CHAPTER 7

Hans Krebs & the Puzzle of Cellular Respiration

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INTRODUCTION

Late in life, Hans Krebs (Figure 7.1) recalled sailing past the white cliffs of Dover during the early morning hours of June 20, 1933. Although he was optimistic about a new life in England, he was experiencing a wrenching forced emigration from his native Germany. As a Jewish scientist, he had been fired from his position as a medical researcher at the University of Freiburg shortly after the Nazis came to power. Like many other German scientists, Krebs quickly fled the country. He spent several years as a refugee working in temporary research positions before becoming a British

FIGURE 7.1 Hans Krebs. *Source:* Courtesy of Philip P. Cohen.

citizen in 1939 just as World War II began. In 1953, he would be knighted and awarded the Nobel Prize for discovering the metabolic cycle that bears his name.

Krebs was more fortunate than many other refugees. Just a year before leaving Germany, he had discovered the chemical steps by which an important waste product (urea) is produced in the liver. This was particularly significant because it was the first example of an important type of cellular process: a biochemical cycle. The discovery brought Krebs international recognition, and after fleeing Germany he was invited to work at Cambridge University, one of the world's leading centers of biochemical research.

Although the Nazis prevented him from taking more than a handful of personal belongings, by a strange twist of fate he was allowed to take some of his laboratory equipment. Thus his finances were in a precarious state when he arrived in England, but Krebs was able to resume his research almost immediately after settling in Cambridge. Less than five years later, he published an article announcing his most famous discovery: the citric acid cycle (Krebs cycle).

THE MAKING OF A BIOCHEMIST

Krebs grew up in Hildesheim, a town that was almost totally destroyed by Allied bombing during World War II. The son of a prominent surgeon, Krebs wanted to follow in his father's footsteps. He entered medical school shortly after the end of World War I but soon realized that he was more interested in research than in practicing medicine. This was a serious decision because Germany was in the midst of a crippling economic depression and research positions were hard to find. Fortunately, after completing his medical degree, Krebs was hired to assist one of Germany's leading physiologists, Otto Warburg.

Warburg was an autocratic leader who refused to allow his assistants to work on independent projects. Despite this dictatorial style, he was an excellent teacher. He set high standards for himself and his assistants. As an outstanding physiologist who made important contributions to the study of photosynthesis, enzyme activity, and the metabolism of cancer cells, Warburg provided a role model for Krebs as the young scientist began his career. Krebs greatly admired his mentor, and he claimed that his own accomplishments were partly driven by a desire to emulate the great physiologist.

Warburg also perfected the instruments that Krebs used in his later research. Like many earlier physiologists, Warburg measured oxygen consumption during respiration with manometers (Figure 7.2). The Warburg manometer was unusual, however, because it could be used to study respiration in cells, rather than in whole animals. This required carefully slicing very thin pieces of animal tissue with a razor. Containing only about ten layers of cells, these slices were so thin that oxygen and carbon dioxide could freely diffuse through them. Placed in the reaction vessel of the Warburg manometer, the cells survived for several hours—plenty of time to do physiological experiments. Warburg's manometer and his "tissue slice method" provided crucial tools for discovering the details of cellular respiration. When he fled Germany in 1933, Krebs carried about 30 of these manometers with him, making his cargo far more valuable than the Nazis realized.

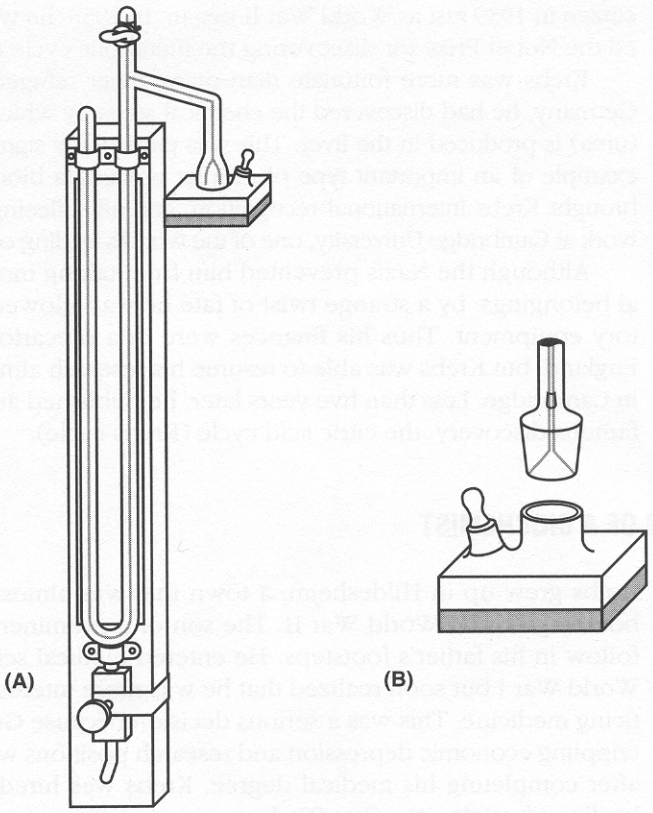

FIGURE 7.2 (A) A Warburg manometer and reaction vessel. A thin slice of tissue was placed in the reaction vessel. As respiration occurred, oxygen was consumed. The carbon dioxide produced by respiration was chemically absorbed by potassium hydroxide, placed in a small container in the reaction vessel. Because both oxygen and carbon dioxide were removed, the air pressure inside the sealed reaction vessel decreased. As a result, fluid was drawn upward into the arm of the manometer tube. Therefore, the change in the height of the fluid measured the rate of respiration. (B) Reaction vessel, showing the ground glass connection with the manometer.

THE PROBLEM OF CELLULAR RESPIRATION

When Krebs began studying respiration around 1930, scientists had already been working on the problem for more than a century. By the end of the 1700s, scientists knew that the breakdown of sugar (glucose) in living organisms was chemically similar to combustion. In both cases, a fuel is burned (oxidized) to produce carbon dioxide and water. Throughout the nineteenth century, physiologists studied this process of organic combustion in whole animals by measuring the oxygen used, the carbon dioxide produced, and the heat generated during respiration. These whole-animal studies provided useful information, but with the rise of cell theory, biologists

realized that respiration must occur inside these tiny structures. Cells rather than whole organisms became the focus of later biochemical studies.

Scientists realized that respiration in cells must occur in a series of small steps, each of which releases a tiny amount of energy. Otherwise, the heat given off by burning sugar molecules would destroy the cell. There must be a kind of "biochemical pathway" leading from glucose, through a series of intermediate compounds, to the final products of respiration: carbon dioxide and water. Discovering the steps in this biochemical pathway became a major goal for twentieth-century biochemists, but they faced a serious technical problem. How can you study chemical reactions happening inside a microscopic cell?

At first, the solution to this problem seemed to be within grasp. In 1897, the German chemist Eduard Buchner demonstrated that fermentation could occur outside the cell. Using an enzyme extracted from crushed yeast cells, Buchner converted sugar to alcohol in a "cell-free" mixture. This was a dramatic scientific accomplishment, for it held out the possibility that all biochemical reactions could be duplicated in the test tube. Unfortunately, this hope was short lived. Attempts to study the oxygen-requiring steps in the breakdown of sugar using Buchner's cell-free approach failed. Unlike fermentation, aerobic respiration only occurred inside the living cell. How could scientists get inside the cell to investigate this process?

Krebs thought that he had an answer. The very thin slices of tissue that Otto Warburg placed in his manometers were really tiny colonies of cells. By experimentally manipulating the chemical environment of these cells, perhaps he could find clues to the separate reactions making up a biochemical pathway. When Krebs suggested this to Warburg, however, the older physiologist rejected the idea. At the time, Warburg was interested in comparing overall rates of respiration in normal cells and cancerous cells. Studying the separate steps in the pathway was a distraction, and Warburg refused to allow Krebs to pursue this new line of research. Studying metabolism would have to wait until Krebs left Warburg's laboratory.

BIOCHEMICAL PUZZLE SOLVING

A few years later, Krebs moved to another laboratory at the University of Freiburg, and he began seriously studying cellular respiration in 1932, shortly before he fled Germany. At the time relatively little was known about the process. Following the lead of Eduard Buchner, scientists realized that something similar to fermentation occurred in all cells. A molecule of glucose was always split into two pieces (pyruvate) during the first stage of the breakdown of sugar. This set of reactions (what we now call *glycolysis*) did not require oxygen. What happened to these glucose fragments during the oxygen-consuming steps of cellular respiration still remained a mystery, although many scientists were working on the problem.

Krebs later claimed that solving the problem of cellular respiration was like putting together a jigsaw puzzle. Brainstorming with pencil and paper, he generated numerous hypothetical pathways. Each chemical step in a pathway had to follow the rules of organic chemistry, but just because it was plausible did not make it correct.

In fact, most of the hypotheses turned out to be wrong. The only way Krebs knew whether a hypothetical step could actually be a piece in the puzzle was to test it with the Warburg manometer. Suppose that a hypothetical chemical compound really was one of the intermediate steps in the chain leading from glucose to carbon dioxide. If so, then adding this substance to the tissue slice in the Warburg manometer ought to increase the rate of oxygen consumption.

The Search for Intermediate Compounds

To visualize this type of experiment, remember that respiration is somewhat similar to combustion. Adding fuel to a fire increases combustion, so more oxygen gets consumed. Following this analogy, glucose is fresh wood, and the intermediate compounds are partially burned embers. Compounds unrelated to cellular respiration are not fuel and so do not burn at all. During the early 1930s, Krebs and other biochemists tested many potential "fuels" to find out whether they would "burn." For example, in one set of experiments Krebs tested three 4-carbon compounds: succinate, fumarate, and malate. Could they be intermediate compounds in the respiratory pathway? Because all three compounds increased oxygen consumption, it seemed likely that they were involved in cellular respiration. Using the combustion analogy, Krebs was adding more embers to the fire, so more oxygen was being used to burn them. When he completed the experiments he discovered the following results:

Substance Added	Oxygen Consumed (μmole)
None (control)	670
Succinate	1,520
Fumarate	1,290
Malate	1,340

Like many experiments, however, these raised more questions than they answered. Succinate, fumarate, and malate increased respiration even more than Krebs expected. Furthermore, all three of the compounds could still be detected in the reaction vessel at the end of the experiment. If they really were intermediate compounds in the slow oxidation of glucose to carbon dioxide, why weren't these extra embers completely burned in the process? If they weren't intermediate compounds, why did respiration increase so much when they were added to the reaction vessel?

The Hungarian biochemist Albert Szent-Györgyi, who later won a Nobel Prize for discovering Vitamin C, proposed one plausible explanation for the unexpected results. He believed that succinate, fumarate, and malate were not reactants in cellular respiration. They acted like catalysts, instead, accelerating the chemical reactions of cellular respiration without being consumed in the process. This explained why they were still detected at the end of the experiment. If this were true, however, biochemists would have to keep looking for the true reactants. Were the three compounds catalysts or reactants? The controversy remained unresolved for almost two years.

A Role for Citric Acid

Many other pieces of the jigsaw puzzle were also missing. Perhaps the most puzzling was citric acid (citrate), a six-carbon molecule. Biochemists could increase cellular respiration by adding citrate to the Warburg manometer, but they were unable to find a logical place for citrate in their hypothetical pathways. Krebs's early attempts to brainstorm a role for citrate had failed, and he never published them.

PROBLEM

Both glucose and citrate are six-carbon molecules. Knowing what they did about glycolysis, why might it have been difficult for biochemists to hypothesize a role for citrate in the later stages of cellular respiration?

In 1937, two German biochemists, Franz Knoop and Carl Martius, published a series of reactions beginning with citrate and ending in another organic acid, oxaloacetate (see Figure 7.3(A)). Prominently placed in this scheme were the three compounds

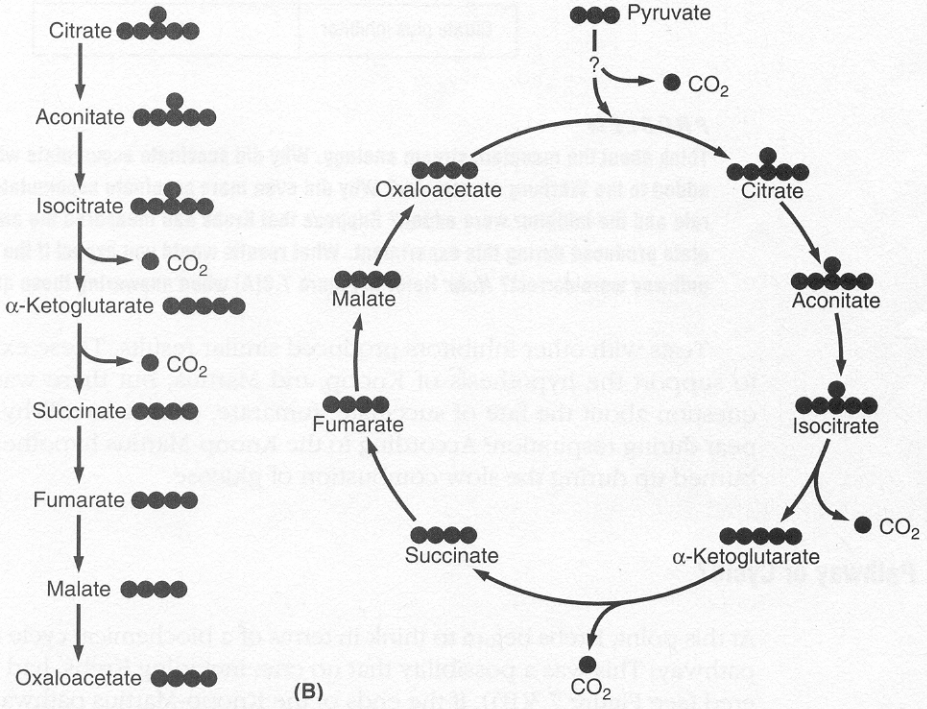

FIGURE 7.3 (A) The hypothetical metabolic pathway proposed by Franz Knoop and Carl Martius. This pathway included all of the steps in the Krebs cycle in the correct order. But Knoop and Martius incorrectly presented the reactions as a linear pathway rather than a metabolic cycle. *Note:* Only the carbon backbones of each intermediate compound are represented in this diagram. (B) A simplified diagram of the Krebs cycle. The crucial step leading from pyruvate into the cycle was not fully explained for a decade after the publication of Krebs's scheme. The linkage between the steps in the Krebs cycle and the production of ATP was also not well understood in 1937.

studied by Krebs and Szent-Györgyi: succinate, fumarate, and malate. Was this a large missing piece in the biochemical pathway leading from glucose to carbon dioxide?

After reading this report, Krebs designed a simple experiment to test this hypothesis. He would try to block one of the reactions in the hypothetical pathway with a chemical inhibitor. If successful, the later reactions in the chain should not occur and the intermediate compound just before the block should accumulate. To visualize the design of this experiment, think about a mountain stream cascading from one pool to another as it flows downstream. If you dam the stream, the water will stop flowing below the dam and will accumulate in the pool just above the dam.

Krebs chose to inhibit the proposed reaction leading from succinate to fumarate (see Figure 7.3(A)). If the Knoop-Martius pathway was correct, succinate should accumulate in the Warburg manometer. At the end of the experiment, Krebs recorded the following results:

Substance Added	Succinate Produced (µl)
None (control)	20.5
Citrate	64.5
Citrate plus inhibitor	387.0

PROBLEM

Think about the mountain stream analogy. Why did succinate accumulate when citrate was added to the Warburg manometer? Why did even more succinate accumulate when both citrate and the inhibitor were added? Suppose that Krebs had measured the amount of oxaloacetate produced during this experiment. What results would you expect if the Knoop-Martius pathway were correct? *Note:* Refer to Figure 7.3(A) when answering these questions.

Tests with other inhibitors produced similar results. These experiments seemed to support the hypothesis of Knoop and Martius, but there was still the nagging question about the fate of succinate, fumarate, and malate. Why didn't they disappear during respiration? According to the Knoop-Martius hypothesis, they should be burned up during the slow combustion of glucose.

Pathway or Cycle?

At this point, Krebs began to think in terms of a biochemical cycle rather than a linear pathway. This was a possibility that no one, including Krebs, had previously considered (see Figure 7.3(B)). If the ends of the Knoop-Martius pathway were connected, several pieces of the jigsaw puzzle would fall into place. A cycle of reactions would explain why succinate, fumarate, and malate did not disappear during cellular respiration—instead of being completely oxidized, they were regenerated with each turn of the cycle. It also explained the crucial role of citric acid as the first intermediate compound in the cycle. Finally, the results of Krebs's experiments with chemical inhibitors fit just as well with a biochemical cycle as they did with a linear pathway.

Could Krebs find experimental evidence that would support his hypothesis rather than the hypothesis of Knoop and Martius? The key would be to demonstrate a reaction that produced citrate from oxaloacetate. In a crucial set of experiments, Krebs found that when both pyruvate and oxaloacetate were added to the Warburg manometer, the amount of citrate increased. This was particularly significant because Krebs knew that pyruvate was the major product of glycolysis. He had firmly tied the two ends of the Knoop-Martius pathway together, forming what we now call the Krebs cycle.

At this point—four months after reading the article by Knoop and Martius—Krebs was ready to report what he called "the citric acid cycle." He chose to submit a short article to *Nature*, one of the world's most widely read scientific journals. Because it is published weekly, scientists often use this journal to quickly communicate important discoveries. Ironically, *Nature* rejected the paper—the only time this ever happened to Krebs. Perhaps we should not make too much of this, because almost every scientist has papers rejected. The editor was not a biochemist, and he had to select among many submitted manuscripts. This anecdote should remind us, however, that sometimes even important scientific discoveries are not immediately recognized.

Stung by the rejection, Krebs sent a longer article to a leading biochemical journal, which quickly published it. Realizing the importance of Krebs's work, biochemists expanded on it. During the years following its discovery, the citric acid cycle was found in a wide variety of organisms, including many bacteria. It played a critical role in the oxidation not only of glucose and other carbohydrates, but also of fats and proteins. What later became known as the "Krebs cycle" was the central energy-releasing pathway in almost all cells.

Krebs liked to compare his accomplishment to piecing together a jigsaw puzzle. As he recognized, however, the Krebs cycle was also part of a much larger puzzle. During his research, Krebs treated the cell as a "black box." Neither he nor anyone else knew exactly where respiration occurred in the cell. Although some scientists suspected it occurred in the mitochondria, this was not demonstrated until a decade after Krebs's discovery. The important role of ATP as the primary energy-carrying molecule was only beginning to be understood when Krebs discovered his cycle. How ATP is synthesized in the mitochondria would not be discovered for another 30 years (see Chapter 8). Although Krebs knew that pyruvate links glycolysis to the citric acid cycle, he did not understand the connection. The important role of coenzyme A in bridging these two pathways was not discovered until a decade after Krebs described his cycle. Recognizing how many of the pieces of the puzzle were still missing in 1937 does not diminish Krebs's accomplishment, but it reminds us that scientific knowledge is often constructed of small increments.

☐ EPILOGUE

How can we explain scientific creativity? What makes one scientist more successful than another? Why was Krebs able to see a biochemical cycle where other scientists still saw a linear pathway? There are no simple answers to these questions, but Krebs's scientific career provides some interesting insights into the nature of creativity. It also dispels a common misconception about how discoveries are made.

Remember that Krebs had discovered another biochemical cycle several years before he completed his work on the citric acid cycle. The urea-producing process was the first example of a biochemical cycle, and its discovery brought Krebs international recognition. Did this early discovery predispose Krebs to look for other biochemical cycles? Is there evidence that at some point in his later research on cellular respiration he had a flash of insight in which he suddenly recognized another cycle? Apparently this kind of "Aha!" or "Eureka!" experience did not occur. Krebs could never pinpoint exactly when the discovery took place, and he always denied having had an "Aha!" experience. Frederic Holmes, a historian who carefully studied Krebs's laboratory notebooks, also claims that there is no evidence for a sudden flash of insight. Like other biochemists, Krebs believed that cellular respiration was a linear pathway, and he only gradually came to see its cyclic nature.

If Krebs's discovery was not due to a flash of insight, what might account for his achievement? Holmes points to several important elements of Krebs's creativity. Like all successful scientists, Krebs was deeply committed to research. Early in his career, he pinpointed the major unsolved problems facing the biochemists of his day. After carefully choosing among these problems, he devoted his life to solving some of them. This usually meant spending six days a week in the laboratory. Despite this rigorous, highly structured approach to work, Krebs remained intellectually very flexible. He quickly abandoned hypotheses when they could not be supported by experimental evidence. This willingness to change direction meant that he rarely got bogged down in fruitless dead ends.

These characteristics help explain Krebs's creative insight. He had been trying to fit the pieces of his puzzle together for several years. Although he did not have a solution to the complete problem, he was very familiar with many of its parts. When he ultimately solved the problem, he was following the same routines that he had used throughout his research. There was no flash of insight, just a reworking of the problem from a slightly different perspective. This fresh perspective emerged after reading the paper of Knoop and Martius. Even so, it took several days of thinking and experimenting before Krebs began to realize that tying the ends of the Knoop-Martius pathway would produce another biochemical cycle. Gradually seeing what no one had previously recognized was Krebs's creative act.

QUESTIONS AND ACTIVITIES

1. What does this case show about the following aspects of doing biology?
- — role of scientific instruments
- — mentors and the training of young scientists
- — positive role of incorrect hypotheses
- — incremental nature of discovery
- — scientific creativity

2. During the 1800s, Eduard Buchner performed experiments using "cell-free" extracts. He demonstrated that fermentation occurred even in the absence of intact cells, but he was unable to detect respiration in cell-free extracts. How can this be explained by the different sites where these processes occur in the cell? Why did respiration occur in tissue preparations that Warburg placed in his manometer?
3. When Krebs added succinate, fumarate, or malate to the Warburg manometer, he was surprised to find that respiration increased even more than predicted. He was also surprised to find that these compounds were not consumed during respiration. How does the Krebs cycle—Figure 7.3(B)—explain these unexpected experimental results? What would you predict would happen if pyruvate were added to the Warburg manometer? Why might pyruvate behave differently than succinate, fumarate, or malate?
4. Krebs compared doing science to putting together a jigsaw puzzle. He stressed the importance of cumulative small steps in problem solving. How did the type of questions Krebs asked, his research style, and his equipment favor a puzzle-solving approach to scientific work?
5. Consider the Krebs cycle as it is presented in your biology textbook. What parts of the process were known before Krebs published his description of the cycle? What contributions did Krebs make? What important parts were discovered after Krebs published his paper?
6. Krebs called his cycle the "tricarboxylic acid cycle" after the family of chemical compounds involved in the reactions. He also used the term "citric acid cycle," after the first intermediate in the cycle. Today most biologists refer to this process as "the Krebs cycle." Does this honorific name give too much credit to one scientist among the many who worked on the problem of cellular respiration? How much credit for the discovery do Knoop and Martius deserve for proposing their hypothesis?

SUGGESTED READING

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