Serendipity in Discovery: From Nitric Oxide to Viagra[1]

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Introduction

A fitting opening to a discussion centered on the biological effects of nitric oxide (NO) is Charles Sheeler's 1930 painting entitled *American Landscape* (Figure 1). In this painting, Sheeler depicts the Ford Motor Company's River Rouge manufacturing plant; a smokestack billowing smoke dominates the center of the painting. Why is this a fitting beginning? NO is a highly toxic gas, and oxides of nitrogen are common constituents of environmental pollutants that could easily have been part of the effluent smoke in Sheeler's painting. In fact, prior to the 1980s, published works on the biological effects of NO largely appeared in journals with a focus on environmental chemistry and toxicology. So it was a great surprise about 30 years ago when NO was found to be an important metabolite made endogenously in humans by an enzyme (nitric oxide synthase). NO is a diatomic gas and is chemically similar to oxygen (O_2) and carbon monoxide (CO), the former being a requirement for aerobic life and the latter a well-known toxin. Compared to CO, NO is even more toxic, and one challenge for nature is to discern the subtle chemical differences among these three diatomic gases.

Two main functions for NO emerged in humans and other animals. The first function turned out to be a role in the immune system, and this perhaps is not a surprise. Studies over many years have established that cells of the immune system, such as macrophages, produce toxins to kill bacterial pathogens and tumor cells. To add NO to this chemical armamentarium of the immune system where high concentrations of toxins are generated locally to kill pathogens does make some sense. A good analogy is when firefighters dig a ditch around a forest fire to prevent it from spreading; NO and other immune system–derived toxins effectively do the same thing by creating a chemical wall that is difficult to cross.

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FIGURE 1. Sheeler, Charles (1883–1965). *American Landscape*. 1930. Oil on canvas, 24 x 31" (61 x 78.8 cm). Gift of Abby Aldrich Rockefeller. The Museum of Modern Art. **Digital Image © The Museum of Modern Art/Licensed by SCALA/ Art Resource, NY.**

The second function for NO is as a signaling molecule (a way for one cell to talk to another cell and elicit a specific response from this second cell). In this signaling role, NO controls some very important physiological processes such as blood vessel dilation. So in short, NO plays a vital role in controlling blood pressure, and, given the toxicity of NO, this is puzzling. This indeed was a surprise to the scientific world. Nature's roll of the evolutionary dice has led to some seemingly irrational choices, and this one certainly falls into that category. Regardless, we were not there when the dice settled so it is up to us to figure out how it is that NO functions in this important capacity without poisoning our own cells. Paracelsus had perhaps the best view of this dialectic when he said, "Poison is in everything, and no thing is without poison. The dosage alone makes it so a thing is not a poison." Clearly nature has evolved the means to handle NO for important tasks and yet keep the dose below the level where NO would be toxic, without question a very difficult challenge.

Why is NO so toxic? Recall the periodic table of the elements and the fact that the periodicity means that elements have increasing

numbers of protons moving from left to right. In the periodic table you will find –C–N–O– in that order; therefore, combinations of these three elements in different ratios will be unique yet show some close similarities to one another. Thus, it is not so surprising that CO can act as an inactive surrogate for O_2 and, in so doing, interfere in the vital life functions of O_2. NO is capable of the same interference with O_2 with the added issue that it is much more chemically reactive compared to O_2 and CO, and consequently creates trouble.

In brief, here is the problem: humans are aerobic organisms, meaning we require O_2 to live, and yet we have evolved to make and use NO to control vital cellular processes. However, NO is toxic to normal O_2 function, so how can we continue to use both NO and O_2 and survive? The answer is actually rather simple—NO works at very low concentrations, as Paracelsus would have expected. NO's safe functions, achieved at very low levels, place stringent demands on the ability to specifically sense NO in the presence of many thousand times more O_2. Indeed these specific sensors have evolved and their discovery was spurred on by investigations seeking answers to questions surrounding NO function in biology.

Key Discoveries

So what were the key discoveries and how were they made? Today we have the benefit of hindsight to tell a story often repeated in science, one of serendipity and curiosity—the mainstays of discovery (Marletta 1989; Marsh and Marsh 2000). The story begins in 1847 when the Italian chemist Ascanio Sobrero (Figure 2), working with Théophile-Jules Pelouze in Paris, discovered nitroglycerin. Chemists' interest in their work often leads them to do things that might seem strange to the non-practitioner, such as smelling what they synthesize (not so surprising) and tasting what they synthesize (requiring a significantly higher level of devotion). Upon tasting nitroglycerin, Sobrero described an intense headache. Several others did experiments with nitroglycerin, including Thomas Lauder Brunton and Constantin Hering, but it was William Murrell who first used it to treat angina pectoris in 1878 (Figure 2). This continued interest came directly from Sobrero's decision to taste nitroglycerin and record his now-famous headache.

It was Alfred Nobel who saw the practical applications of nitroglycerin for explosives, but safe handling was the impediment that would need to be overcome. He was able to accomplish this but not without the personal loss of his brother Emil Oskar Nobel in an 1864 factory explosion. As he progressed in age, Alfred Nobel himself came to suffer from angina pectoris. And then in a great "irony of fate,"
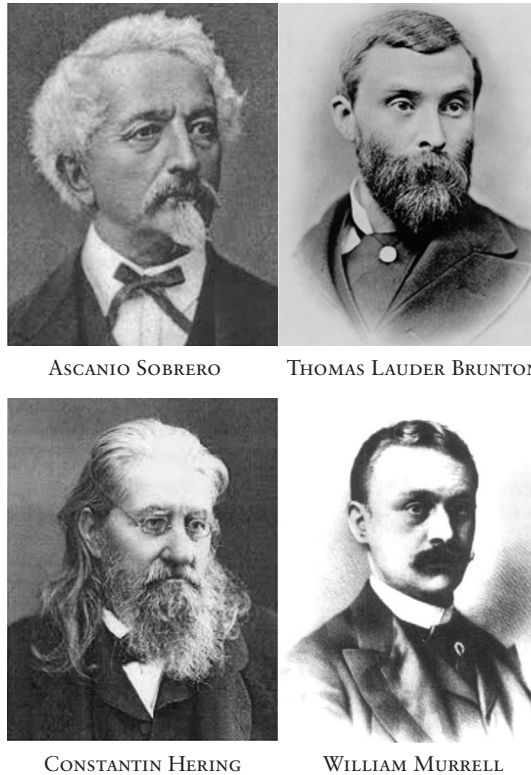
ASCANIO SOBRERO   THOMAS LAUDER BRUNTON

CONSTANTIN HERING   WILLIAM MURRELL

FIGURE 2. Central figures in the early discoveries surrounding "nitrate drugs" and the treatment of angina pectoris. Ascanio Sobrero discovered nitroglycerin in 1847. Thomas Lauder Brunton and Constantin Hering followed up Sobrero's report of a headache induced by nitroglycerin. William Murrell was the first to treat angina pectoris patients with nitroglycerin in 1878.
Sources: Wellcome Library, London. Photograph by G. Jerrard, 1881; and National Library of Medicine, Bethesda, MD.

Nobel was prescribed nitroglycerin to relieve his suffering from angina (Figure 3).

To appreciate our current understanding of NO function in biology, we turn to two ostensibly unrelated tracks of investigation: one in pharmacology involving a search for how blood vessels dilate and the other, seemingly quite distant from that, involving the biosynthesis of nitrates in humans. To place the pharmacological studies in context, it had become known that a metabolite called cyclic guanosine monophosphate (cGMP), made by the enzyme guanylate cyclase, was directly involved in blood vessel dilation. (Recall that enzymes are proteins that catalyze chemical reactions in living organisms.) Further, it was known that guanylate cyclase could be activated (turned on) by organic nitrates
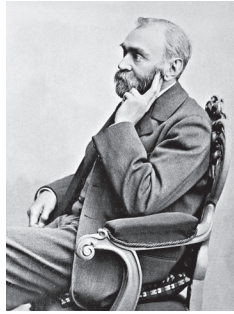
ALFRED NOBEL

"My heart trouble will keep me here in Paris for another few days at least, until my doctors are in complete agreement about my immediate treatment. Isn't it the irony of fate that I have been prescribed N/G 1, to be taken internally! They call it Trinitrin, so as not to scare the chemist and the public."

FIGURE 3. Alfred Nobel and his letter to Ragnar Sohlman dated October 25, 1896. Sources: www.imagebank.sweden.se, Gösta Florman/The Royal Library; and https://www.nobelprize.org/alfred_nobel/biographical/articles/ringertz/.

like nitroglycerin and other related vasodilators, but how did these drugs activate this enzyme? In 1977, Ferid Murad and colleagues were studying this and, in the course of their studies, found that the simplest of nitrates, namely NO, could directly activate guanylate cyclase (Arnold et al. 1977; Figure 4). While an interesting "lab finding," scientific common sense said this could not be biologically relevant since NO was so toxic.

In parallel, Robert Furchgott and his colleagues were following their long-standing interest in how the endothelium causes blood vessels to relax. In a simplistic sense, a blood vessel can be thought of as a garden hose (Figure 4). A transverse slice of the hose reveals an inner lining; that lining can be thought of as the endothelium and the outer part of the hose as smooth muscle. When the smooth muscle relaxes, the blood vessel will dilate, and when it tightens, the vessel will contract. Furchgott showed that the endothelium produced a substance that freely diffused into the smooth muscle causing the muscle to relax. He did this by simply scraping the endothelium off a piece of rabbit aorta and showing that the smooth muscle left could no longer relax. He coined the term EDRF for endothelium-derived relaxation factor and the race was on to identify it (Furchgott and Zawadzki 1980).

Meanwhile, while the pharmacologists were chasing EDRF, the second track of investigation began to take shape and impact the nitric
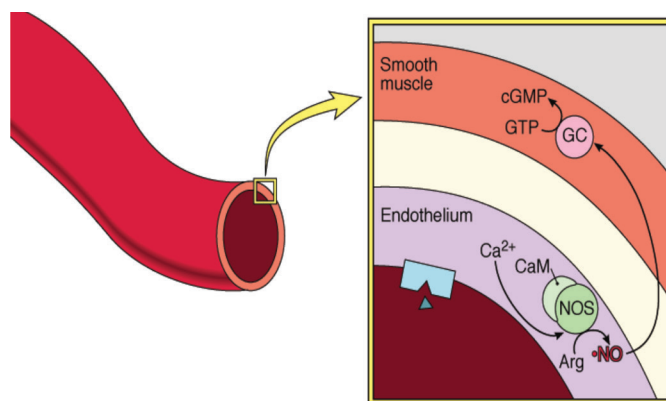
FIGURE 4. A schematic representation of a blood vessel. The larger inset shows the endothelium layer and contained within it is the enzyme nitric oxide synthase (NOS) that synthesizes nitric oxide (NO) from the amino acid arginine. NOS activity is tightly controlled by an on- and off-switch involving calcium (Ca^{2+}) and another protein, calmodulin (CaM). NO is a gas that freely diffuses into the smooth muscle layer. NO then activates the enzyme guanylate cyclase (GC) to synthesize cyclic guanosine monophosphate (cGMP). The small blue triangle in the lumen of the blood vessel is a representation of a molecule that is a signal to dilate the vessel; once bound to its receptor (shown in light blue), calcium is released, NOS is turned on, and NO is made.

oxide story. Steven Tannenbaum and colleagues at the Massachusetts Institute of Technology (MIT) were following their interest in nitrate biosynthesis. That interest stemmed not from blood vessel dilation, but instead from a focus on chemical carcinogenesis. The association of environmental exposure to nitrates with cancer was thought to be due to the chemical reaction of nitrates with endogenous molecules (amines that are ubiquitous), thus forming a carcinogenic molecule (Figure 5). This nitrate association with cancer led to the concern about dietary intake of cured meats such as bacon and salami and other foods high in nitrates. Tannenbaum convincingly showed that nitrates were formed biosynthetically as natural metabolites in human, rats, mice, and other animals (Tannenbaum et al. 1978). Nitrate formation in humans and other animals was unprecedented and so the source was unknown. A biochemist was needed and my laboratory answered the call having just joined the MIT faculty.

Where do you start to identify the source of a molecule like nitrate when there is little in the way of precedent to guide you? My students and I were developing what we hoped would be good and productive ideas when serendipity intervened. Tannenbaum and colleagues were continuing to study nitrate biosynthesis in humans. It was not difficult
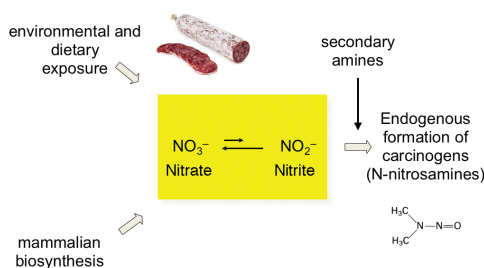
FIGURE 5. Nitrates and carcinogenesis. Nitrates (nitrite and nitrate as shown) are interconvertible; however, in aerobic organisms nitrate is the predominate species. Concern about exposure to nitrates was always thought to be environmental (including cured meats) but once mammalian synthesis was shown to occur, interest shifted to the source of nitrates. Nitrite reacts with amines under the acidic conditions in the stomach and carcinogenic N-nitrosamines are formed.

since nitrate is excreted in the urine, so sample collection was easy; however, nitrate levels are diet-dependent and vary widely, so animal subjects in such a study would be placed on a low-nitrate diet. The experimental design was also uncomplicated—mammals (humans, rats, and mice) were put on a low-nitrate diet and nitrate excretion was measured. The result was clear—both rats and humans that were tested excreted more nitrate than they ingested. Thus somehow both animals were making nitrate (Tannenbaum et al. 1978; Figure 6).

These studies were extended to compare normal rats with germ-free rats (no microbiome) and the results were the same, thus ruling out any contribution of the microbiome in the formation of nitrate. The low-nitrate diet itself was not the most appetizing so when daily analysis showed a very large (10-fold) increase in nitrate excretion in one of the subjects (an MIT undergraduate), they called her to ask about "cheating" on the diet (Figure 6). Their thinking was that she tired of the low-nitrate diet and went out for a hamburger. In fact, she was dedicated to the project and explained even though she developed a bad case of diarrhea, she continued to collect her urine despite her illness (Wagner and Tannenbaum 1982).

There were two hypotheses for the increase in nitrate excretion. The first was that the "bug" colonizing her intestinal tract was making nitrate. This hypothesis was quite reasonable since bacterial synthesis of nitrate was well-known. The second was that her immune system was synthesizing nitrate in response to fighting the colonizing bug. This second hypothesis connecting nitrate synthesis to immune system function was without precedent. In fact, the second hypothesis turned out to be the answer. In a brilliant experiment, the Tannenbaum research
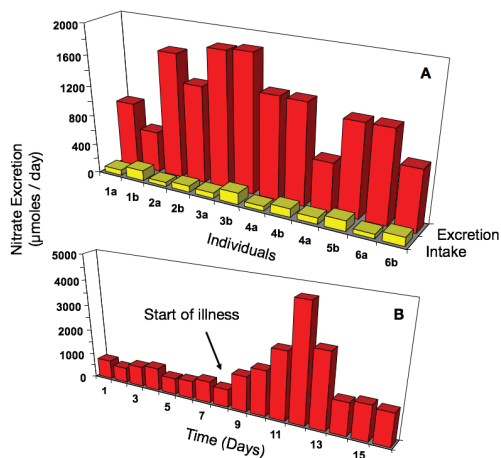
FIGURE 6. Nitrate excretion in human subjects. Panel A shows the results of six human subjects on a low-nitrate diet (yellow bars) and their respective urinary nitrate levels at 12-hour intervals (a and b; red bars). Panel B shows the increase in nitrate excretion in a human subject who became ill during the course of the experiment. This subject was on the same low-nitrate diet as in panel A.

group showed it was the immune system that was responsible for nitrate synthesis. Rats treated with an isolated component of the bacteria (lipopolysaccharide [LPS]) generate an immune response since the cells in the immune system sense the administered LPS and think that bacteria have invaded. They become activated but without any live bacteria. Rats treated with LPS make high levels of nitrate just like the student who became ill (Wagner, Young, and Tannenbaum 1983).

My laboratory had begun to address the biochemistry of nitrate biosynthesis and we were in the midst of testing several hypotheses (fishing expeditions, actually) when we learned of the serendipitous observation of elevated nitrate in this student. We saw, firsthand, the results from the rat-LPS experiment and the clear conclusion of immune system involvement. We first focused on finding which cell type in the immune system was responsible for nitrate synthesis. Fortunately, immunologists had long been interested in LPS responses and, in the course of those studies, generated mice with specific immune cell type defects in response to LPS treatment. Using these mice, we showed that macrophages (an immune cell type, a so-called white blood cell) were solely responsible for nitrate synthesis (Stuehr and Marletta 1987). Macrophages' main function in the immune system is to kill cells including invading bacteria and tumor cells; however, they need to be activated to carry out this killing function. LPS is one of the agents that

will activate this killing function in macrophages. We found it was only activated macrophages that made nitrate—indeed, macrophages activated by LPS. Macrophages can be grown and activated in cell culture, thus greatly simplifying subsequent experiments. The culture media that contains constituents for growth and bathes the cell in culture can be manipulated. For example, when LPS was added to this media, those macrophages made nitrate, which was easily detected in a sample of the culture media. With this cell culture model of nitrate biosynthesis, we could address the fundamental question of the source of nitrate; more specifically, what was the chemical precursor for this unprecedented metabolite that appeared to be linked to activation of a key cell in the immune response?

To develop a hypothesis essentially out of the blue is a formidable task. An important guidepost for us was the fact that, once activated, macrophages go on to kill invading organisms but they themselves die in the process. So we developed the hypothesis that these cells had a unique cellular metabolism that, for whatever reason, caused them to make nitrate. Since the activation process led to their downfall, we further assumed they might degrade internal protein as a source of nutrients—eating their own seed corn so to speak. It is not possible to "extract" all the protein from the inside of a cell without destroying it; however, it is possible to limit the building blocks of proteins, namely the 20 naturally occurring amino acids. The experiment was simple in design: (*i*) treat macrophages with LPS to activate nitrate synthesis, (*ii*) remove conventional culture media and replace with a series of media each lacking one of the 20 amino acids, and finally (*iii*) measure nitrate synthesis. We made "educated guesses" based on the chemical composition of each amino acid and developed a priority ranking to remove each of the 20 amino acids. Through this we found that nitrate was exclusively derived from arginine (Iyengar, Stuehr, and Marletta 1987). A culturable cell type and a single precursor were all we needed to define the chemical steps that convert arginine to nitrate, and it turned out that, in addition to nitrate, the rest of the arginine molecule ended up as another amino acid, citrulline. Typical of science, others were onto this as well, including another very important player in the discoveries, John Hibbs at the University of Utah (Hibbs, Taintor, and Vavrin 1987).

Why would an activated macrophage—a cell that is tuned to kill invading bacteria and tumor cells—convert arginine to citrulline and nitrate? The answer in part came from Hibbs. His interest in infectious disease drew him to study the killing function of activated macrophages. Hibbs's experimental model was to coculture macrophages with tumor cells. Once the macrophages were activated, for example

with LPS, they would destroy the tumor cells. Hibbs was testing what media components were needed by macrophages and found that if arginine was removed from the culture medium, it could no longer kill tumor cells (Hibbs, Vavrin, and Taintor 1987). So it became clear that an integral function of activated macrophages was dependent on the arginine to nitrate pathway—but why? The chemistry carried out on arginine was without precedent and indicated that there must be a novel enzyme involved.

Meanwhile, the pharmacologists were frantically at work on the chemical identity of EDRF. As is the case with almost every paradigm-shifting discovery, there was controversy. Recall that work with the nitrovasodilator drugs like nitroglycerin drew investigators to simpler molecules and then Murad, as noted above, showed that NO itself had properties like that of EDRF (Arnold et al. 1977). Lou Ignarro at the University of California, Los Angeles carried out a series of experiments that indirectly pointed to NO as EDRF (Ignarro et al. 1987). However, Salvador Moncada at the Wellcome Research Laboratories in the United Kingdom definitively showed that endothelial cells made NO (Palmer, Ferrige, and Moncada 1987). This finding was truly remarkable. The idea that cells, human cells in fact, would make and use such a toxic molecule to control essential physiological processes caught the scientific world by surprise. And the stage was now set for the story to come full circle.

Moncada turned his attention to the source of NO and he found our papers detailing the conversion of arginine to nitrate in activated macrophages. He then tested the idea that NO in endothelial cells was derived from arginine, and indeed it was (Palmer, Ashton, and Moncada 1988). Meanwhile, we saw his NO paper and, recalling that NO is unstable and decomposes to nitrate, tested the idea that NO was an intermediate in the synthesis of nitrate that we measured in macrophages, and that turned out to be the case as well (Marletta et al. 1988). In an instant, two very disparate fields of investigation merged into one with the focus on this novel metabolic reaction of arginine to citrulline and NO with the subsequent decomposition of NO to nitrate.

While endothelial cells and activated macrophages carry out the same reaction, namely the formation of NO from arginine, the cellular usage and function is dramatically different. Endothelial cells use NO to dilate blood vessels whereas macrophages use it to kill pathogens and tumor cells. So how does biology use this toxic molecule to bring about such different responses? For this we return to Paracelsus: it is all about the dose. After the discovery of NO biosynthesis, the enzyme responsible for it was discovered and called nitric oxide synthase (NOS). There are two different forms of NOS (called isoforms) and

they are controlled differently. The NOS isoform that is in endothelial cells contains an on- and off-switch that very carefully controls how much NO is made. That switch is transiently turned on so that very small amounts of NO are made (Figure 4). The amounts are very small—just high enough to dilate blood vessels but low enough not to be toxic. The macrophage isoform also contains the on- and off-switch; however, the switch is permanently turned on. Consequently, NO levels generated are high and toxic and thus fit with killing function of macrophages.

Drug Discovery

Efforts to use what was learned about NO function in medicine have come quickly with serendipitous aspects as well. Recall that the effect of NO in the cardiovascular system is to dilate blood vessels, which in turn lowers blood pressure. The first idea was in principle an easy one: use NO itself to treat pulmonary hypertension in infants. Warren Zapol at the Massachusetts General Hospital championed this idea and since NO gas could be purchased, it would be easy to test. However, as one could imagine, serious safety questions were raised before anyone could treat people, much less infants, with a toxic gas like NO. How did Zapol convince the human use committee at the Massachusetts General Hospital to allow him to set up a clinical trial? He turned to Occupational Safety and Health Administration (OSHA) standards established for short-order cook exposure to nitrogen oxides, products that come from the gas grilling process. Zapol used those exposure levels established for adults and extrapolated to infant body weight and came up with a dosage over a specified time. It was good that he was persistent as this therapeutic approach is now used in every medical center in the world to treat acute respiratory distress syndrome (Rossaint, Lewandowski, and Zapol 2014).

The most famous therapeutic result to come from discoveries of NO function is sildenafil (Viagra) and the drugs that followed it. Sildenafil was discovered by scientists at Pfizer trying to develop a blood pressure–reducing drug without the tolerance that people develop to nitroglycerin and related drugs (Campbell 2000). Penile erections are a vasodilatory effect and every tissue has a specific way to terminate the signal. So while trying to increase the vasodilation in blood vessels to control systemic blood pressure, the Pfizer scientists ended up selectively increasing vasodilation in the penis. Luckily, male subjects in the clinical study complained of a "side effect," namely erections, and then the "side effect" became the therapeutic end point. In 2013, Bayer completed Pfizer's original goal to develop a blood pressure–reducing

drug by gaining FDA approval for a medication called riociguat (Adempas) to treat pulmonary hypertension in adults (Schermuly et al. 2011).

As mentioned, there was some controversy surrounding the discoveries and, not surprisingly, that controversy was centered on those awarded the Nobel Prize in Physiology or Medicine in 1989. The Nobel Committee decided to focus the award on discoveries of NO action in the cardiovascular system with the presumed reasoning that the medical impact (sildenafil) was already very clear and significant. With that as their starting point, due to the three-person limit to shared Nobel Prizes, they then needed to pare down four key players to three, and in so doing awarded the prize to Murad, Furchgott, and Ignarro, leaving Moncada out. To my mind, Moncada's definitive result showing that endothelial cells make NO was more compelling than the indirect experiments carried out by Ignarro, but it certainly was a close call.

Most discoveries in science have twists and turns, unexpected results, and serendipitous observations. The discoveries surrounding NO function in biology are no exception. As noted at the beginning of this paper, prior to the mid- to late 1980s, if you were experienced in the discipline and someone told you that NO was a vital metabolite in human physiology, you would have likely walked away thinking the science police should be called and the perpetrator arrested. Obviously, no crime was committed. The discoveries have withstood the continual scrutiny that comes with the territory in science. Better yet, important practical applications have been developed and novel therapies to treat human disease have been realized. All in all it is an impressive tale of discovery.

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