

## **Mini-Series: Significant Contributions to Biological Chemistry Over the Past 125 Years**

### **Landmark Discoveries in the Trail from Chemistry to Cellular Biochemistry, with Particular Reference to Mileposts in Research on Bioenergetics\***

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*“The fascination of a growing science lies in the work of the pioneers at the very borderland of the unknown, but to reach this frontier one must pass over well traveled roads” [1].*

**Molecular oxygen was discovered in 1775 by Joseph Priestley. He also made the important observations that oxygen is produced by plants and that gases (O<sub>2</sub> and CO<sub>2</sub>) mediate the interdependence of plant and animal life. The biological experiments of Priestley dealt with aerobes, the most advanced forms of life. It was the research of Louis Pasteur, however, on fermentation of sugar by anaerobic microbes that provided a system that was the first to reveal significant clues to the biochemistry of bioenergetics. Analysis of the detailed mechanism of anaerobic sugar fermentation by yeast during the first third of the 20th century and the later study of energy-yielding aerobic respiration and energy conversion in photosynthesis required development of many new techniques. These became the tools that were exploited to yield the basic outlines of cell biochemistry. Included were chromatography, metabolic gas manometry, spectrophotometry, the use of stable and radioactive isotopes as tracers of intermediary metabolism, and procedures for purification of proteins and other macromolecules. Noteworthy advances included discovery and characterization of the (Krebs) tricarboxylic acid cycle, “activated” intermediates such as acetyl coenzyme A, electron carriers and coenzymes necessary for energy conversion and reductive biosynthesis, ATP (the universal energy “currency”) and a multitude of enzymes involved in catabolic and biosynthetic metabolism.**

The founding of the American Chemical Society was closely related to discoveries that had great importance in the history of research in biological chemistry. I refer, in particular, to discoveries made by the English minister Joseph Priestley (1733–1804). Priestley was a self-taught polymath who discovered molecular oxygen in 1775 and demonstrated that the gas is produced by green plants. Using the mouse as an experimental system, Priestley also did clever experiments showing that the oxygen produced by plants was essential for the life of animals [2].

In 1794, Priestley left the United Kingdom and emigrated to Northumberland, PA, where he continued research in a combination home/laboratory. In 1874, 77 chemists met at Priestley’s house to commemorate the centenary of his discovery of O<sub>2</sub> and discussed the possibility of forming a national association. The idea was rejected, because many at the meeting felt there would never be enough chemists in the United States to support such an organization. Two

years later, in 1876, some of the same group met again and voted to establish the American Chemical Society. There are now more than 163,000 members! In the Chemical & Engineering News of August 8, 1994 (p. 9), there is a photograph showing Priestley House on the occasion of its dedication in 1994 as the third National Historic Chemical Landmark.

After Priestley’s discoveries, about 100 years elapsed before it was recognized that there are microorganisms that do not require molecular oxygen and, in fact, are inhibited by oxygen. The so-called “anaerobic” life style became the focus of the research of Louis Pasteur in the 1870s, and this had far-reaching consequences. Pasteur concluded that anaerobic fermentation of sugars by organisms such as yeast was the result of life without air and was always intimately associated with cell growth. In other words, he believed that fermentation occurred only in living cells.

Pasteur died in 1895. If he had lived two more years, he would have been stupefied by an accidental discovery made in 1897 by Hans and Eduard Buchner. The Buchners were trying to make cell-free protein preparations from yeast for use in immunological experiments and decided to add sugar to a batch of yeast “juice” to act as a

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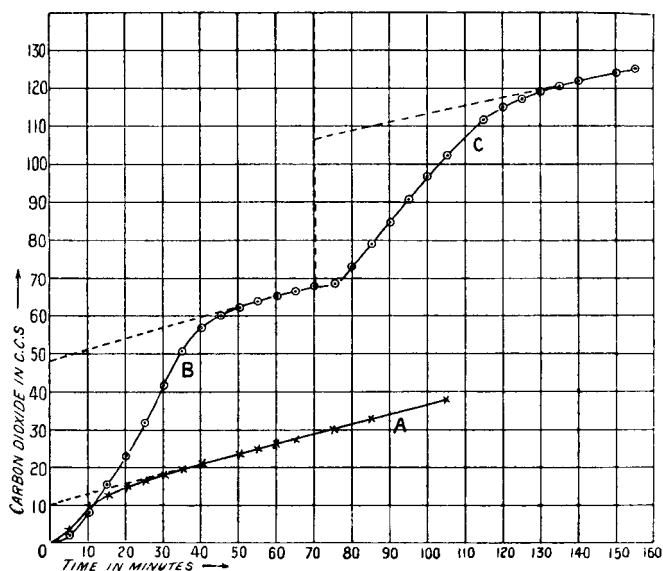


FIG. 1. Effect of inorganic phosphate on production of CO<sub>2</sub> during fermentation of sugar by yeast juice. For details see Ref. 4.

preservative. They were surprised to observe a vigorous production of CO<sub>2</sub> and the formation of ethanol. Announcement of the cell-free fermentation, by “zymase,” was astonishing. In many ways, this discovery was the opening wedge leading to modern biochemistry [3].

Soon after the serendipitous discovery of cell-free yeast juice fermentation, Arthur Harden (United Kingdom) began a systematic analysis of the system. Eduard Buchner had observed that the production of CO<sub>2</sub> by yeast juice declined rapidly with time, long before the sugar was exhausted. Harden looked into this and found that addition of inorganic phosphate resulted in an immediate increase in the rate of fermentative CO<sub>2</sub> production. This is illustrated in Fig. 1 [4]. Curve A is a control with no added phosphate. In B, 0.03 M phosphate was added, and after 70 min another addition of phosphate was made. Harden and his colleagues found that during the course of fermentation, the inorganic phosphate was esterified into organic forms, and they isolated three esters, glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate. The isolation of the sugar phosphates eventually led to the unraveling of the molecular details of the intermediary steps of sugar fermentation and glycolysis.

By 1924, it became apparent that the sequence of reactions in fermentation of glucose by yeast was virtually identical to the sequence of reactions in glycolysis, the conversion of glucose to lactic acid in mammalian muscle. It also became clear that the purpose of these anaerobic sequences was the regeneration of ATP, the universal energy currency used by all types of cells for biosynthesis and other energy-requiring processes. ATP was first isolated in 1929, from muscle, by Fiske and Subbarow, and in the same year Sir Arthur Harden was awarded a Nobel Prize in Chemistry.

It is reasonable to believe that fermentation and glycolysis were very ancient mechanisms in the evolution of biological energy conversion systems. The anaerobic mechanisms are widespread, even in contemporary organisms, from microbes to muscle tissue of humans. An

example of an organism that obtains all of its energy by anaerobic glycolysis of sugar is the bacterium *Streptococcus pneumoniae*, formerly known as the pneumococcus. This bacterium is responsible for many millions of cases of pneumonia and bronchitis, and antibiotic-resistant strains are now of great concern. The search is on for new kinds of effective antibiotics.

*S. pneumoniae* holds a unique place in the history of biochemical research. During the 1940s, studies by Oswald Avery and his colleagues at the Rockefeller Institute on the genetics of polysaccharide formation by this bacterium showed for the first time that the genetic material is DNA. This was clearly demonstrated in 1944 when the conventional wisdom was that genes are made of protein [5]. It is remarkable to recall that between 1944 and 1950, the Avery's conclusions were considered very dubious. I remember hearing a seminar in 1949 that seriously questioned Avery's identification of DNA as the genetic material.

Going back a bit in the chronology, the 1930s was an important decade in the development of the fundamental background of cell biochemistry. There was a renewal of interest in the mechanism of aerobic respiration. Otto Warburg, in Berlin, isolated and characterized flavoproteins and the pyridine nucleotide electron carriers NAD and NADP. He also developed manometric methods for assay of metabolic gases, and the so-called Warburg manometric apparatus soon became a standard fixture in biochemistry and microbiology laboratories. Equally important was Warburg's development of the methodology of spectrophotometric assays of coenzymes and enzyme activities.

During the same period, Albert Szent-Györgyi in Hungary found that fumarate and succinate played an important role in respiration and proposed that triose phosphate from sugar breakdown was oxidized aerobically by a C-4 dicarboxylic acid cycle. The methods that Warburg developed and Szent-Györgyi's observations provided Hans Krebs in Sheffield, United Kingdom with some tools and clues he needed to develop and prove a brilliant conception, namely the citric acid cycle, otherwise known as the tricarboxylic or Krebs cycle. This is the primary metabolic machinery that furnishes the reducing power for energy-yielding respiration of aerobic organisms.

In 1937, *Nature* magazine rejected the brief paper in which Krebs first described the cycle; the form rejection letter is reproduced in an autobiographical memoir published by Krebs in 1981 [6]. When we learned years later that this paper had been rejected it greatly encouraged the hopes of many young scientists who had negative dealings with *Nature*. Fig. 2 (from Ref. 6) shows the Krebs cycle, which generates CO<sub>2</sub> and hydrogen atoms from major foodstuffs. The cycle occurs in virtually every kind of animal and plant and in the majority of ordinary bacteria. The hydrogen atoms derived from reactions of the cycle constitute the fuel for regeneration of ATP by the aerobic respiratory system. One question Krebs was unable to answer was the exact nature of the C<sub>2</sub> compound that is funneled into the cycle from carbohydrates, fats, and proteins. It was obviously similar to acetate, but what was it really? The answer was mainly provided by Fritz Lipmann,

## DISCOVERY OF THE CITRIC ACID CYCLE (1936–1937) 115

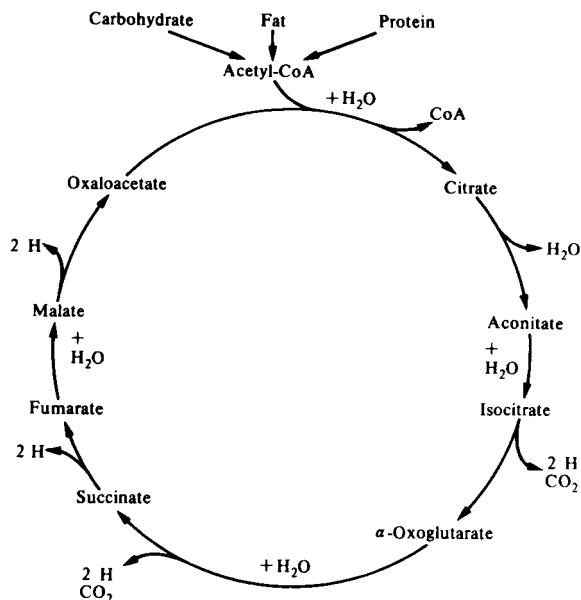


FIG. 2. **The Krebs tricarboxylic acid cycle (also known as the citric acid cycle).** Carbohydrate, fat, and protein all form acetic acid attached to CoA. CoA is regenerated when citrate is formed; thus it acts as a catalyst, and only small amounts are needed. For details see Ref. 6.

who discovered coenzyme A, the coenzyme of acetylation, in 1947. Acetyl-CoA is the actual energy source for the cycle. Lipmann also introduced the concept of energy-rich phosphoryl groups and made other important contributions to biochemistry, and he shared the 1953 Nobel Prize in Physiology or Medicine with Krebs.

## INTERMEDIARY METABOLISM

When research accelerated after the end of World War II, many biochemists began exploring metabolic pathways of carbon and nitrogen in animal and plant tissues and in microorganisms. <sup>14</sup>C was first produced in the Berkeley cyclotron by Sam Ruben and Martin Kamen in 1940 and started to become commercially obtainable about 1950. The availability of this isotope had a profound effect on research in intermediary metabolism. Among many other applications, <sup>14</sup>C provided the means for elucidation of the Calvin/Benson cycle of photosynthetic CO<sub>2</sub> reduction [7].

Biosynthesis of amino acids and proteins became "hot" topics, and studies on properties of enzymes led to important discoveries on the regulation of enzyme activity. An example of the latter was negative feedback inhibition of aspartokinase activity [8]; the kinase is the first enzyme of the branched pathway leading to isoleucine synthesis. Horizons of biochemistry were expanding rapidly, and there were spectacular advances in our knowledge of the complexity of metabolic pathways.

The explosion of new information on cell biochemistry condensed in the metabolic pathways map was greatly facilitated by the study of biochemical mutants of bacteria and *Neurospora*. Mutational interruption of individual steps in complex pathways led to accumulation of transient intermediates that could be isolated and identified. There was another very important development during the

1940s. Namely, the establishment of the Sigma Chemical Company in St. Louis, MO. Sigma began producing valuable biochemicals, sparing investigators arduous, time-consuming labor needed to make the reagents for experiments. Before Sigma came into existence, I personally recall spending weeks isolating NAD or NADP from a large mass of yeast cells, ending up with preparations that were no more than 30% pure. Later, when I was a faculty member at Washington University (St. Louis, MO) in the 1960s, I could get the pure compounds delivered from Sigma by taxi within an hour. Incidentally, Sigma now makes available the latest metabolic pathways map on an impressive chart, 33 × 50 inches, for the bargain price of \$6.95 (product M 3907).

## BIOENERGETICS

In the background of the flood of new biochemical information being generated during the 1940s to 1960s, another great research current was forming. I am referring to investigation of the details of how electron flow in aerobic respiration and photosynthesis drives regeneration of ATP from adenosine diphosphate and inorganic phosphate. This was a persistent topic of symposia at biochemistry meetings for some time.

The involvement of cytochromes and the terminal electron-transporting heme proteins in energy conversion had a curious history. The cytochromes were first discovered in a great variety of animal tissues in 1884 by Charles MacMunn, a practicing Irish physician. MacMunn had a small laboratory in a hay loft over his stables and did research in his spare time. Later he built a small laboratory in his garden and had a horizontal iron pipe built into the wall. Through the pipe he could see which patients were coming up the path. If he didn't want to be interrupted, he would warn the maid to say that he was out. MacMunn's research publications were strongly criticized by some eminent chemists, notably Felix Hoppe-Seyler. The main criticism, which was completely erroneous, was that MacMunn was simply observing breakdown products of hemoglobin (see Ref. 9 for details of the early history).

For the next 40 years, MacMunn's findings were ignored. Then, in 1925, the cytochromes were rediscovered by David Keilin, who spent his entire career working on the redox behavior and distribution of cytochromes in cells of many kinds. Most of Keilin's observations were made with the naked eye using a microspectroscope. Fig. 3, from Keilin's classic book [9], shows the appearance of reduced cytochrome absorption bands in various organisms as he observed them. Keilin examined only a few microorganisms, including a strictly anaerobic *Clostridium*. He could not detect any cytochrome in the latter, and this led him to generalize that cytochromes are absent from anaerobes. Keilin's conclusion seemed reasonable at the time, but he was outwitted by the diversity of metabolic types in the microbial world. In 1954, John Postgate in the United Kingdom discovered a c-type cytochrome in a strictly anaerobic sulfate-reducing bacterium, and in 1953, Leo Vernon found c-type cytochromes in anaerobic purple photosynthetic bacteria. In the purple bacteria, the cytochromes function as carriers in the electron flow that drives photophosphorylation. Since 1954, both b- and c-

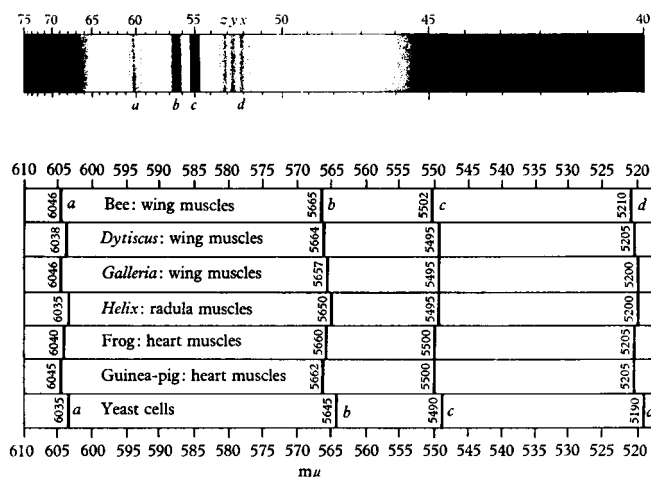


FIG. 3. Absorption bands of reduced cytochromes in various organisms, as observed with a spectroscope. For details see Ref. 9.

type cytochromes have been observed in a variety of anaerobes where they function as electron carriers in anaerobic energy conversion processes that are analogous to aerobic respiration.

The actual mechanism of the energy-requiring coupling of ADP and inorganic phosphate baffled investigators for a long time. It remained for Peter Mitchell, in the United Kingdom, to develop a totally new concept of how this occurs. This was the chemiosmotic theory. Mitchell endured a long battle with what can be called the entrenched "Oxidative Phosphorylation Establishment." The Establishment consisted of about six prominent investigators who dominated almost all symposia of the biochemical societies for years. In my own laboratory, I referred to them as the Traveling Vaudeville Show. In 1963, Mitchell resigned his position at the University of Edinburgh, bought a very old manor house in the remote moors of Cornwall, United Kingdom, and remodeled half of it as a laboratory. Eventually, Mitchell's "protonmotive force" toppled all the old ideas. Mitchell's persistence and insights were recognized by the 1978 Nobel Prize in Chemistry.

Efraim Racker [10] described the history of research on oxidative phosphorylation with the clever cartoon shown in Fig. 4. His summary was that "The history of oxidative phosphorylation had its beginning in Europe. David Keilin (1925) in England deserves credit for the concept of the respiratory chain. Single-handed, literally, with a hand spectroscope, he deciphered the *a-b-c* of the cytochrome chain. In Russia, Vladimir Engelhardt (1930) conceived the idea that phosphorylation is linked to oxidative processes. But oxidative phosphorylation became a respectable field only when Severo Ochoa (1943) established that for each atom of oxygen three molecules of phosphate are esterified. Lehninger (1951) made the crowning discovery that NADH is the primary hydrogen donor for the respiratory chain in isolated mitochondria."

In the cartoon, Edward Slater (1953) represents part of the Establishment that kept busy, for years, searching for the mythical "A squiggle X," the supposed precursor of "X squiggle P," which supposedly gave rise to ATP. Finally, Mitchell is dumping "A squiggle X" into a waste basket. The mechanism of ATP formation by ATP synthase is still

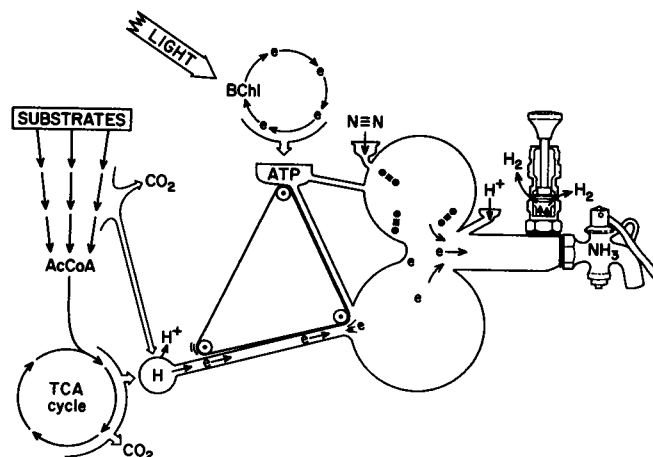


FIG. 5. A representation of metabolic conversions occurring in photosynthetic bacteria during photoproduction of  $H_2$  catalyzed by nitrogenase. If  $N_2$  becomes available  $H_2$  is not produced, and reducing power is used instead for reduction of  $N_2$  to ammonia.

under active study, and we now know that the enzyme is an exquisite "machine" consisting of two interconnected molecular motors that are driven by protonmotive force (see Ref. 11).

#### $N_2$ FIXATION AND PHOTOSYNTHESIS

Before 1949, only three kinds of free-living bacteria were known to have the capacity to use  $N_2$  as a nitrogen source for growth. These were identified before 1900, and it was generally believed that this metabolic ability was very restricted in nature. Serendipity, however, proved otherwise. While I was a graduate student at Washington University (St. Louis, MO), I spent the summer of 1948 doing research at the Hopkins Marine Station of Stanford University, located in Pacific Grove, CA. I was testing the growth of purple photosynthetic bacteria in various media, as a preliminary to research on the possible involvement of ATP and phosphorylation in photosynthetic energy conversion. One morning, I was astonished to observe that in one particular medium, the experimental organism, *Rhodospirillum rubrum*, had produced copious amounts of molecular hydrogen during photosynthetic growth.

After I returned to St. Louis, I conducted many experiments trying to demonstrate  $H_2$  formation by illuminated resting cells of *R. rubrum* derived from  $H_2$ -producing cultures. The cells were resuspended in a dilute solution of buffered mineral salts under an atmosphere of  $N_2$ . In experiment after experiment, not a trace of  $H_2$  was produced. After many trials, one day in January, 1949 I performed an experiment in which I deliberately replaced the supposedly inert gas phase of 100%  $N_2$  with 100%  $H_2$  or 100% helium, and for the first time observed light-dependent production of  $H_2$ . It was evident that  $N_2$  was not inert in the metabolism of *R. rubrum*. The obvious conclusion that *R. rubrum* could fix  $N_2$  was quickly substantiated [12, 13], and subsequent research has shown that virtually all anoxygenic phototrophs have the same capacity.

A key fact leading to the discovery of  $N_2$  fixation by *R. rubrum* was that the medium in the culture showing  $H_2$  production contained glutamate as the sole nitrogen

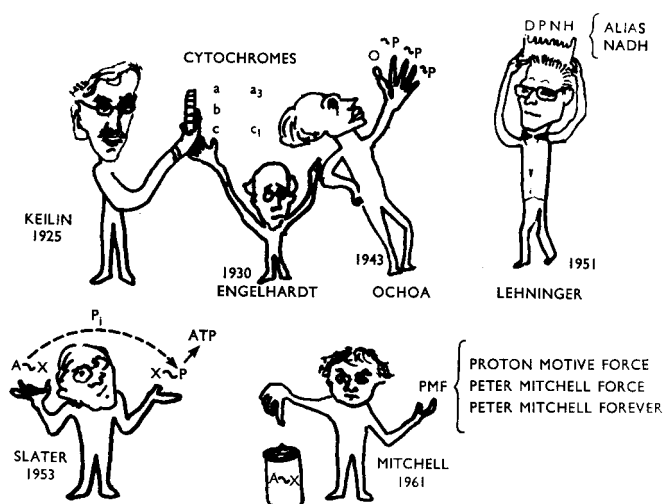


FIG. 4. Highlights in the history of research on oxidative phosphorylation. For details see Ref. 10.

source, rather than an ammonium salt. In all previous research with photosynthetic bacteria,  $\text{NH}_4^+$  was routinely provided as the nitrogen source. It has since been found that ammonium salts repress synthesis, as well as activity of the nitrogenase complex. In my glutamate medium, the nitrogenase complex was derepressed. This set the stage for hydrogen production, because in the absence of  $\text{N}_2$  and  $\text{NH}_4^+$ , all nitrogenases show the alternative activity of energy-dependent reduction of protons to  $\text{H}_2$ . Experiments with *R. rubrum*, in fact, gave the first indications that the nitrogenase complex can catalyze  $\text{H}_2$  formation [14, 15].

Fig. 5 depicts the flow of carbon and electrons in purple photosynthetic bacteria producing  $\text{H}_2$  during photoheterotrophic growth. The double-bulbed device on the right represents the nitrogenase complex. In the absence of ammonia, the nitrogenase is derepressed, and when  $\text{N}_2$  is also absent, the complex functions as a hydrogen-evolving catalyst. With glutamate as the nitrogen source, the supplies of ATP from photophosphorylation, and electrons from organic substrates, are evidently in excess relative to the requirements of the biosynthetic machinery. Under these conditions, protons are reduced yielding molecular hydrogen, which is shown here as being discarded by a "hydrogen relief valve." If molecular nitrogen is added, hydrogen evolution stops, because ATP and the electron supply are used for the formation of ammonia, which is rapidly consumed for the production of amino acids and other nitrogenous compounds. The hydrogen relief valve is construed as a control device that permits "energy idling" when this is required by the balance between generation of ATP and reducing power on one hand and overall biosynthetic rate on the other [16, 17].

#### EPILOGUE

In reviewing the history of research in biochemistry from 1876 to approximately 1960, it soon became clear to me that it would be very difficult to present a comprehensive view of the highlights in a short article. The 80-odd-year period witnessed a cornucopia of advances that was

equivalent to erection of the main girders of the skyscraper of biochemistry and also to the design and validation of its basic operating machinery. The metaphor of constructing a grand edifice to depict the development of a scientific discipline was deftly used by Lewis and Randall in their classic book on thermodynamics [1],

"There are ancient cathedrals which, apart from their consecrated purpose, inspire solemnity and awe. Even the curious visitor speaks of serious things, with hushed voice, and as each whisper reverberates through the vaulted nave, the returning echo seems to bear a message of mystery. The labors of generations of architects and artisans has been forgotten, the scaffolding erected for their toil has long since been removed, their mistakes have been erased, or have become hidden by the dust of centuries. Seeing only the perfection of the completed whole, we are impressed as by some superhuman agency. . . . Science has its cathedrals, built by the efforts of a few architects and of many workers."

I have noted some of the chief architects. There were many more. Their achievements, summarized here, are described in historical accounts by Florkin and Stotz [3] and Fruton [18, 19].

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