck showed that the large family of odorant receptors belongs to the G protein-coupled receptors (GPCR).

All the odorant receptors are related proteins but differ in certain details, explaining why they are triggered by different odorous molecules. Each receptor consists of a chain of amino acids that is anchored into the cell membrane and traverses it seven times. The chain creates a binding pocket where the odorant can attach. When that happens, the shape of the receptor protein is altered, leading to G protein activation.

**One type of odorant receptor in each olfactory receptor cell**

Independently, Axel and Buck showed that every single olfactory receptor cell expresses one and only one of the odorant receptor genes. Thus, there are as many types of olfactory receptor cells as there are odorant receptors. It was possible to show, by registering the electrical signals coming from single olfactory receptor cells, that each cell does not react only to one odorous substance, but to several related molecules – albeit with varying intensity.

Buck’s research group examined the sensitivity of individual olfactory receptor cells to specific odorants. By means of a pipette, they emptied the contents of each cell and showed exactly which odorant receptor gene was expressed in that cell. In this way, they could correlate the response to a specific odorant with the particular type of receptor carried by that cell.
Most odors are composed of multiple odorant molecules, and each odorant molecule activates several odorant receptors. This leads to a combinatorial code forming an "odorant pattern" - somewhat like the colors in a patchwork quilt or in a mosaic. This is the basis for our ability to recognize and form memories of approximately 10,000 different odors.

**Olfactory receptor cells activate micro regions in the olfactory bulb**

The finding that each olfactory receptor cell only expresses one single odorant receptor gene was highly unexpected. Axel and Buck continued by determining the organization of the first relay station in the brain. The olfactory receptor cell sends its nerve processes to the olfactory bulb, where there are some 2,000 well-defined microregions, glomeruli. There are thus about twice as many glomeruli as the types of olfactory receptor cells.

Axel and Buck independently showed that receptor cells carrying the same type of receptor converge their processes into the same glomerulus, and Axel’s research group used sophisticated genetic technology to demonstrate in mice the role of the receptor in this process. The convergence of information from cells with the same receptor into the same glomerulus demonstrated that also glomeruli exhibit remarkable specificity (see figure).

In the glomeruli we find not only the nerve processes from the olfactory receptor cells but also their contacts with the next level of nerve cells, the mitral cells. Each mitral cell is activated only by one glomerulus, and the specificity in the information flow is thereby maintained. Via long nerve processes, the mitral cells send the information to several parts of the brain. Buck showed that these nerve signals in turn reach defined micro regions in the brain cortex. Here the information from several types of odorant receptors is combined into a pattern characteristic for each odor. This is interpreted and leads to the conscious experience of a recognizable odor.

**Pheromones and taste**

The general principles that Axel and Buck discovered for the olfactory system appears to apply also to other sensory systems. Pheromones are molecules that can influence different social behaviors, especially in animals. Axel and Buck, independent of each other, discovered that pheromones are detected by two other families of GPCR, localized to a different part of the nasal epithelium. The taste
buds of the tongue have yet another family of GPCR, which is associated with the sense of taste.


**Odorant Receptors and the Organization of the Olfactory System**

Information for the Public
Nobel Prize in Chemistry
6 October 2004

Discovery of Ubiquitin-Mediated Protein Degradation

A human cell contains some hundred thousand different proteins. These have numerous important functions: as accelerators of chemical reactions in the form of enzymes, as signal substances in the form of hormones, as important actors in the immune defense and by being responsible for the cell’s form and structure. This year’s Nobel Laureates in chemistry, Aaron Ciechanover, Avram Hershko and Irwin Rose, have contributed ground-breaking chemical knowledge of how the cell can regulate the presence of a certain protein by marking unwanted proteins with a label consisting of the polypeptide ubiquitin. Proteins so labeled are then broken down - degraded - rapidly in cellular "waste disposers" called proteasomes.

Animation (Plug in requirement: Flash Player 6) »

Through their discovery of this protein-regulating system Aaron Ciechanover, Avram Hershko and Irwin Rose have made it possible to understand at molecular level how the cell controls a number of very important biochemical processes such as the cell cycle, DNA repair, gene transcription and quality control of newly-produced proteins. New knowledge of this form of controlled protein death has also contributed to explaining how the immune defense functions. Defects in the system can lead to various diseases including some types of cancer.

Proteins labeled for destruction

Degradation needs no energy – or does it?

While great attention and much research have been spent on understanding how the cell controls the synthesis of a certain protein - at least five Nobel Prizes have been awarded in this area - the reverse, the degradation of proteins, has long been considered less important. A number of simple protein-degrading enzymes were already known. One example is trypsin, which in the small intestine breaks down proteins in our food to amino acids. Likewise, a type of cell organelle, the lysosome,
in which proteins absorbed from outside are broken down, had long been studied. Common to these processes is that they do not require energy in order to function.

Experiments as long ago as the 1950s showed, however, that the breakdown of the cell’s own proteins does require energy. This long puzzled researchers, and it is precisely this paradox that underlies this year’s Nobel Prize in Chemistry: that the breakdown of proteins within the cell requires energy while other protein degradation takes place without added energy. A first step towards an explanation of this energy-dependent protein degradation was taken by Goldberg and his co-workers who in 1977 produced a cell-free extract from immature red blood cells, reticulocytes, which catalyze the breakdown of abnormal proteins in an ATP-dependent manner (ATP = adenosine triphosphate – the cell’s energy currency).

Using such an extract Aaron Ciechanover, Avram Hershko and Irwin Rose, in a series of epoch-making biochemical studies in the late 1970s and early 1980s, succeeded in showing that protein degradation in cells takes place in a series of step-wise reactions that result in the proteins to be destroyed being labeled with the polypeptide ubiquitin. This process enables the cell to break down unwanted proteins with high specificity, and it is this regulation that requires energy. As distinct from reversible protein modifications such as phosphorylation (Nobel Prize in Physiology or Medicine 1992), regulation through polyubiquitination is often irreversible since the target protein is destroyed. Much of the work was done during a series of sabbatical leaves that Avram Hershko and Aaron Ciechanover of the Technion (Israel Institute of Technology) spent with Irwin Rose at the Fox Chase Cancer Center in Philadelphia, USA.

The label is ubiquitin

The molecule that would later prove to be the label that marks out a protein for degradation was isolated as early as 1975. This 76-amino-acid-long polypeptide was isolated from calf sweetbread and was assumed to participate in the maturation of white blood cells. Since the molecule was subsequently found in numerous different tissues and organisms – but not in bacteria – it was given the name ubiquitin (from Latin *ubique*, "everywhere") (fig. 1).
The discovery of ubiquitin-mediated protein degradation

After taking his doctorate, Avram Hershko had studied energy-dependent protein degradation in liver cells, but decided in 1977 to transfer to the reticulocyte extract described above. This extract contained large quantities of hemoglobin, which upset the experiments. In their attempts to remove the hemoglobin using chromatography, Aaron Ciechanover and Avram Hershko discovered that the extract could be divided into two fractions, each inactive on its own. But it turned out that as soon as the two fractions were recombined, the ATP-dependent protein degradation restarted. In 1978 the researchers reported that the active component of one fraction was a heat-stable polypeptide with a molecular weight of only 9000 which they termed APF-1 (active principle in fraction 1). This protein later proved to be ubiquitin.

The decisive breakthrough in the research was reported in two works that Ciechanover, Hershko and Rose published in 1980. Until that time the function of APF-1 was entirely unknown. In the first work it was shown that APF-1 was bound covalently, i.e. with a very stable chemical bond, to various proteins in the extract.

In the second work it was further shown that many APF-1 molecules could be bound to the same target protein; the latter phenomenon was termed polyubiquitination. We now know that this polyubiquitination of substrate proteins is the triggering signal that leads to degradation of the protein in the proteasome. It is this reaction that constitutes the actual labeling, the "kiss of death" if you will.
At a stroke, these entirely unanticipated discoveries changed the conditions for future work: it now became possible to concentrate on identifying the enzyme system that binds ubiquitin to its target proteins. Since ubiquitin occurs so generally in various tissues and organisms, it was quickly realized that ubiquitin-mediated protein degradation must be of general significance for the cell. In addition, the researchers guessed that the energy requirement in the form of ATP enabled the cell to control the specificity of the process.

The field was now open and between 1981 and 1983 Ciechanover, Hershko, Rose and their post docs and students developed "the multistep ubiquitin-tagging hypothesis" based on three newly-discovered enzyme activities they termed E1, E2 and E3 (fig. 2). We now know that a typical mammalian cell contains one or a few different E1 enzymes, some tens of E2 enzymes and several hundred different E3 enzymes. It is the specificity of the E3 enzyme that determines which proteins in the cell are to be marked for destruction in the proteasomes.

![Fig 2. Ubiquitin-mediated protein degradation](image)

1. The E1 enzyme activates the ubiquitin molecule. This reaction requires energy in the form of ATP.
2. The ubiquitin molecule is transferred to a different enzyme, E2.
3. The E3 enzyme can recognize the protein target which is to be destroyed. The E2-ubiquitin complex binds so near to the protein target that the actual ubiquitin label can be transferred from E2 to the target.
4. The E3 enzyme now releases the ubiquitin-labeled protein.
5. This last step is repeated until the protein
has a short chain of ubiquitin molecules attached to itself.

6. This ubiquitin chain is recognized in the opening of the proteasome. The ubiquitin label is disconnected and the protein is admitted and chopped into small pieces.

All the studies up to this point had been done in cell-free systems. To be able to study the physiological function of ubiquitin-mediated protein degradation as well, Avram Hershko and his co-workers developed an immunochemical method. By using antibodies to ubiquitin, ubiquitin-protein-conjugate could be isolated from cells where the cell proteins had been pulse-labeled with a radioactive amino acid not present in ubiquitin. The results showed that cells really break down faulty proteins using the ubiquitin system, and we now know that up to 30% of the newly-synthesized proteins in a cell are broken down via the proteasomes since they do not pass the cell's rigorous quality control.

The proteasome - the cell’s waste disposer

What is a proteasome? A human cell contains about 30,000 proteasomes: these barrel-formed structures can break down practically all proteins to 7-9-amino-acid-long peptides. The active surface of the proteasome is within the barrel where it is shielded from the rest of the cell. The only way in to the active surface is via the "lock", which recognizes polyubiquitinated proteins, denatures them with ATP energy and admits them to the barrel for disassembly once the ubiquitin label has been removed. The peptides formed are released from the other end of the proteasome. Thus the proteasome itself cannot choose proteins; it is chiefly the E3 enzyme that does this by ubiquitin-labeling the right protein for breakdown (fig. 3).
More recent research

While the biochemical mechanisms underlying ubiquitin-labeled protein degradation were laid bare around 1983 its physiological significance had not yet been fully understood. That it is of importance in destroying defective intracellular proteins was known but, to proceed, a mutated cell was needed in the ubiquitin system. By studying in detail how the mutated cell differs from a normal cell under various growth conditions, it was hoped to gain a better idea of what reactions in the cell depend on the ubiquitin system.

A mutated mouse cell had been isolated in 1980 by a research group in Tokyo. Their mouse-cell mutant contained a protein that, because of the mutation, was sensitive to temperature. At lower temperatures the protein functioned as it should, but not at higher. Cells cultured at the higher temperature stopped growing. In addition, they showed defective DNA synthesis and other erroneous functions at the higher temperature. Researchers in Boston quickly showed that the heat-sensitive protein in the mutant mouse cell was the ubiquitin-activating enzyme E1. Obviously, ubiquitin activation was necessary for the cell to function and reproduce itself at all. Controlled protein breakdown was not only important for degrading incorrect proteins in the cell but it probably also took part in control of the cell cycle, DNA replication and chromosome structure.
Since the late 1980s a number of physiologically important substrates for ubiquitin-mediated protein breakdown have been identified. Only a few of the most important will be mentioned here.

**Prevention of self-pollination in plants**

Most plants are bisexual, hermaphroditic. Self-pollination leads to a gradual decline in genetic diversity which in the long run can cause the whole species to die out. To prevent this, plants use ubiquitin-mediated degradation to reject "own" pollen. The exact mechanism has not yet been clarified but the E3 enzyme has been encountered and when proteasome inhibitors have been introduced, the rejection has been impaired.
Regulation of the cell cycle

When a cell is to make a copy of itself, many chemical reactions are involved. In a human being, six thousand million base pairs must be duplicated in DNA. These are gathered in 23 chromosome pairs that must be copied. Ordinary cell division, mitosis, and the formation of sex cells, meiosis, have many points of contact with the subjects of this year’s Nobel Prize. The E3 enzyme responsible, a protein complex termed the "anaphase-promoting complex" (APC) checks that the cell goes out of mitosis. This enzyme complex has also proved to play an important role in the separation of the chromosomes during mitosis and meiosis. A different protein complex acts like a rope around the chromosome pair, holding it together. At a given signal, the APC labels an inhibitor of a certain protein-degrading enzyme, whereupon the inhibitor is carried to the proteasome and destroyed. The enzyme is released, is activated and cuts the rope around the chromosome pair. Once the rope is gone, the chromosome pair can be separated. Incorrect chromosome division during meiosis is the commonest cause of spontaneous miscarriage during pregnancy, and an extra chromosome 21 in humans leads to Down's syndrome. Most malignant tumors have cells with changed numbers of chromosomes as a result of incorrect chromosome division during mitosis.
DNA repair, cancer and programmed cell death

Protein p53 has been dubbed "the guardian of the genome" and it is a tumor-suppressor gene. This means that as long as a cell can produce p53 the development of cancer is hampered. Sure enough, the protein is mutated in at least 50% of all human cancer. The amount of protein p53 in a normal cell is low in consequence of continual production and breakdown. The breakdown is regulated through ubiquitination and the E3 enzyme responsible forms a complex with protein p53. Following DNA injury, protein p53 is phosphorylated and can no longer bind to its E3 enzyme. The breakdown stops and the quantity of p53 in the cell rises rapidly. Protein p53 acts as a transcription factor, i.e. a protein that controls the expression of a certain gene. Protein p53 binds to and controls genes that regulate DNA repair and programmed cell death. Raised levels of protein p53 lead first to interruption of the cell cycle to allow time for repair of DNA damage. If the damage is too extensive the cell triggers programmed cell death and "commits suicide".

Infection with human papilloma virus correlates strongly to the occurrence of cervical cancer. The virus avoids the protein p53 control function through one of its proteins activating and changing the recognition pattern of a certain cellular E3 enzyme, E6-AP, which is tricked into ubiquitinating the protein p53, which is totally destroyed. In consequence of this the infected cell can no longer repair DNA damage in a normal manner or trigger programmed cell death. The DNA mutations increase in number and this can ultimately lead to the development of cancer.

Immune and inflammatory reactions

A certain transcription factor regulates many of the genes in the cell that are important for immune defense and inflammatory reactions. This protein, the transcription factor, occurs bound to an inhibitor protein in the cytoplasm of the cell, and the bound form of the transcription factor lacks activity. When cells are exposed to bacteria or various signal substances, the inhibitor protein is phosphorylated, and this results in its being ubiquitinated and broken down in the proteasome. The released transcription factor is transported to the cell nucleus where it binds to, and activates the expression of, specific genes.

The ubiquitin-proteasome system also produces the peptides that are presented by the immune defense on the surface of a virus-infected cell by breaking down virus
proteins to suitable sizes. T lymphocytes recognize these peptides and attack the cell as an important part of our defense against virus infections.

**Cystic fibrosis (CF)**

The hereditary disease cystic fibrosis, CF, is caused by a non-functioning plasma membrane chloride channel called CFTR, the "cystic fibrosis transmembrane conductance regulator". Most CF patients have one and the same genetic damage, loss of the amino acid phenylalanine in the CFTR protein. The mutation causes faulty folding of the protein and this in turn leads to the protein being retained in the cell’s control system for protein quality. This system ensures that the incorrectly folded protein is destroyed through ubiquitin-mediated protein breakdown instead of being transported out to the cell wall. A cell with no functioning chloride channel can no longer transport chloride ions through its wall. This affects secretion in, among other organs, the lungs and leads to the accretion of thick phlegm in the lungs which impairs their function, greatly increasing the risk of infection.

The ubiquitin system has become an interesting area of research for medicines against various diseases. Such preparations can be aimed at components of the ubiquitin-mediated breakdown system to prevent the degradation of specific proteins. They can also be designed to cause the system to destroy unwanted proteins. A medicine already being tested clinically is the proteasome inhibitor Velcade (PS341) which is used against multiple myeloma, a cancer disease that affects the body's antigen-producing cells.

This year’s Laureates have explained the molecular background to a protein regulation system of great importance for all higher cells. New cell functions controlled by ubiquitin-mediated protein degradation are being discovered all the time and this research is being conducted in numerous laboratories all over the world.

**The Laureates**
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Israeli citizen. Born 1947 (57 years) in Haifa, Israel. Doctor’s degree in medicine in 1975 at Hebrew University of Jerusalem, and in biology in 1982 at the Technion (Israel Institute of Technology), Haifa. Distinguished Professor at the Center for Cancer and Vascular Biology, the Rappaport Faculty of Medicine and Research Institute at the Technion, Haifa, Israel.

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Israeli citizen. Born 1937 (67 years) in Karcag, Hungary. Doctor’s degree in medicine in 1969 at the Hadassah and the Hebrew University Medical School, Jerusalem. Distinguished Professor at the Rappaport Family Institute for Research in Medical Sciences at the Technion, Haifa, Israel.

Irwin Rose
Dept. of Physiology and Biophysics
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American citizen. Born 1926 (78 years) in New York, USA. Doctor’s degree in 1952 at the University of Chicago, USA. Specialist at the Department of Physiology and Biophysics, College of Medicine, University of California, Irvine, USA.
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Illustrations: Typoform


Fimbriae, Fibrils, Sex and Fuzzy Coats

The Limitation of Light

One of the frustrating aspects of working with bacteria is that they are so small that it is almost impossible to see anything other than their shape when looking down even the very best of optical microscopes. Even then, their refractive index is so similar to that of water that they have to be stuck to a glass slide, killed and stained before even their shape is revealed. Microscopes which can make use of polarized light (Phase contrast microscopy) can be used to see living bacteria but apart from the added ability of seeing some species happily swimming around they add little to what we can see using conventional staining techniques.

The fact that some species could move quite rapidly intrigued many early microbiologists and eventually some special staining procedures lead to the discovery of thin whip-like appendages which they called flagellae and conferred motility. This is not to say that light microscopy is not useful. It remains an essential tool in any bacteriology laboratory but it should be recognized that the information obtained, although extremely helpful in routine work, is limited.

Electron Microscopy Reveals More

The invention of the electron microscope revealed much more detail of bacteria. Compared to the fascinating structures uncovered in eukaryotic cells, bacteria, both inside and out, were pretty uninteresting. It wasn’t until the early 1960s that some interesting surface features of some bacterial species were noticed. This delay was partly due to the electron microscopy techniques in use at that time. The convention at the time was to use ultra-thin sections of tissue, far thinner than sections used for light microscopy. It seemed normal then to prepare bacteria in the same way. Using these techniques, the outer surfaces of bacteria seemed fairly barren but the technique did reveal some of the double membrane-like composition of Gram-negative bacteria.

Shadow-Casting Reveals Still More
Although thin sections of bacteria did not allow flagella to be seen in their entirety it did reveal interesting cross-sections which showed their internal structure. It also enabled detail of flagella attachment to be demonstrated. It was not until electron microscopes were used to look at whole cells rather than ultra-thin sections that more progress was made. This change required the development of new staining techniques known as shadow-casting where bacterial surfaces were sprayed with electron-dense material such as gold or carbon at an angle. This highlighted the fine surface structures in a way exactly analogous to light falling on a stone surface at an angle reveals more detail than light falling on it at right angles.

**Shadows, Flagellae and Fimbriae**

Once shadow-casting techniques had been developed the whip-like flagellae were the first to be examined in detail but one researcher in particular noticed the presence of previously undreamed of structures on the surface of some species. The person who first described these structures which he found on strains of *Escherichia coli* and *Salmonella* was Professor James Duguid. He called them fimbriae.

**What are Fimbriae?**

Fimbriae are thin, hair-like, projections made of protein sub-units. A number of different types have been described (about 7 at the last count, labeled Types I-VII) which can be distinguished by their size (length and diameter) and the type of antigens they carry. They are characteristic of some Gram-negative bacteria such as *Escherichia coli* and *Salmonella spp* and were first described back in the 1960s by JP Duguid who was the Professor of Microbiology at the University of Dundee. Later, it was discovered that these fimbriae would re-grow after they had been broken off e.g. by vigorous shaking and that this re-growth was from pre-formed protein sub-units which were stored inside the cells. Fimbriae originate in the cytoplasm of the cell and project through the cell membrane and the cell wall.

<table>
<thead>
<tr>
<th>A Controversy</th>
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<tbody>
<tr>
<td>A short while after Duguid published his findings an American called Robert Brinton published much the same stuff and called them pili. What followed was a pretty acrimonious exchange of letters in the</td>
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scientific press about what they should be called.

It was all pretty good fun but to this day our American cousins, and anybody who doesn’t know any better, call them pili whereas all right-thinking, clear-minded and fair microbiologists refer to them as fimbriae.

So What do Fimbriae Actually do?

Over the years we have learned quite a lot about fimbriae and right from the very early days it was thought that they were involved in helping the bacteria adhere to surfaces. There is now a substantial body of evidence in support of this much of it in relation to pathogenic strains of E. coli.

Type I Fimbriae are Pathogenicity Factors

It’s clear these days that Type I fimbriae are involved in bacterial adhesion and the very best example are those carried by pathogenic strains of E. coli. These come in a variety of forms including plain old EnteroPathogenic E. coli (EPEC), EnteroToxigenic E. coli (ETEC), EnteroInvasive E. coli (EIEC) and VeroToxogenic E. coli (VTEC). These E. coli strains use Type I fimbriae to adhere to gut mucosal cells which is the first step in the pathogenic process. Without the fimbriae their capacity to cause disease is greatly diminished or abolished completely.

Type IV fimbriae are particularly interesting. These have also been referred to as "bundle forming pili" because of their ability to aggregate into bundles. These fimbriae are thought to be connected with the ability of EPEC strains to form microcolonies on tissue monolayers and mutants lacking this ability show reduced virulence. Type IV fimbriae have also been shown to be involved in the remarkable phenomenon of bacterial twitching motility which allows bacterial cells to crawl over a surface.

Type VII Fimbriae, Viruses and the Sex Bit

Type VII fimbriae are the conduit for DNA transfer between bacterial mating strains. As it happens they also provide a binding site for certain bacteriophages. The significance of this is a mystery but it does enable Type VII to be seen clearly
because when some of the bacteriophage is added to a suspension of cells, the phage coat the Type VII fimbriae. In the electron microscope picture above right you can clearly see little particles stuck on two of the fimbriae which are much longer than the rest because size does matter, at least to *E.coli*. In a generous attempt to resolve the fimbriae/pili argument it was proposed that Type VII fimbriae were named the "sex pilus".

The photograph above was taken using a transmission electron microscope. The Type I fimbriae are the thin projections sticking out from the surface of the cell. Some of the fimbriae have broken off indicating that they are quite brittle.

**Surfaces of Streptococci**

Back in the days before we knew much about fimbriae researchers looking at ultra-thin sections of the serious pathogen *Streptococcus pyogenes* noticed that the very outside of the cells had a fuzzy appearance. In a fit of imagination it was called "fuzzy coat". Later, when they learned about shadow-casting whole cells they applied this technique but it did not help to resolve any particular structures like fimbriae.
Even today we have not resolved any definite structure to the *S. pyogenes* "fuzzy-coat". We do know, however, that it consists partly of a substance called "M-protein" which is a major pathogenicity factor of this species.

### Negative Staining Reveals Surface Fibrils on Some Streptococci

Towards the late 1970s a rather different technique which made use of a special type of stain called a "negative stain" revealed very thin, delicate, hair-like structures on some oral streptococci such as *Streptococcus sanguis* and *Streptococcus salivarius*. Take a look at the photograph on the right. This is an electron micrograph of the surface of a *Streptococcus salivarius* cell and although it may not be terribly clear on this reproduction, the original shots showed two types of these thin hair-like structures, long ones and short ones. This negative-staining technique could not, by the way, reveal anything hair-like on the surface of *Streptococcus pyogenes* which had the fuzzy coat.

### Fibrils are not Fimbriae

More research using lots of different strains of different species of oral streptococci showed these "hairs" came in all sorts of lengths and some cells carried more than one type. They were very thin and flexible. Although some fimbriae on *E.coli* can be very thin, "flexible" is not a term normally associated with fimbriae. To begin with these hairs were called "fibrils" and there is a fair amount of evidence to suggest they are made of protein and some evidence which suggests
that some are even made of glycoprotein although glycoproteins are generally considered pretty rare beasts in bacteria. As far as fibril synthesis goes, we don’t know much. Generally speaking they are difficult to remove, probably because they are so flexible, so it’s not possible to say whether they can re-grow like fimbriae. The analogy was taken a stage further when a role in adhesion was postulated and, in fact, there is fairly good evidence to back this up, at least for the *S. salivarius* fibrils.

Unfortunately at this point the waters got a bit muddy when some people started referring to the long fibrils as "fimbriae" and the short ones as "fibrils". Since they are kind of like fimbriae this wasn’t so surprising but what was surprising was that they were never referred to as pili!
tufts of fibrils and looked rather like punk-rockers with Mohican hairstyles. Later these were grouped together into a new species and given the rather elegant name *Streptococcus cristae*.

**Fibril Tufts and Co-aggregation**

There is evidence that these may also be involved in adhesion, this time to rod-shaped bacteria to make the structures commonly found in mature dental plaque called "corn-cob-configuration". When bacteria of the same species stick to each other it’s known as "aggregation". In this case the bacteria are from different species and it’s known as "CO-aggregation".

**"Corn Cobs" in Dental Plaque**

And finally

You may have guessed by now that I'm a bit skeptical about using the term "fimbriae" to describe the surface structures of these oral streptococci. I prefer to describe them all as fibrils but I'll probably end up in the minority. Sooner or later this is all going to be resolved but for the time being it’s probably best to keep the term "fimbriae" reserved for those brittle hair-like, proteinaceous surface projections of Gram-negative rods like *Escherichia* and *Salmonella* and call everything else "fibrils".
Just remember pili are fimbriae and fibrils are different and you won’t go far wrong.

<table>
<thead>
<tr>
<th>SUMMARY</th>
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<tbody>
<tr>
<td>1. Fimbriae are appendages which have been seen on the surfaces of a range of Gram-negative rods such as <em>E.coli</em> and various species of <em>Salmonella</em></td>
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<tr>
<td>2. Fimbriae come in 7 different types (I-VII) distinguished by their length and width</td>
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<td>3. Fimbriae are thought to be important in adhesion and have been shown to be pathogenicity factors in pathogenic strains of <em>E.coli</em>.</td>
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<tr>
<td>4. Type VII fimbriae allow DNA transfer between mating strains of certain species such as <em>E.coli</em></td>
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<tr>
<td>5. Fibrils are found on streptococci</td>
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<tr>
<td>6. Fibrils are different from fimbriae, they are thinner and appear to be more flexible</td>
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<tr>
<td>7. Some fibrils have been shown to function in adhesion <em>e.g.</em> in corn-cob-formations found in dental plaque</td>
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</tbody>
</table>

[http://www.ncl.ac.uk/dental/oralbiol/oralenv/tutorials/fimbriae.htm](http://www.ncl.ac.uk/dental/oralbiol/oralenv/tutorials/fimbriae.htm)
Revolutionary Analytical Methods for Biomolecules

The Nobel Prize in Chemistry for 2002 is being shared between scientists in two important fields: mass spectrometry (MS) and nuclear magnetic resonance (NMR). The Laureates, John B. Fenn and Koichi Tanaka (for MS) and Kurt Wüthrich (for NMR), have contributed in different ways to the further development of these methods to embrace biological macromolecules. This has meant a revolutionary breakthrough, making chemical biology into the "big science" of our time. Chemists can now rapidly and reliably identify what proteins a sample contains. They can also produce three-dimensional images of protein molecules in solution. Hence scientists can both "see" the proteins and understand how they function in the cells.

Why study biological macromolecules?
All living organisms - bacteria, plants and animals - contain the same types of large molecules, macromolecules, which are responsible for what we call life. Events in the cells are controlled by nucleic acids (such as DNA) that may be termed the cells' "directors", while the various proteins are the cells' leading actors. Each protein has a biological function that may vary with its environment. The protein hemoglobin, for example, transports oxygen to all the cells in the body.

Protein research itself is not new, but proteomics, i.e. studies of how different proteins and other substances act together in the cell, is a relatively new field of research that has grown enormously in the past few years. As the gene sequences of more and more organisms have been mapped and the research frontier has advanced, new questions have cropped up: how can it be that man's 30,000-or-so genes code for hundreds of thousands of different proteins? What happens if a gene is damaged or is missing? How do diseases such as Alzheimer's or mad cow disease originate? Can the new chemistry be used to diagnose and treat more quickly the diseases that are threatening mankind?

To be able to tackle questions such as these chemists are in constant pursuit of more knowledge of proteins and how they function together with each other and with other molecules in the cells. This is because small variations in a protein's structure determine its function. The next step is to study the dynamics: what do...
protein molecules look like at the very moment when they are interacting with one another? What happens at the decisive moments? To understand, we need to see.

Fig 1. This protein consists of a long chain of amino acids that is pleated, folded and wound together like a ball of wool. It is this three-dimensional image of the protein one needs to achieve to be able to understand the function of that protein. This protein molecule, which was one of the first to have its structure determined with NMR, has a diameter of approximately one millionth of a centimeter ($10^{-8}$ m).

**Mass spectrometry – a method of identifying molecules**

*Mass spectrometry* now allows us to identify a substance in a sample, rapidly, on the basis of its mass. This technique has long been used by chemists on small and medium-sized molecules. The method is so sensitive that it is possible to trace very small quantities of each type of molecule. Doping and drug tests, foodstuff control and environmental analysis are examples of areas where mass spectrometry is now in routine use.

The foundations of mass spectrometry were already in place at the end of the nineteenth century. The first analyses of small molecules were reported in 1912 by Joseph J. Thompson. Several of the Nobel Prizes of the twentieth century
depended directly on mass-spectrometric analysis. Examples are Harold Urey’s
discovery of deuterium (Nobel Prize in Chemistry 1934) and the discovery of the
fullerenes, "carbon footballs" that gave Robert Curl, Sir Harold Kroto and Richard
Smalley the Nobel Chemistry Prize in 1996.

The goal of using mass spectrometry for macromolecules as well long attracted the
scientists. During the 1970s a number of successes were achieved in transferring
macromolecules to ions in the gas phase, termed desorption technology. These have
formed the basis for the revolution in this field during the past twenty years.

Macromolecules may be large in comparison with other molecules but we are
nevertheless dealing here with incredibly small structures. Hemoglobin molecules,
for example, have a mass of a tenth of a thousand-millionth of a thousand-millionth
of a gram (10\(^{-19}\) g). How to weigh something that is so small? The trick is to cause
the individual protein molecules to let go of each other and spread out as a cloud of
freely hovering, electrically charged protein ions. A common method of
subsequently measuring the mass of these ions - and hence identifying the proteins
- is to accelerate them in a vacuum chamber where their time of flight (TOF) is
measured. They "reach their targets" in an order determined partly by their charge
and partly by their mass. The fastest ones are those that are lightest and
have the highest charge.

Today there are two principles for causing proteins to transform into the gas
phase without losing their structure and form, and it is the discoverers behind
these methods that are being rewarded jointly with half the Nobel Prize in
Chemistry. In one of these methods, of which John B. Fenn is the originator, the
sample is sprayed using a strong electrical field to produce small, charged, freely
hovering ions. The other method, instead, uses an intense laser pulse. If this is
done under suitable conditions (as to the energy, structure and chemical
environment of the sample) the test molecules take up some of the energy of the
laser pulse and become released as free ions. The first person to show that this
phenomenon, soft laser desorption, could be used for large molecules such as
proteins was Koichi Tanaka.

Fenn’s contribution – hovering through spraying
During 1988 John B. Fenn published two articles that were to mean a breakthrough
for mass spectrometry with "electrospray" for macromolecules. In the first,
studies of polyethylene glycol molecules of unknown mass showed that the method
could handle large molecule masses with high charges. The second publication reported the use of the method on medium-sized whole proteins as well. The release of ions is achieved by spraying the sample using an electrical field so that charged droplets are formed. As the water gradually evaporates from these droplets, freely hovering "stark naked" protein molecules remain. The method came to be called electrospray ionization, ESI.

As the molecules take on strong positive charges, the mass/charge ratio becomes small enough to allow the substances to be analyzed in ordinary mass spectrometers. Another advantage is that the same molecule causes a series of peaks, since each can take up a varying number of charges. While this complicates the pattern, at first confusing the researchers, it also gives information that makes identification easier.

Fig 2. The principles for mass spectrometry of biomolecules.

Tanaka’s contribution – hovering through blasting
At the same time exciting things were happening in another part of the world. At the Japanese Shimadzu instrument company in Kyoto, a young Japanese engineer, Koichi Tanaka, reported an entirely different technique for the first critical stage. At a symposium in 1987 and a year later in print, Tanaka showed that the protein molecules could be ionized using soft laser desorption (SLD). A laser pulse strikes the sample which, unlike in the spray method, is in a solid or viscous phase. When the sample takes up the energy from the laser pulse it is "blasted" into small bits. The molecules let go of one another, released as intact hovering molecule ions with low charge which are then accelerated by an electrical field and detected as described above by recording their time of flight. Tanaka was the first to demonstrate the applicability of laser technology to biological macromolecules. The principle is fundamental for many of today’s powerful laser desorption methods, particularly the one abbreviated MALDI (Matrix-Assisted Laser Desorption Ionization) but also SELDI (Surface Enhanced Laser Desorption Ionization) and DIOS (Direct Ionization on Silicon).
Applications of mass spectrometry

Both electrospray ionization (ESI) and soft laser desorption (SLD) have many areas of application. The sophisticated biochemical analyses now possible were but dreams a few years ago. Interactions between proteins are very important to study in order to understand the signal systems of life. Such non-covalent biomolecule complexes can be examined with ESI. The method is superior to other methods in the rapidity, sensitivity and identification of the actual interaction. Mass spectrometric analytical methods are relatively cheap, enabling them to spread quickly to laboratories all around the world. Today soft laser desorption (in the form of MALDI) and electrospray are standard methods for structure analyses of peptides, proteins and carbohydrates which make it possible to quickly analyze the protein content of intact cells and living tissue. The following examples of current fields of research gives a picture of the application versatility generated by this year’s Nobel Prize. Applications include:

Pharmaceuticals development

The early phase of pharmaceuticals development has undergone a paradigm shift. Combined with fluid separation, ESI-MS has made it possible to analyze several hundreds of compounds per day.

Malaria

Scientists have recently discovered new ways of studying the spreading of malaria. Early diagnosis is possible thanks to the soft laser desorption method. The oxygen-bearing part of human hemoglobin is used here to absorb the energy of the laser pulse.

Ovarian, breast and prostate cancer

New methods for early diagnosis of different forms of cancer have been reported at a rapid rate during the past year. By having a surface that cancer cells adhere to - and then analyzing this with soft laser desorption - chemists can discover cancer faster than doctors can.

Foodstuff control

ESI technology has also made progress for small molecules. During the past few months we have learned that preparation of the food we eat can give rise to a number of substances hazardous to health, e.g. acrylamide which can cause cancer. With mass spectrometry, food is analyzed rapidly at various stages of production. By modifying the temperature and the ingredients, the harmful substances can be
NMR for biological macromolecules

Where mass spectrometry gives answers to questions about e.g. a protein, such as "what?" and "how much?". NMR in one sense answers the question "what does it look like?" Even the largest proteins are too small to be studied at sufficient resolution with any type of microscope. To be able to form a picture of what a protein really looks like, then, other methods must be used. NMR (Nuclear Magnetic Resonance) is one such method. By interpreting the peaks in an NMR spectrum one can draw a three-dimensional picture of the molecule being studied. One finesse is that the sample can be in a solution, in the case of proteins their natural environment in the cell.

Before the advent of NMR, X-ray crystallography was the only method available for determining the three-dimensional structure of the substance. In 1957 the first true three-dimensional structure of a protein, myoglobin, was presented. This was rewarded with a Nobel Prize in Chemistry to Max Perutz in 1962. X-ray crystallography is based on the diffraction of X rays in protein crystals, and has since contributed to a further series of Nobel Prizes. As a complement to X-ray crystallography, chemists long sought a method that would also function in a solution, i.e. an environment that better resembles the one the biomolecules surround themselves with naturally.

The physicists Felix Bloch and Edward Purcell discovered as early as in 1945 that some atom nuclei, through what is called their nuclear spin, absorb radio waves of a certain frequency when placed in a powerful magnetic field. This was rewarded with the Nobel Prize in Physics in 1952. A few years earlier it was discovered that the frequency for nuclear resonance depended not only on the strength of the magnetic field and the type of atom but also on the chemical environment of the atom. In addition, the nuclear spins of different nuclei could affect each other, generating fine structures, i.e. a further number of peaks in the NMR spectrum.
The applicability of the NMR method was initially limited by its low sensitivity: it required incredibly concentrated solutions. But in 1966 the Swiss chemist Richard Ernst (Nobel Prize in Chemistry 1991) showed that this sensitivity could be increased dramatically if, instead of slowly varying the frequency, the sample was exposed to short and intense radio frequency pulses. He also contributed, during the 1970s, to the development of a way of determining what nuclei were adjacent to one another in a molecule, e.g. two atoms bound to each other. By interpreting the signals in an NMR spectrum it was thus possible to gain an idea of the appearance of the molecule, its structure. The method was successful for relatively small molecules but, for larger ones, it was hard to differentiate between the resonances of the different atom nuclei. An NMR spectrum of this kind could look like a grass lawn in section - thousands of peaks where it was impossible to decide which peak belonged to which atom. The scientist who finally solved this problem was the Swiss chemist Kurt Wüthrich.

Kurt Wüthrich - showed that NMR was possible for proteins
At the beginning of the 1980s, Kurt Wüthrich developed an idea about how NMR could be extended to cover biological molecules such as proteins. He invented a systematic method of pairing each NMR signal with the right hydrogen nucleus (proton) in the macromolecule (see fig. 4). The method is called sequential
Assignment and is today a cornerstone of all NMR structural investigations. He also showed how it was subsequently possible to determine pair wise distances between a large number of hydrogen nuclei and use this information with a mathematical method based on distance-geometry to calculate a three-dimensional structure for the molecule.

Fig 4. If one knows all the measurements of a house one can draw a three-dimensional picture of the house. In the same way, by measuring a vast number of short distances in a protein it is possible to create a three-dimensional picture of its structure, as shown schematically in the figure.

The first complete determination of a protein structure with Wüthrich’s method came in 1985. At present 15-20% of all the thousands of known protein structures have been determined with NMR. The structures of the others have been determined chiefly with X-ray crystallography; a few with other methods such as electron diffraction or neutron diffraction.

Areas of application for NMR with macromolecules
In many respects, the NMR method complements X-ray crystallography for structural determination. If the same protein is investigated with both methods, in the one case in solution and in the other crystallized, the same result is generally obtained, with the exception of certain superficial areas that are affected by the environment in both cases - in the crystals by the tightly packed protein molecules, in solution by the surrounding molecules of the solvent. While the strength of X-ray crystallography lies in being able to determine accurately really large three-dimensional structures, the NMR method has other unique advantages. The fact that the investigation takes place in a solution means that physiological conditions
can be approximated. A particular strength of NMR is its ability to demonstrate unstructured and very mobile parts of a molecule. It is possible to elucidate the mobility, the dynamics, and how it varies along a protein chain. Isotope labeling can also be used to facilitate the identification of the atoms.

One example of NMR-determined protein structures comes from studies of the prion proteins involved in the development of a number of dangerous diseases such as mad cow disease (Nobel Prize in Medicine to Stanley Prusiner in 1997). Here Wüthrich and coworkers have shown with NMR methodology that the healthy form of prion proteins has two parts: approximately half of the protein chain assumes a well-ordered, fairly rigid three-dimensional structure in a water solution (121-231 in the picture below), while the other half is without structure and very mobile (23-120).

NMR can also be used in studies of structure and dynamics of other biological macromolecules such as DNA and RNA.

![Structure of prion protein, determined with NMR. Half of the protein chain (23-120) is disordered and quite flexible in water solution.](image)

NMR is also used in the pharmaceuticals industry to determine the structure, and hence the properties, of proteins and other macromolecules that can be interesting target molecules for new pharmaceuticals. Pharmaceutical molecules
are designed to fit into the structure of the protein - like a key in a lock. The perhaps most important industrial use of NMR is in the search for small potential pharmaceutical molecules that can interact with a given biological macromolecule. If the small molecule binds to the large one, the NMR spectrum of the large molecule is normally changed. This may be used to "screen" a large number of pharmaceuticals candidates at an early stage of the development of a new drug.

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Nobel Prize in Medicine 2002

Genetic Regulation of Organ Development and Programmed Cell Death

Introduction

Sydney Brenner, Robert Horvitz and John Sulston’s discoveries concerning the genetic regulation of organ development and programmed cell death have truly opened new avenues for biological and medical research. We have all begun our lives in a seemingly modest way - as the fertilized egg cell, a tenth of a millimeter in size. From this small cell, the adult human being develops, with its hundred thousand billion cells, through cell division, cell differentiation and by formation of the various organs. To only make new cells is however not sufficient, certain cells must also die at specific time points as a natural part of the growth process. Think for example about how we for a short period during fetal life have web between our fingers and toes, and how this is removed by cell death.

The importance of cell differentiation and organ development was understood by many, but progress was slow. This was largely an effect of our complexity, with the large number of cells and many cell types - the forest could not be seen because of all the trees. Could the task to find the genetic principles be made simpler? Were there a species simpler than humans, but still sufficiently complex to allow for general principles to be deduced?
Sydney Brenner in Cambridge, UK, took on the challenge, and his choice was the nematode *Caenorhabditis elegans*. This may at first seem odd, a spool-shaped approximately 1 millimeter long worm with 959 cells that eats bacteria, but Brenner realized in the early 1960s that it was, what we today would call, "loaded with features". It was genetically amenable and it was transparent, so that every cell division and differentiation could be directly followed in the worm under the microscope. Brenner demonstrated in 1974 that mutations could be introduced into many genes and visualized as distinct changes in organ formation. Through his visionary work, Brenner created an important research tool. The nematode had made into the inner circle of research.

John Sulston came to Brenner’s laboratory in 1969. He took advantage of that cell divisions could be followed under the microscope and assembled the cell lineage in the worm, showing which cells are siblings, first and second cousins. He found that cell divisions occurred with a very high degree of precision, the cell lineage was identical between different individuals. He also realized that certain cells in the lineage always died at a certain time point. This meant that programmed cell death was not a stochastic process, but rather occurred with a very high degree of precision. During the course of this work Sulston identified the first gene important for the cell death process: *nuc-1*.

Robert Horvitz came to work with Brenner and Sulston in 1974. Horvitz started a systematic search for genes controlling programmed cell death. He identified the key genes for the cell death process proper. The discovery of these central death genes, *ced-3*, *ced-4* and *ced-9*, changed the view on programmed cell death from something rather obscure to a process with a strict genetic program. Horvitz also showed that there are human homologues to the death genes in the worm and that those have corresponding functions - the cell death machinery had deep evolutionary roots.

This year's Nobel Prize celebrates the Joy of Worms. Brenner’s almost prophetic visions from the early 1960s of the advantages of this model organism have been fulfilled. It has given us new insights into the development of organs and tissues and why specific cells are destined to die. This knowledge has proven valuable, for instance, in understanding how certain viruses and bacteria attack our cells, and how cells die in heart attack and stroke.

*Cell lineage – from egg to adult*
All cells in our body are descendents from the fertilized egg cell. Their relationship can be referred to as a cellular pedigree or cell lineage. Cells differentiate and specialize to form various tissues and organs, for example muscle, blood, heart and the nervous system. The human body consists of several hundreds of cell types, and the cooperation between specialized cells makes the body function as an integrated unit. To maintain the appropriate number of cells in the tissues, a fine-tuned balance between cell division and cell death is required. Cells have to differentiate in a correct manner and at the right time during development in order to generate the correct cell type.

It is of considerable biological and medical importance to understand how these complicated processes are controlled. In unicellular model organisms, e.g. bacteria and yeast, organ development and the interplay between different cells cannot be studied. Mammals, on the other hand, are too complex for these basic studies, as they are composed of an enormous number of cells. The nematode *C. elegans*, being multi-cellular, yet relatively simple, was therefore chosen as the most appropriate model system, which has then led to characterization of these processes also in humans.

**Programmed cell death**

Normal life requires cell division to generate new cells but also the presence of cell death, so that a balance is maintained in our organs. In an adult human being, more than a thousand billion cells are created every day. At the same time, an equal number of cells die through a controlled "suicide process", referred to as programmed cell death.

Developmental biologists first described programmed cell death. They noted that cell death was necessary for embryonic development, for example when tadpoles undergo metamorphosis to become adult frogs. In the human foetus, the interdigital mesoderm initially formed between fingers and toes is removed by programmed cell death. The vast excess of neuronal cells present during the early stages of brain development is also eliminated by the same mechanism.

The seminal breakthrough in our understanding of programmed cell death was made by this year’s Nobel Laureates. They discovered that specific genes control the cellular death program in the nematode *C. elegans*. Detailed studies in this simple
model organism demonstrated that 131 of totally 1090 cells die reproducibly during development, and that this natural cell death is controlled by a unique set of genes.

**The model organism C. elegans**

**Sydney Brenner** realized, in the early 1960s, that fundamental questions regarding cell differentiation and organ development were hard to tackle in higher animals. Therefore, a genetically amenable and multicellular model organism simpler than mammals, was required. The ideal solution proved to be the nematode *Caenorhabditis elegans*. This worm, approximately 1 mm long, has a short generation time and is transparent, which made it possible to follow cell division directly under the microscope.

Brenner provided the basis in a publication from 1974, in which he broke new ground by demonstrating that specific gene mutations could be induced in the genome of *C. elegans* by the chemical compound EMS (ethyl methane sulphonate). Different mutations could be linked to specific genes and to specific effects on organ development. This combination of genetic analysis and visualization of cell divisions observed under the microscope initiated the discoveries that are awarded by this year's Nobel Prize.

**Mapping the cell lineage**

**John Sulston** extended Brenner’s work with *C. elegans* and developed techniques to study all cell divisions in the nematode, from the fertilized egg to the 959 cells in the adult organism. In a publication from 1976, Sulston described the cell lineage for a part of the developing nervous system. He showed that the cell lineage is invariant, i.e. every nematode underwent exactly the same program of cell division and differentiation.

As a result of these findings Sulston made the seminal discovery that specific cells in the cell lineage always die through programmed cell death and that this could be monitored in the living organism. He described the visible steps in the cellular death process and demonstrated the first mutations of genes participating in programmed cell death, including the *nuc-1* gene. Sulston also showed that the protein encoded by the *nuc-1* gene is required for degradation of the DNA of the dead cell.

**Identification of "death genes"**
Robert Horvitz continued Brenner’s and Sulston’s work on the genetics and cell lineage of *C. elegans*. In a series of elegant experiments that started during the 1970s, Horvitz used *C. elegans* to investigate whether there was a genetic program controlling cell death. In a pioneering publication from 1986, he identified the first two bona fide "death genes", *ced-3* and *ced-4*. He showed that functional *ced-3* and *ced-4* genes were a prerequisite for cell death to be executed.

Later, Horvitz showed that another gene, *ced-9*, protects against cell death by interacting with *ced-4* and *ced-3*. He also identified a number of genes that direct how the dead cell is eliminated. Horvitz showed that the human genome contains a *ced-3*-like gene. We now know that most genes that are involved in controlling cell death in *C. elegans*, have counterparts in humans.

**Of importance for many research disciplines**

The development of *C. elegans* as a novel experimental model system, the characterization of its invariant cell lineage, and the possibility to link this to genetic analysis have proven valuable for many research disciplines. For example, this is true for developmental biology and for analysis of the functions of various signaling pathways in a multicellular organism. The characterization of genes controlling programmed cell death in *C. elegans* soon made it possible to identify related genes with similar functions in humans. It is now clear that one of the signaling pathways in humans leading to cell death is evolutionarily well conserved. In this pathway *ced-3*-, *ced-4*-, and *ced-9*-like molecules participate. Understanding perturbations in this and other signaling pathways controlling cell death are of prime importance for medicine.

**Disease and programmed cell death**

Knowledge of programmed cell death has helped us to understand the mechanisms by which some viruses and bacteria invade our cells. We also know that in AIDS, neurodegenerative diseases, stroke and myocardial infarction, cells are lost as a result of excessive cell death. Other diseases, like autoimmune conditions and cancer, are characterized by a reduction in cell death, leading to the survival of cells normally destined to die.

Research on programmed cell death is intense, including in the field of cancer. Many treatment strategies are based on stimulation of the cellular "suicide program". This is, for the future, a most interesting and challenging task to
further explore in order to reach a more refined manner to induce cell death in cancer cells.

Using the nematode *C. elegans* this year's Nobel Laureates have demonstrated how organ development and programmed cell death are genetically regulated. They have identified key genes regulating programmed cell death and demonstrated that corresponding genes exist also in higher animals, including man. The figure schematically illustrates the cell lineage (top left) and the programmed cell death (below) in *C. elegans*. The fertilized egg cell undergoes a series of cell divisions leading to cell differentiation and cell specialization, eventually producing the adult organism (top right). In *C. elegans*, all cell divisions and differentiations are invariant, i.e. identical from individual to individual, which made it possible to construct a cell lineage for all cell divisions during development. 1090 cells are generated, but precisely 131 of these cells are eliminated by programmed cell death. This results in an adult nematode (the hermaphrodite), composed of 959 somatic cells.

The mega-nosed fly (*Moegistorhynchus longirostris*) of southern Africa, like its literary counterpart, Pinocchio, has a bizarre appearance that reveals an underlying truth. Its proboscis, which looks like a nose but is actually the longest mouthpart of any known fly, protrudes as much as four inches from its head—five times the length of its bee-size body. In flight the ungainly appendage dangles between the insect’s legs and trails far behind its body.

To an airborne fly, an elongated proboscis might seem a severe handicap (imagine walking down the street with a twenty-seven-foot straw dangling from your mouth). Apparently, though, the handicap can be well worth its aerodynamic cost. The outlandish proboscis gives the mega-nosed fly access to nectar pools in long, deep flowers that are simply out of reach to insects with shorter mouthparts.

But that poses a conundrum: why would natural selection favor such a deep tube in a flower? After all, nectar itself has evolved because it attracts animals that carry pollen, the sperm of the floral world, from one plant to another. And since pollinators perform such an essential service for the flower, shouldn’t evolution have favored floral geometries that make nectar readily accessible to the pollinators?

Yet the story of the long proboscis of the mega-nosed fly and the long, deep tubes of the flowers on which it feeds is not quite so straightforward. There are subtle advantages; it turns out, to making nectar accessible to only a few pollinators, and nature factors those advantages into the evolutionary equation as well. In fact, the evolution of those two kinds of organisms, pollinator and pollinated, presents an outstanding example of an important evolutionary phenomenon known as
coevolution. Coevolution can explain the emergence of bizarre or unusual anatomies when no simple evolutionary response to natural selection is really adequate. It can help conservationists identify species that could be vital in maintaining a given habitat. And it can help naturalists investigating novel plants predict what kinds of animals might pollinate their flowers.

The coevolution of the mega nosed fly and the plants it pollinates is a tale of extreme specialization. Each species has adapted to changes in the other in ways that have left each of them, to some degree, reliant on the other. The idea that a plant species might become dependent for pollination on a single species of animal goes back to the writings of Charles Darwin. For example, Darwin noted, the flower spur of the Malagasy orchid (*Angraecum sesquipedale*) contains a pool of nectar that is almost a foot inside the opening of the flower. (A flower spur is a hollow, hornlike extension of a flower that holds nectar in its base.) In pondering the evolutionary significance of those unusual flowers, Darwin predicted that the orchid must be adapted to a moth pollinator with a long proboscis.

Critical to Darwin’s prediction was his suspicion that pollination could take place only if the depth of a plant’s flowers matched or exceeded the length of a pollinator’s tongue. Only then would the body of the pollinator be pressed firmly enough against the reproductive parts of the flower to transfer pollen effectively as the pollinator fed. Thus, as ever deeper flowers evolved through enhanced reproductive success, moths with ever longer proboscises would also, preferentially, live long enough to reproduce, because they would most readily reach the available supplies of nourishing nectar. Longer proboscises would lead yet again to selection for deeper flower tubes.

The result would be the reciprocal evolution of flowers and pollinator mouthparts. That coevolutionary process would cease only when the disadvantages of an exaggerated trait balanced or outweighed its benefits. Given enough time, the process might even produce new species: an insect the specializes in feeding on nectar from deep flowers, and a deep-flowered plant specialized for being pollinated by insects with long mouthparts.

In the early twentieth century it seemed that Darwin’s prediction had been borne out. A giant hawk moth from Madagascar, *Xanthopan morganii praedicta*, was captured, with a proboscis that measured more than nine inches long. Although no one has actually seen the insect feeding on the flower, the discovery is still remarkable, and strongly suggestive of the coevolution of the orchid and moth.
Other insects that have relationships with highly specific plants, such as the mega
nosed fly and other, related long-nosed fly species of southern Africa, provide
even better evidence of the reciprocal links between planes and their pollinators.

Darwin would have been amazed that some flies in southern Africa have longer
tongues than most hawk moths do. After all, the flies' bodies are several times
smaller than the hawk moths' are. Flies are described as long-nosed if their
mouthparts are longer than three quarters of an inch. By that criterion, more than
a dozen long-nosed fly species are native to southern Africa. They belong to two
families. The nemestrinids, or tangle-veined flies (which include the mega-nosed
fly), feed solely on nectar, whereas the tabanids, or horseflies, feed mostly on
nectar, though female tabanids have separate mouthparts to suck blood for their
developing eggs.

Like all other long-nosed flies, the mega nosed fly is the sole pollinator to a group
of unrelated plant species: such a group is known as a guild. The plant guild of the
mega nosed fly includes species from a wide variety of plant families, including
geranisms, irises, orchids, and violets.

Even though guild members may be only distantly related, all of them have roughly
the same characteristics. For example, plants in the long-nosed fly guild all have
long, straight floral tubes or spurs; brightly colored flowers that are open during
the day; and no scent. The defining traits of a guild together form what botanists
call a pollination syndrome. For example, bird-pollinated flowers are typically large,
red, and unscented, whereas moth-pollinated flowers are more likely to be long,
narrow, white, and scented in the evening.

The most important trait in the pollination syndrome of the long-nosed fly (and
indeed, in all pollination syndromes of long-nosed insects) is a deep, tubular flower
or floral spur. One of us (Johnson) and Kim E. Steiner of the Compton Herbarium in
Claremont, South Africa, studied the orchid Disadraconis, a southern African plant
with a deep, tubular floral spur. The two investigators artificially shortened the
spurs of some orchids in a habitat where the only pollinators present were long-
nosed flies. The plants whose spurs remained long got more pollen, and were more
likely to produce fruits, than the ones whose spurs were shortened.

Yet short floral spurs are not necessarily a reproductive disadvantage. Shorter
spurs would make it possible for a wider range of pollinators to access the nectar,
if various potential pollinators are present. Instead, longer spurs only seem to be
an advantage when long-tongued insects are the sole pollinators. Johnson and Steiner found that differences in spur length among populations cannot be blamed on differences in moisture or temperature, thus reinforcing their conclusion that spur length was an adaptation to the local distributions of long-tongued flies.

Not only does spur length correlate statistically with pollinator traits, but a direct causal connection can be demonstrated. Johnson and Ronny Alexandersson, a botanist at Uppsala University in Sweden, studied South African Gladiolus flowers pollinated by long-tongued hawk moths. When the hawk moth proboscises were long compared to the length of the flower tube, the hawk moths did not efficiently pick up pollen, and the flowers did not reproduce well. When the hawk moth proboscises were relatively short, pollen was more readily transferred, and the plants were more likely to be fertilized and bear fruit. Thus the length of the pollinator’s proboscis exerts a strong pressure on the reproductive success of the flowers.

Those studies and others suggest that what Darwin predicted of the Malagasy orchid is a rather general phenomenon: hawk moths and long-nosed flies coevolved with their plant partners. As floral tubes became longer, so did the pollinators’ proboscises, and those led, in turn, to even longer flowers. As the lengths of the flower tube and the insect proboscis converge, a remarkable degree of specialization develops. The plants come to rely for pollination on the few insect species that can reach their flowers’ nectar supplies.

There are advantages for the specialists on both sides of this relationship. The long-nosed flies obviously get privileged access to pools of nectar. And the plants pollinated by long-nosed flies benefit from a near-exclusive pollen courier service—or at least one that minimizes the risk of delivery to the wrong address. But specializing can also be a risky strategy for the plants if the pollinators are less interested in fidelity than the plants are. Long-nosed flies could not survive on the nectar they could get by visiting just one plant species; the flies must visit several plant species to gather the energy they need. Johnson and Steiner observed mega nosed flies visiting at least four species with deep flowers.

Such promiscuous behavior could be detrimental to the plants. A fly might end up carrying pollen from one species to a different species in the guild, thereby wasting the pollen. Worse, the foreign pollen could end up clogging the stigmata, the female reproductive structures, of the receiving flowers, preventing them from getting the "right" pollen. But the stigmata of plants in the guild of the mega nosed fly do not clog, because among those plants yet another clever adaptation to
specialized pollination has evolved. Each plant species arranges its anthers, the male reproductive structures, in a characteristic position. That way, the pollen from each species sticks to the pollinator’s body in a distinct but consistent, plant-specific location. The fly becomes an even more efficient courier, carrying pollen from various plant species simultaneously, say, on its head, legs, and thorax.

The risks of specialization are not confined to the flowers. Just as the flies are unfaithful partners, some flowers are dishonest about signaling a nectar reward. The orchid D. draconis, for instance, is not the mutualistic partner it seems. The flower attracts the mega-nosed fly because it looks like other members of the fly’s guild. But, whereas the fly carries the orchid’s pollen, the orchid offers no nectar in return.

The risk of falling for such a trick seems a small price for the flies to pay for the benefits of specialization. But specialization also carries a much graver risk—in fact the ultimate risk—for both members of the partnership because the disappearance of either partner is likely to doom the other one, as well. Some plant species have mechanisms, such as vegetative reproduction or self-pollination, that may help sustain their populations in the short run. But in the long run, without their pollinators, the species will slowly and irrevocably decline. Pollinating insects may be more flexible in some cases, but are still vulnerable if a key food source disappears.

Unfortunately, in southern Africa that is just what is happening to many plants and their long-nosed fly partners. Often not even closely related insect species can help in pollination. For affected plants, the loss of a single fly species means extinction. And examples of that gloomy cascade have already been observed. Peter Goldblatt of the Missouri Botanical Garden in St. Louis and John C. Manning of the Compton Herbarium have reported that many populations of long-nosed flies are threatened by the loss of their wetland breeding habitat, and also, possibly, by the loss of other insects they parasitize during their larval stages. In some habitats, flowers in the long-nosed fly guild already produce no seeds, because their pollinator is locally extinct.

Naturalists have accepted the concepts of guilds and pollinator syndromes for many years, and predicting which pollinators regularly visit which plants has become something of a cottage industry. But just how common is pollinator specialization in southern Africa? Promiscuity could turn out to be a more successful—and more
In recent years ecologists have discovered that just because plants and insects appear to form a pollination guild does not guarantee they never venture outside it. For example, ecologists have noted that in years when hummingbird populations are low, flowers ordinarily pollinated by hummingbirds can fill up with nectar and become pollinated effectively by bees. Likewise, bees once thought to specialize in only one or two plant species turn out to forage on a variety of plants.

The take-home lesson has been that the syndrome concept is no substitute for careful field observation. Some investigators even think that the concept has caused botanists to overlook generalists. In the Northern Hemisphere, for instance, studies suggest that generalization is the norm, not the exception. Johnson and Steiner recently completed a study showing that members of the orchid and asclepiad families in the Northern Hemisphere tend to rely on between three and five pollinators each. In contrast, plants from the same families in the Southern Hemisphere rely on just one pollinator each.

So why might generalization be more common in the Northern Hemisphere than it is in the Southern Hemisphere? Perhaps the reason is that social bees, which are largely opportunistic, dominate pollinator faunas in northern regions. In the Southern Hemisphere, by contrast, social bees are mostly absent, replaced instead by more specialized pollinators such as the long-nosed flies and hawk moths.

But that is just a broad generalization itself. More data on the geographic distribution of pollinator specialization needs to be gathered, particularly in tropical countries. The data is vital, not only to advance the specialization debate, but also to protect as many of these unique species and relations as possible, lest they disappear forever.
17 October 1978

The Royal Swedish Academy of Sciences decided to award the 1978 Nobel Prize in Chemistry to Dr Peter Mitchell, Glynn Research Laboratories, Bodmin, Cornwall, UK, for his contribution to the understanding of biological energy transfer through the formulation of the chemiosmotic theory.

Chemiosmotic Theory of Energy Transfer

Introduction

Peter Mitchell was born in Mitcham, in the County of Surrey, England, on September 29, 1920. His parents, Christopher Gibbs Mitchell and Kate Beatrice Dorothy (née) Taplin, were very different from each other temperamentally. His mother was a shy and gentle person of very independent thought and action, with strong artistic perceptiveness. Being a rationalist and an atheist, she taught him that he must accept responsibility for his own destiny, and especially for his failings in life. That early influence may well have led him to adopt the religious atheistic personal philosophy to which he has adhered since the age of about fifteen. His father was a much more conventional person than his mother, and was awarded the O.B.E. for his success as a Civil Servant.

Peter Mitchell was educated at Queens College, Taunton, and at Jesus college, Cambridge. At Queens he benefited particularly from the influence of the Headmaster, C. L. Wiseman, who was an excellent mathematics teacher and an
accomplished amateur musician. The result of the scholarship examination that he took to enter Jesus College Cambridge was so dismally bad that he was only admitted to the University at all on the strength of a personal letter written by C. L. Wiseman. He entered Jesus College just after the commencement of war with Germany in 1939. In Part I of the Natural Sciences Tripos he studied physics, chemistry, physiology, mathematics and biochemistry, and obtained a Class III result. In part II, he studied biochemistry, and obtained a II-I result for his Honours Degree.

He accepted a research post in the Department of Biochemistry, Cambridge, in 1942 at the invitation of J. F. Danielli. He was very fortunate to be Danielli's only Ph.D. student at that time, and greatly enjoyed and benefited from Danielli's friendly and unauthoritarian style of research supervision. Danielli introduced him to David Keilin, whom he came to love and respect more than any other scientist of his acquaintance.

He received the degree of Ph.D. in early 1951 for work on the mode of action of penicillin, and held the post of Demonstrator at the Department of Biochemistry, Cambridge, from 1950 to 1955. In 1955 he was invited by Professor Michael Swann to set up and direct a biochemical research unit, called the Chemical Biology Unit, in the Department of Zoology, Edinburgh University, where he was appointed to a Senior Lectureship in 1961, to a Readership in 1962, and where he remained until acute gastric ulcers led to his resignation after a period of leave in 1963.

From 1963 to 1965, he withdrew completely from scientific research, and acted as architect and master of works, directly supervising the restoration of an attractive Regency-fronted Mansion, known as Glynn House, in the beautiful wooded Glynn Valley, near Bodmin, Cornwall - adapting and furnishing a major part of it for use as a research laboratory. In this, he was lucky to receive the enthusiastic support of his former research colleague Jennifer Moyle. He and Jennifer Moyle founded a charitable company, known as Glynn Research Ltd., to promote fundamental biological research and finance the work of the Glynn Research Laboratories at Glynn House. The original endowment of about £250,000 was donated about equally by Peter Mitchell and his elder brother Christopher John Mitchell.

In 1965, Peter Mitchell and Jennifer Moyle, with the practical help of one technician, Roy Mitchell (unrelated to Peter Mitchell), and with the administrative help of their company secretary, embarked on the programme of research on
chemiosmotic reactions and reaction systems for which the Glynn Research Institute has become known. Since its inception, the Glynn Research Institute has not had sufficient financial resources to employ more than three research workers, including the Research Director, on its permanent staff. He has continued to act as Director of Research at the Glynn Research Institute up to the present time. An acute lack of funds has recently led to the possibility that the Glynn Research Institute may have to close.

Mitchell studied the mitochondrion, the organelle that produces energy for the cell. ATP is made within the mitochondrion by adding a phosphate group to ADP in a process known as oxidative phosphorylation. Mitchell was able to determine how the different enzymes involved in the conversion of ADP to ATP are distributed within the membranes that partition the interior of the mitochondrion. He showed how these enzymes' arrangement facilitates their use of hydrogen ions as an energy source in the conversion of ADP to ATP.

Chemiosmotic hypothesis: Proposed by Peter Mitchel (1970) to explain how NADH oxidation is coupled to ATP synthesis.

As electrons are passed down the chain, protons are pumped across the membrane (between the inner membrane and outer membrane of the cristae or thylakoids). This results in a pH and electrical gradient. The protons move back into the matrix through a pore created by ATP synthetase allowing the enzyme to make ATP at the expense of this gradient.

Peter Mitchell's 1961 paper introducing the chemiosmotic hypothesis started a revolution which has echoed beyond bioenergetics to all biology, and shaped our understanding of the fundamental mechanisms of biological energy conservation, ion and metabolite transport, bacterial motility, organelle structure and
biosynthesis, membrane structure and function, homeostasis, the evolution of the
eukaryote cell, and indeed every aspect of life in which these processes play a role.
The Nobel Prize for Chemistry in 1978, awarded to Peter Mitchell as the sole
recipient, recognized his predominant contribution towards establishing the
validity of the chemiosmotic hypothesis, and ipso facto, the long struggle to
convince an initially hostile establishment.

NOBEL PRIZE IN CHEMISTRY FOR BIOLOGICAL ENERGY TRANSFER

Mitchell’s research has been carried out within an area of biochemistry often
referred to in recent years as 'bioenergetics', which is the study of those
chemical processes responsible for the energy supply of living cells. Life processes,
as all events that involve work, require energy, and it is quite natural that such
activities as muscle contraction, nerve conduction, active transport, growth,
reproduction, as well as the synthesis of all the substances that are necessary for
carrying out and regulating these activities, could not take place without an
adequate supply of energy.

It is now well established that the cell is the smallest biological entity capable of
handling energy. Common to all living cells is the ability, by means of suitable
enzymes, to derive energy from their environment, to convert it into a biologically
useful form, and to utilize it for driving various energy requiring processes. Cells of
green plants as well as certain bacteria and algae can capture energy by means of
chlorophyll directly from sunlight - the ultimate source of energy for all life on
Earth - and utilize it, through photosynthesis, to convert carbon dioxide and water
into organic compounds. Other cells, including those of all animals and many
bacteria, are entirely dependent for their existence on organic compounds which
they take up as nutrients from their environment. Through a process called cell
respiration, these compounds are oxidized by atmospheric oxygen to carbon
dioxide and water.

During both photosynthesis and respiration, energy is conserved in a compound
called adenosine triphosphate, abbreviated as ATP. When ATP is split into
adenosine diphosphate (ADP) and inorganic phosphate (Pi), a relatively large amount
of energy is liberated, which can be utilized, in the presence of specific enzymes,
to drive various energy-requiring processes. Thus, ATP may be regarded as the
universal 'energy currency' of living cells. The processes by which ATP is formed
from ADP and Pi during photosynthesis and respiration are usually called
'photophosphorylation' and 'oxidative phosphorylation', respectively. The two
processes have several features in common, both in their enzyme composition - both involve an interaction between oxidizing (electron-transferring) and phosphorylating enzymes - and in their association with cellular membranes. In higher cells, photophosphorylation and oxidative phosphorylation occur in specific membrane-enclosed organelles, chloroplasts and mitochondria, respectively; in bacteria, both these processes are associated with the cell membrane.

The above concepts had been broadly outlined by about the beginning of the 1960s, but the exact mechanisms by which electron transfer is coupled to ATP synthesis in oxidative phosphorylation and in photophosphorylation remained unknown. Many hypotheses were formulated, especially with regard to the mechanism of oxidative phosphorylation; most of these postulated a direct chemical interaction between oxidizing and phosphorylating enzymes. Despite intensive research in many laboratories, however, no experimental evidence could be obtained for any of these hypotheses. At this stage, in 1961, Mitchell proposed an alternative mechanism for the coupling of electron transfer to ATP synthesis, based on an indirect interaction between oxidizing and phosphorylating enzymes. He suggested that the flow of electrons through the enzymes of the respiratory or photosynthetic electron-transfer chains drives positively charged hydrogen ions, or protons, across the membranes of mitochondria, chloroplasts and bacterial cells. As a result, an electrochemical proton gradient is created across the membrane. The gradient consists of two components: a difference in hydrogen ion concentration, or pH, and a difference in electric potential; the two together form what Mitchell calls the 'protonmotive force'. The synthesis of ATP is driven by a reverse flow of protons down the gradient. Mitchell's proposal has been called the 'chemiosmotic theory'.

This theory was first received with scepticism; but, over the past 15 years, work in both Mitchell's and many other laboratories have shown that the basic postulates of his theory are correct. Even though important details of the underlying molecular mechanisms are still unclear, the chemiosmotic theory is now generally accepted as a fundamental principle in bioenergetics. This theory provides a rational basis for future work on the detailed mechanisms of oxidative phosphorylation and photophosphorylation. In addition, this concept of biological power transmission by protonmotive force (or 'proticity', as Mitchell has recently began to call it in an analogy with electricity) has already been shown to be applicable to other energy-requiring cellular processes. These include the uptake of nutrients by bacterial cells, cellular and intracellular transport of ions and metabolites, biological heat production, bacterial motion, etc. In addition, the
chloroplasts of plants, which harvest the light-energy of the sun, and the mitochondria of animal cells, which are the main converters of energy from respiration, are remarkably like miniaturized solar- and fuel-cell systems. Mitchell's discoveries are therefore both interesting and potentially valuable, not only for the understanding of biological energy-transfer systems but also in relation to the technology of energy conversion.
