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The Road to Stockholm: [Dr. Bruce Merrifield]

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THE ROCKEFELLER UNIVERSITY

RESEARCH PROFILES

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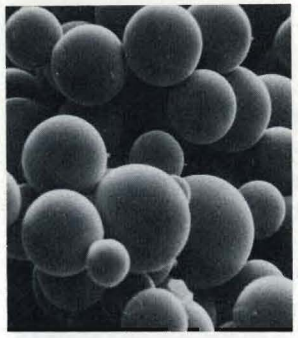


Something to hang your peptide on.

The Road to Stockholm

In his Nobel Lecture last fall, Bruce Merrifield, John D. Rockefeller Jr. Professor of The Rockefeller University, quoted these words written in 1906 by the great German chemist Emil Fischer:

"Whereas cautious professional colleagues fear that a rational study of this class of compounds, because of their complicated structure and their highly inconvenient physical characteristcs, would today still uncover insurmountable difficulties, other optimistically endowed observers, among which I count myself, are inclined to the view that an attempt should at least be made to besiege this virgin fortress. . . . "

If Fischer's rhetoric sounded convoluted and old-fashioned, particularly as spoken by a Texas-born American, his message was visionary. The compounds he was referring to were proteins, and he knew that within their daunting complexities and "inconvenient characteristics" lay the answers to some of the most central questions in biology and medicine.

Fischer himself helped to open the path toward the "virgin fortress." Others pushed farther along the trail. Then, in the 1960s, Bruce Merrifield veered off in a different direction and came up with a simple, ingenious strategy for storming the citadel. He was awarded the 1984 Nobel Prize in Chemistry for the development of "chemical synthesis on a solid matrix," a totally novel technology that is helping scientists penetrate and manipulate biological molecules with a precision Fischer could only dream of. His work, stated the Royal Swedish Academy of Sciences, "has created completely new possibilities in the field of peptide and protein chemistry . . . as well as in the field of nucleic acid chemistry where other researchers have applied Merrifield's ideas."

Stockholm, December 10, 1984. Bruce Merrifield greets his family after the Nobel ceremony.

The news of the prize was greeted with particular pride on the campus where the achievement honored had been born and nurtured. Dr. Merrifield has been at Rockefeller since 1949, an emblematic year in which, says his wife, Elizabeth, "he got his Ph.D. from UCLA on a Sunday, we got married on Monday, and we left for New York on Tuesday." The new celebrity — a quiet, shirtsleeves man whose nonworking pleasures center on camping trips with his family and fiercely competitive Ping-Pong — anticipated with some trepidation the trial by white tie and tails awaiting him in Stockholm. In retrospect he confesses, "We had a wonderful time."

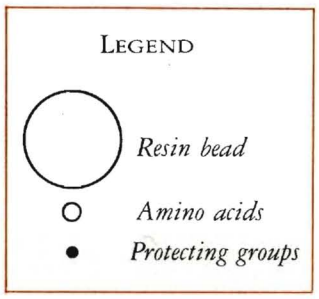
Dr. Merrifield in his Rockefeller office. On the wall behind him, a photograph of his mentor, the late D.W. Woolley.

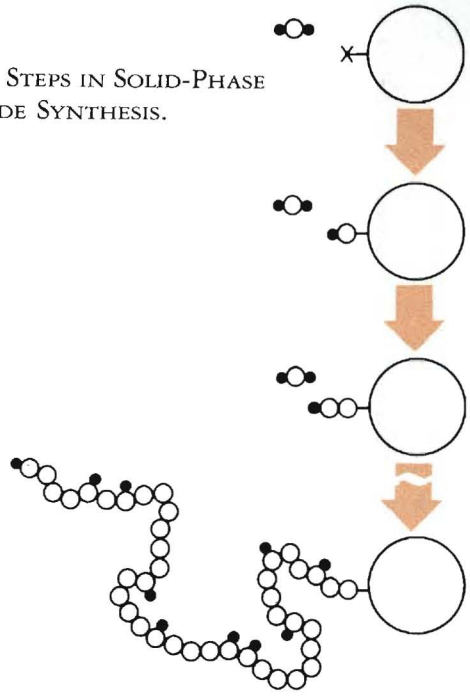
TINY PLASTIC BEADS

Living tissue is built primarily of proteins. The all-important enzymes, tens of thousands in each cell mobilizing the chemical reactions that fuel and sustain life, are proteins. Most of the hormones that regulate these reactions are proteins or peptides, smaller versions of proteins. One way scientists study these molecules is by isolating them from their natural habitat, not always easy or practical to do since some are as rare or unstable as they are crucial. Another way is by chemical synthesis: making them in the laboratory.

Amino acids are the basic constituents of peptides and proteins, which are huge polypeptides. With twenty different amino acids, Nature effortlessly effects millions of combinations, from tiny dipeptides — two amino acids — to sinuous proteins thousands of amino acids long. For the biochemist seeking to emulate Nature the task is a little like playing three-dimensional chess in the dark without a board.

The major problem lies in the formation of the bonds that couple amino acids. Amino acids contain different reactive chemical groups: an amino group, a carboxyl group, and often another group on a side chain. In forming a peptide chain, these groups must be chemically "protected," or blocked, against unwanted combinations. To prepare a dipeptide, the amino group of one unit and the carboxyl group of the other must be blocked. The carboxyl group of the first can then be activated to form a bond with the free amino group of the second. To add a third unit, it is necessary to "deprotect" one of the blocked groups in the dipeptide.



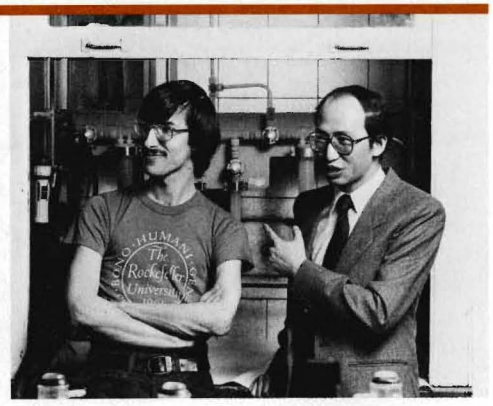


BASIC STEPS IN SOLID-PHASE PEPTIDE SYNTHESIS.

Emil Fischer was the first to synthesize dipeptides. His student Max Bergmann, together with Leonidas Zervas, made the key discovery (of the carbobenzoxy group that reversibly blocks the amino group), which made the so-called classical method of synthesis broadly available to peptide chemists. Bergmann was also responsible for bringing peptide chemistry to this country. He worked at Rockefeller from 1934 until his death in 1944. (His old office is now Dr. Merrifield's.) Classical methods were still in use when Dr. Merrifield began synthesizing peptides as a junior member of the Rockefeller laboratory of D. W. Woolley. "They were effective," he says, "but they were laborious and time-consuming. Depending on the size and complexity of the peptide, the process could take months or even years. For a beginner like me it was extremely frustrating."

The coupling steps, carried out in solution, required that each time a new amino acid was added to the growing chain the products had to be removed from the reaction vessel and purified. However, when crystallization, the standard purification procedure, was used with long chains, it frequently yielded amorphous material contaminated with by-products, necessitating further purification. "Plodding along," Bruce Merrifield began to wonder whether there might be another way. Then, in 1959, he got an idea.

Dr. Merrifield likens the amino acids in a peptide to the boxcars of a train. The idea that came to him was to put the train on a track. "My thought," he says, "was to assemble peptide chains by attaching the amino acids to one another on a solid support. The first amino acid would be attached to the support by one end, leaving only the desired end receptive to the next amino acid. Since the growing chain was anchored within the

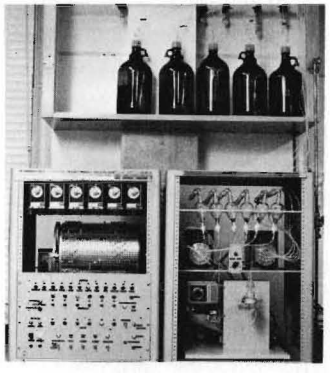


IMMEDIATE RIGHT. *Elizabeth Merrifield.*

CENTER. *Emil Kaiser.*

FAR RIGHT. *William Heath, left, and James Tam at an apparatus in which a hydrogen fluoride mixture deprotects and cleaves peptides. With Dr. Merrifield they designed major improvements in the chemistry of the process, resulting in purer synthetic products. Dr. Tam, a member of the laboratory since 1976, is studying a tumor growth factor and cancer genes and is working to develop a synthetic malaria vaccine.*

The first automatic peptide synthesizer. The synthetic chemistry takes place in the right-hand cabinet. The programmer is on the left side.



matrix of the insoluble support, the peptide would also be insoluble. After each synthetic reaction, the mixture could be filtered and washed to remove excess reactants and by-products *without* having to be removed from the reaction vessel. The growing chain could be purified step-by-step by a simple, rapid procedure rather than by tedious crystallization." With a nod from Dr. Woolley, he began looking for the track.

"I originally had cellulose in mind because it had been useful for chromatography of proteins," he explains. "But cellulose is unstable with some reagents, and when I used it everything turned to goo. So, then, I decided to try plastics. I really didn't know much about polymer chemistry at the time and I sweated through several dead-end attempts before I got to polystyrene. I did know I would have to modify the material — put a reacting group on it — because otherwise it would be inert. Well, there are a lot of ways to activate polystyrene and I managed to pick all the wrong ones before I finally found a reaction that worked."

The beads of polystyrene resin Dr. Merrifield used are two-thousandths of an inch in diameter, gigantic compared to peptides. And they blow up like balloons when immersed in solvent. Each bead potentially can support a trillion peptide chains. The "track" is in fact a porous gel, a mesh structure that allows penetration of reagents as it swells. Once the peptide is synthesized, it can be removed from the support.

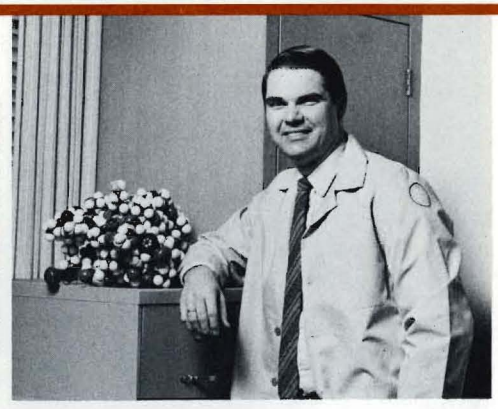
Proving his concept feasible, which he had thought would take about three months, took three years. "During all that time, I produced no publications and it didn't appear I was making progress. In some other places," he reflects, "I think there would have been raised eyebrows."

In allowing his young colleague to continue working unhampered on an unconventional idea he thought had merit, Wayne Woolley followed an institutional precedent which, by and large, has given considerable leeway to talented researchers. Woolley himself tended to wander wherever his interests led him. In a series of brilliant studies, he helped to clarify the function of vitamins, particularly the B vitamins, and of antimetabolites that interfere with vitamin activity, and to establish the cause and treatment of pellagra. From nutritional concerns, he turned to the role of the hormone serotonin in hypertension, and from there to a recognition of the influence of serotonin on mental processes. His book, *The Biochemical Bases of Psychoses,* published in 1962, presaged what was to become one of the most fertile fields in contemporary science.

Woolley, whom Dr. Merrifield remembers as "the brightest man I ever knew," was blind through most of his working life, the result of diabetes. He died from the effects of the disease in 1966 at the age of fifty-two. "He lost his sight shortly after he came here," Dr. Merrifield says. "However, Herbert Gasser, then the director of Rockefeller, recognizing Woolley's gifts, set him up in a lab and let him work."

THE MERRIFIELD MACHINE AND THE ROCKEFELLER ENZYME

Dr. Merrifield's first modest success with the method that came to be known as solid-phase peptide synthesis was a tetrapeptide: a simple chain of four amino acids. (The classical method is now called solution-phase synthesis.) After working out some bugs in the chemistry, he attempted to grow a real-life compound, bradykinin, a nine amino-acid hypotensive hormone. He completed its synthesis in less than a week. John Stewart, another member of Woolley's group, who had spent a year synthesizing three analogs of bradykinin by solution-phase, applied solid-phase and prepared nearly fifty analogs during the following year. Not long after, Garland Marshall, Dr. Merrifield's first graduate student, synthesized angiotensin, a polypeptide hor-



FAR LEFT. *In a return to ribonuclease, first synthesized in 1969, Nagarajan Chandramouli operates a manual synthesizer to make the enzyme with modified activity. With him, research assistant Guadalupe Gautier.*

CENTER. *David Andreu, left, acquaints visiting scientist Li, Zong-qu from the People's Republic of China with the operation of a computerized synthesizer. Dr. Andreu is making a polypeptide similar to glucagon, which will block receptor sites for the hormone in the liver.*

NEAR LEFT. *Bruce Erickson with a model of betabellin, a 65 amino-acid protein he created to show that folding patterns could be predicted, a first step toward the design of proteins with predetermined functions.*

mone that raises blood pressure. "I had no idea how long a peptide we could make," Dr. Merrifield says. "We just kept inching up."

From the beginning, he had been thinking about the possibility of automating his process. "I even tried to set up a simple apparatus myself," he says, "but I couldn't get it to work. After bradykinin, John Stewart offered to help. He was a radio buff, so he handled the wiring while I took care of the plumbing. The valves were machined by Nils Jernberg, the University's instrument maker. John and I constructed the synthesizer at night in the basement at home."

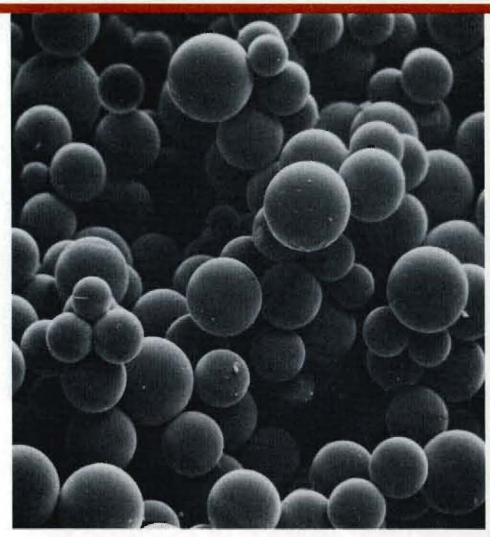
In 1965, they completed a working model of an automatic peptide-making machine. When Dr. Merrifield and a student, Arnold Marglin, applied it to the synthesis of insulin, the smallest polypeptide that qualifies as a protein, they obtained results in a fraction of the time previously required with classical methodology.

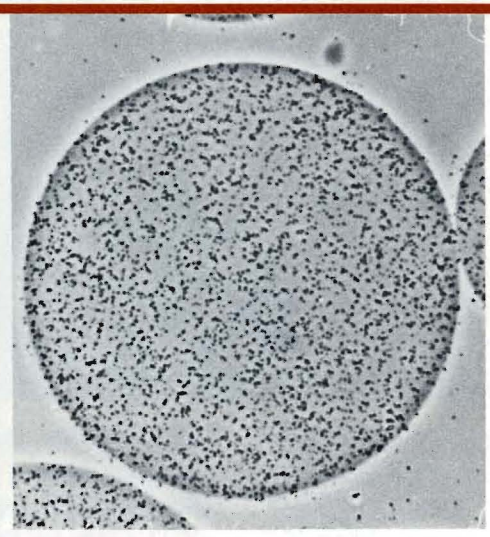
The challenge still looming was an enzyme. The one Dr. Merrifield selected, for scientific and sentimental reasons, was ribonuclease A, which catalyzes the breakdown of ribonucleic acid (RNA). He chose it because it is one of the smallest enzymes — 124 amino acids long — and its properties were well known, mostly because of research done at Rockefeller. It had been isolated and named by René Dubos and crystallized by Moses Kunitz. Its molecular weight had been determined by Alexandre Rothen and its chemical structure mapped in the laboratory of Stanford Moore and William Stein, both of whom began at Rockefeller under Max Bergmann and went on to share a Nobel Prize in Chemistry in 1972.

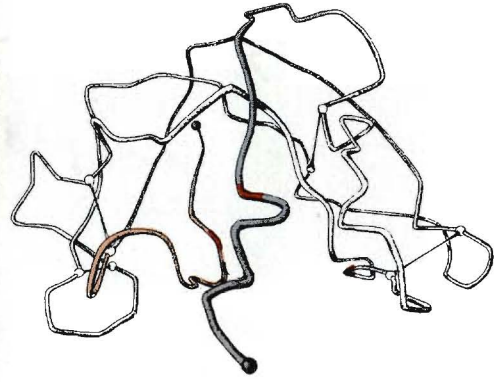
Three hundred and sixty-nine chemical reactions and eleven thousand nine hundred and thirty-one mechanical steps later, Bernd Gutte (now of the University of Zurich) and Dr. Merrifield had a chain. However, biologically born enzymes twist and bend, and their shapes are critical to their function. It had been discovered a few years earlier in Christian B. Anfinsen's laboratory at the National Institutes of Health that when natural ribonuclease A is unfolded, thereby losing its three dimensional structure and enzymatic activity, it refolds by itself and regains activity if allowed to stand in solution. The question was, Would the synthetic enzyme also fold spontaneously into the natural structure? The answer was yes. However, it had chemical impurities.

"The goal in synthesis," Dr. Merrifield explains, "is to come as close as possible to a one-hundred percent yield. That is, each time a new amino acid is added the coupling should be complete. But if, for example, the second amino acid couples with only ninety-eight percent of the first, the third will react not only with the dipeptide but also with the unreacted two percent of the first amino acid, to produce a contaminating by-product. Even with the slightest deviations the accumulation in a long chain can add up considerably, and some of our early products were pretty crude. Although we had succeeded in making an enzyme with eighty percent of expected ribonuclease activity — and I was convinced the yields could be improved — a lot of people were skeptical." (One distinguished peptide chemist, Dr. Merrifield remembers, assessed solid-phase as "amusing.")

For the past twenty years Dr. Merrifield has dedicated a major part of the effort in his laboratory to confirming his conviction, refining the chemistry and developing new support materials, tackling ever more difficult synthetic challenges and higher molecular numbers. "We're at the point," he says, "where we can make almost any peptide we want with a good level of purity." Among the compounds his group has synthesized are the hormones glucagon, important in blood sugar regulation, and thymosin, involved in white blood cell development, and a variety of growth factors, antibiotics, and toxins. Anaphy-







IMMEDIATE RIGHT. *Scanning electron micrograph of cross-linked polystyrene beads used in solid-phase peptide synthesis.*

Center. Autoradiograph of a cross section of a bead showing distribution of radioactively labelled peptide chains.

Far right. Ribonuclease. After synthesizing the whole ribonuclease molecule, Dr. Merrifield's laboratory went on to study the enzyme by synthesizing the peptides at each end. The carboxyl end is shown in light orange, the amino end in gray. The dark orange areas represent catalytic sites.

latoxins, a group of inflammation mediators, have been a special interest of lab member Bruce Erickson, who is seeking to design anti-inflammatory drugs for use in such conditions as rheumatoid arthritis.

One current project is the synthesis of interferon, a protein of one hundred sixty-six amino acids, which is produced by the body to fight viral infection and may have anti-cancer properties. The work is being conducted by a former student of Dr. Merrifield's from his days as a lab instructor at UCLA, who interrupted her studies to marry the teacher and rear five daughters and a son. Mrs. Merrifield resumed her scientific career five years ago. "I'm at the hard part now," she says, "purifying the molecule and getting it to fold right."

Dr. Merrifield has had a number of apt students. George Barany, sixteen years old and fresh out of high school when he was accepted as a Ph.D. candidate at Rockefeller, has devoted considerable attention to methods for making difficult sulfur-containing compounds. Now leading his own group at the University of Minnesota, Dr. Barany is continuing to develop new solid-phase chemistry. Last December he was included in a selection by *Science Digest* of the country's brightest scientists under forty.

WHY MAKE PEPTIDES?

For basic researchers, the reason for synthesizing molecules is the hope of understanding biology better. "Sometimes," says Dr. Merrifield, "you come upon a new entity. You want to find out what it is, what its properties are." Until recently, biochemists had known of a number of important peptide hormones, but, in general, they had assumed peptides to be relatively minor players on the biochemical stage. They found out they were wrong. One of the exciting events in the past decade was the discovery that neuropeptides, hormones in the brain, bear major responsibility for mediating activity in the nervous system. Much of the pioneer research on these compounds depended on solid-phase synthesis.

Peptides and proteins also serve as growth factors, a major area under study for some time by James Tam and more recently by graduate fellow William Heath. During his first couple of years in the lab, Bill Heath worked with Dr. Merrifield and Dr. Tam on ways to eliminate unwanted synthetic side reactions. "Now," he says, "I'm using the improvements we made to put together epidermal growth factor. It's a complicated structure, at least half the amino acids in it are considered problems, so it really tests the methodology to the utmost. That makes it interesting technically, but this particular peptide is also exciting because part of its receptor molecule is an oncogene — a cancer gene."

For thirty years, Dr. Merrifield has lived in the suburban town of Cresskill, New Jersey, and for all those years he has driven to work with a neighbor and Rockefeller colleague, Vincent Allfrey. "Bruce drives and I read," says Dr. Allfrey. "We've gone through four volumes of Churchill's memoirs and all the Lewis and Clark journals — and a number of cars." They also talk shop. Dr. Allfrey studies proteins in the nucleus of cells that affect the activity of DNA. "Bruce and I have collaborated

on a number of projects. For example, his people and mine have been creating peptides to help us ask about how enzymes work in the nucleus and how certain proteins are programmed to get in and out of there. All kinds of interesting questions."

Another reason for making biological molecules is medical application. In 1966 Emil Kaiser was working at Armour Pharmaceuticals in Chicago. "Armour had wanted to make peptide drugs for a long time," he says, "but we had been discouraged by the difficulties and the cost of solution-phase." After conferring with Dr. Merrifield at a scientific meeting, he became a "true believer." A small solid-phase trial was set up at Armour. Within a few years the group had successfully synthesized several medically important compounds, including calcitonin, a thirty-two amino acid hormone effective in treating Paget's disease and other bone conditions. "Today," says Dr. Kaiser, "calcitonin is a multimillion dollar product."

Dr. Kaiser left Armour in 1982, at the age of 80, to come East with his son, E. Thomas Kaiser, also a biochemist, who had been invited to establish a group at Rockefeller. During a reception in Dr. Merrifield's laboratory to welcome the newcomers, the senior Dr. Kaiser was offered a place in the lab to do research on deprotecting methods. "So thanks to the party," he says, "I didn't have to retire after all."

Since the days of ribonuclease synthesis, molecular biologists have learned to make proteins by means of genetic engineering; splicing out the genes that code for their natural manufacture and using recombinant DNA techniques to clone copies of them. This remarkable technology has thus made many large proteins readily available. DNA, the genetic material, and RNA, which transcribes and transmits its message, are nucleic acids. In studying nucleic acids, researchers also have eagerly incorporated solid-phase chemistry, adapting the principle originally designed for peptides to string together the nucleotide units of nucleic acids. Initially trained as a nucleic acid chemist, Dr. Merrifield had himself anticipated the valuable potential of this application and worked on the initial phases of its development.

In research on smaller peptides, solid-phase synthesis remains the most efficient methodology. Another very important advantage of the technique is that it can be manipulated to effect structural and functional modifications in molecules. "Most of the people who use our method," Dr. Merrifield explains, "are trying to make compounds with some special quality. They want to make derivatives in which they have added or omitted something, or made something more active, more specific, or longer lived. A good example is the work on vasopressin by Maurice Manning, who used to be with Dr. Woolley and is now at the Medical College of Ohio. Among vasopressin's properties, it's an anti-diuretic, but it also raises blood pressure. Dr. Manning has made hundreds of variants. He's now come up with an analog that has good anti-diuretic properties but no adverse blood pressure effects. He's also made an *anti*-anti-diuretic. The first compound is for people who can't retain fluid, a terrible problem in diabetes insipidus, and the second for people who retain too much.

"Name almost any peptide hormone and you'll find someone has been working with it in this way. Another area generating a good deal of interest is the possibility of making synthetic peptide vaccines modified to eliminate the danger of inadvertent infection that exists with vaccines made from the killed or attenuated infectious organisms themselves."

The sleek, computerized solid-phase synthesizers in use today are manufactured commercially. Dr. Merrifield's original Model T, exhibited for a number of years in a small museum of historic scientific instruments at Rockefeller, is currently being readied for inclusion in the permanent collection of the Smithsonian Institution in Washington, D.C. Meanwhile, its inventor has demonstrated his fitness for future challenges.

In addition to delivering learned lectures and attending state functions, Nobel Prize winners have the special honor of being inducted into The Order of the Perpetually Leaping and Smiling Frogs during the Santa Lucia Ball at the University of Stockholm. As part of the initiation the inductees are ordered, appropriately, to leap like frogs. "One of my kids got a picture of me," says Bruce Merrifield. "My glasses are flying and my feet are clear off the ground."

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