The Pyruvate Carboxylase Paradox

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The content presented here originated from a question a student asked during a Biochemistry lecture I gave to undergraduate Chemistry students at Sapienza University in the 2022-23 academic year. The topic of that lecture was gluconeogenesis.

Gluconeogenesis, a biochemically significant process, stands as the anabolic pathway dedicated to the *de novo* synthesis of glucose — the primary cellular fuel — from non-sugar precursors. This sequence of enzymatic reactions, antithetical to glycolysis, unfolds through a series of chemical transformations marked by exquisite molecular elegance. Starting from substrates like pyruvate, lactate, or glucogenic amino acids, this metabolic pathway traverses distinct subcellular compartments, orchestrating a symphony of conversions that culminates in the generation of the much-coveted hexose. The critical steps, catalyzed by enzymes of refined specificity such as pyruvate carboxylase and phosphoenolpyruvate carboxykinase, proceed with stereochemical inversions and strategic phosphorylations. The conclusion of this biochemical odyssey sees the intervention of glucose-6-phosphatase, an enzyme that, with a definitive catalytic gesture, releases the newly synthesized glucose, ready to fulfill its primary role in the cellular and organismic energy landscape. This metabolic pathway, regulated with fine precision by multiple allosteric effectors and post-translational modifications, stands as a bastion of glycemic homeostasis, shaping the physiological response to energetic fluctuations with unmatched biochemical mastery.

The first enzyme in this biosynthetic pathway is pyruvate carboxylase (PC, a biotin-dependent ligase, EC 6.4.1.1), which catalyzes the following reaction:

$$\text{Pyruvate} + HCO_3^- + \text{ATP} + H_2O \rightarrow \text{Oxaloacetate} + \text{ADP} + P_i$$

PC, a tetrameric enzyme found in the mitochondrial matrix, catalyzes this reaction in both directions and is composed of four domains (Figure 1): the biotin carboxylase (BC) domain, the carboxyltransferase (CT) domain containing the

two active sites of the enzyme, the biotin carboxyl carrier protein (BCCP) domain, and the allosteric domain that binds the activator acetyl-CoA. In my lecture, which I remind you was aimed at chemistry students, I described the accepted mechanism of the reaction in detail. The biotin-mediated carboxylation reactions occur in two phases, generally catalyzed at separate active sites, BC and CT, of the enzyme. In the first phase, bicarbonate is converted into the more reactive CO_2, with an unstable phosphorylated intermediate then used to carboxylate biotin.

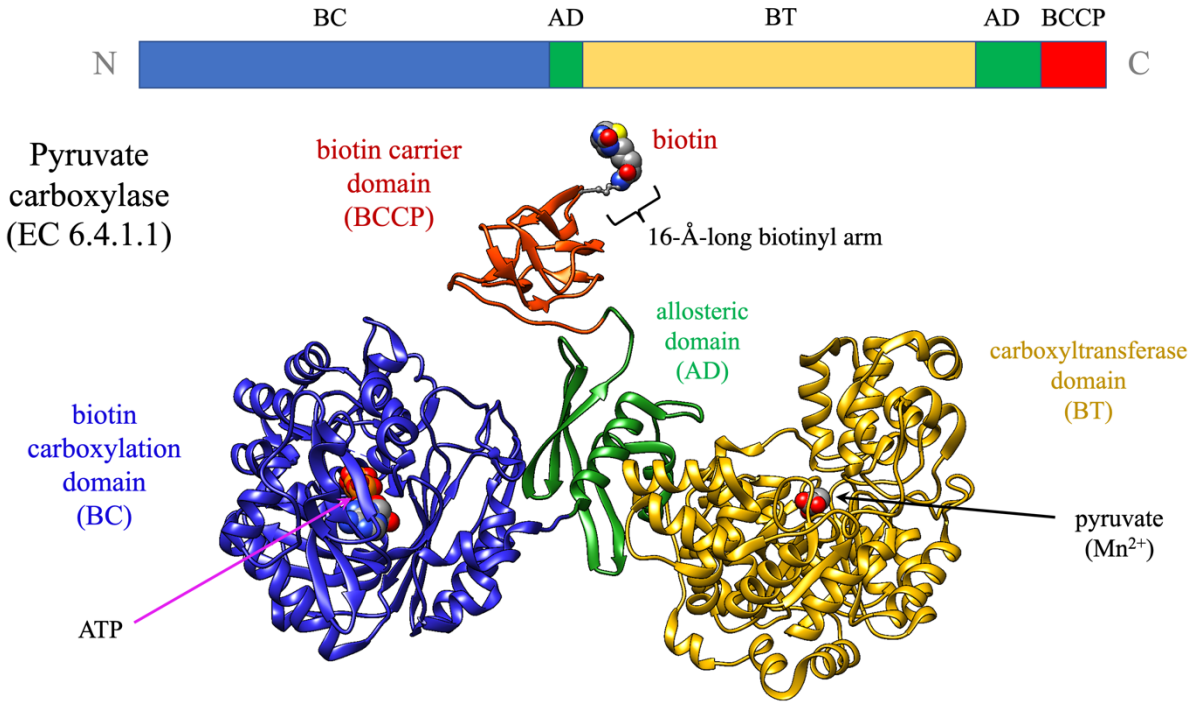

Figure 1. Structure of PC from *S. aureus* and H. sapiens. PDB: 3BG5. Image generated with UCSF Chimera and modified from (2).

Biotin, covalently bound to a lysine residue present in the biotin carrier domain, acts as a carrier for the CO_2 transport from one active site to another on an adjacent monomer of the tetrameric enzyme, from the biotin carboxylation domain to the carboxyltransferase domain. In the second phase, catalyzed in this second active site, CO_2 reacts with pyruvate to form oxaloacetate.

At the end of the lesson, a visibly shy student approached the podium and asked me a question: "Professor, why did nature decide that one ATP molecule must be consumed to generate the intermediate carboxyphosphate, which then decomposes into CO_2 and phosphate? Wouldn't it have been simpler to use CO_2 directly?"

I reflected for a moment before replying, "In the mitochondrial matrix, the concentration of CO_2 is low or negligible due to the alkaline pH (around 8) maintained by the proton pumps of the electron transport chain during oxidative phosphorylation. Nature seems to have solved this problem by creating an alternative pathway for the *in situ* synthesis of CO_2." The student, visibly satisfied with the explanation, thanked me with a smile.

Despite the student's satisfaction, I was not completely satisfied with my response. A sense of curiosity and uncertainty gripped me: who had actually demonstrated this theory? Biochemistry, with its vastness and complexity, makes it difficult for anyone to possess complete and in-depth knowledge of every aspect. During my research, I encountered a recurring challenge in the academic world: the difficulty of tracing the original source of scientific information. This problem, known as "citation chaining", occurs when authors improperly cite works that, in turn, refer to other publications, creating an intricate labyrinth of references. This practice, although common, can make it extremely difficult to trace the actual origin of experimental data or a discovery. The situation is further complicated by the vastness of modern scientific literature and the rapidity with which new research is published.

In an era where access to information is broader than ever, paradoxically, establishing the exact authorship of experimental data or a specific theory can prove to be a surprisingly complex task. This phenomenon not only hinders the verification of sources but can also lead to the propagation of erroneous interpretations or the loss of important nuances present in the original work.

I decided to delve deeper into the matter. I began with a meticulous search on PubMed, hoping to find studies that definitively demonstrated what the true substrate of PC was. In particular, I sought confirmation of the hypothesis that it was bicarbonate and not CO_2. However, despite hours of research, I couldn't find a definitive answer. This initial frustration, rather than discouraging me, further fueled my scientific curiosity. I decided to change strategy: instead of directly seeking the information, I focused on the most prominent researchers studying PC.

After a careful evaluation of the scientific literature, one name emerged as particularly relevant: Professor Paul Attwood from the University of Western

Australia in Perth. His reputation in the field and his significant contributions to the study of PC made him the ideal candidate to answer my questions.

With a mixture of trepidation and hope, I composed a detailed email to Professor Attwood. In the message, I explained my dilemma, provided the context of the student's question, and asked for clarification on the nature of PC's substrate.

Professor Attwood's response proved enlightening, providing me with the key to resolving my bibliographic dilemma. The work he suggested (1) had escaped my initial search for an unexpected reason: the crucial experiments that established the currently accepted mechanism for carboxyphosphate synthesis had not been conducted on PC, as I had mistakenly assumed, but on propionyl-CoA carboxylase! PC and propionyl-CoA carboxylase are structurally related enzymes. They belong to the family of biotin-dependent carboxylases, enzymes that utilize biotin as a cofactor to catalyze carboxylation reactions through a common kinetic mechanism.

This seemingly minor detail had a significant impact on my ability to find the original source. The study in question was ingeniously designed: the researchers had mixed the enzyme with ATP, bicarbonate labeled with the isotope ^{18}O, and pyruvate. The revealing aspect of the experiment was the incorporation of ^{18}O into the phosphate product (1), as shown in Figure 2. This discovery highlighted

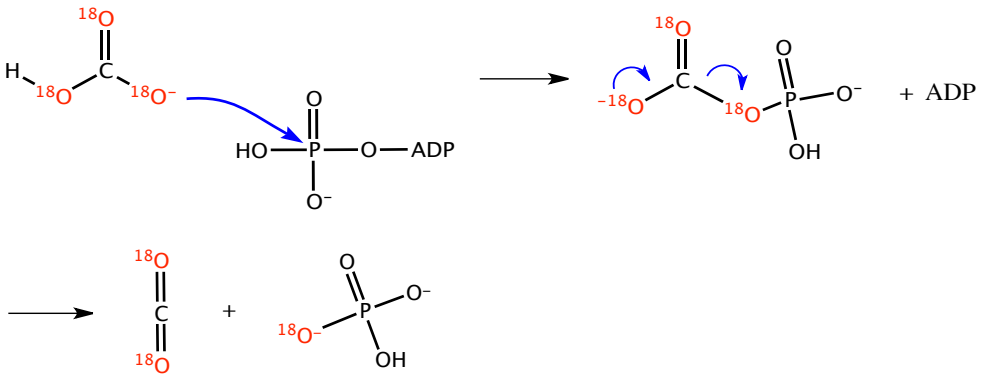

Figure 2. The exchange of ^{18}O between [^{18}O]carboxyphosphate and phosphate through carboxyphosphate decomposition. The half-life of carboxyphosphate is estimated to be ca. 70 ms (Clymer, T., 2015. Computational Characterization of Carboxyphosphate. Doctoral dissertation, Duquesne University. Retrieved from https://dsc.duq.edu/etd/75)

not only the complexity of metabolic pathways but also the importance of broadening the scope of research when investigating biochemical mechanisms.

Often, seemingly unrelated enzymes can share similar mechanisms, and discoveries in one area can shed light on processes in another. Without Professor Attwood's suggestion, this valuable information would have remained hidden, demonstrating how communication between researchers can bridge crucial gaps in our understanding.

To ultimately arrive at a comprehensive solution to the problem discussed here, it is necessary to demonstrate that the concentration of CO_2 within the mitochondrial matrix is negligible. Figure 3A illustrates the equilibria to which CO_2 in our body is subject, including the portion produced by the Krebs cycle. These equilibria include: the dissolution of CO_2, the hydration reaction catalyzed by carbonic anhydrase, the first and second ionization of carbonic acid, as well as the reaction of CO_2 with the hydroxide anion, which becomes predominant at pH values above 8.

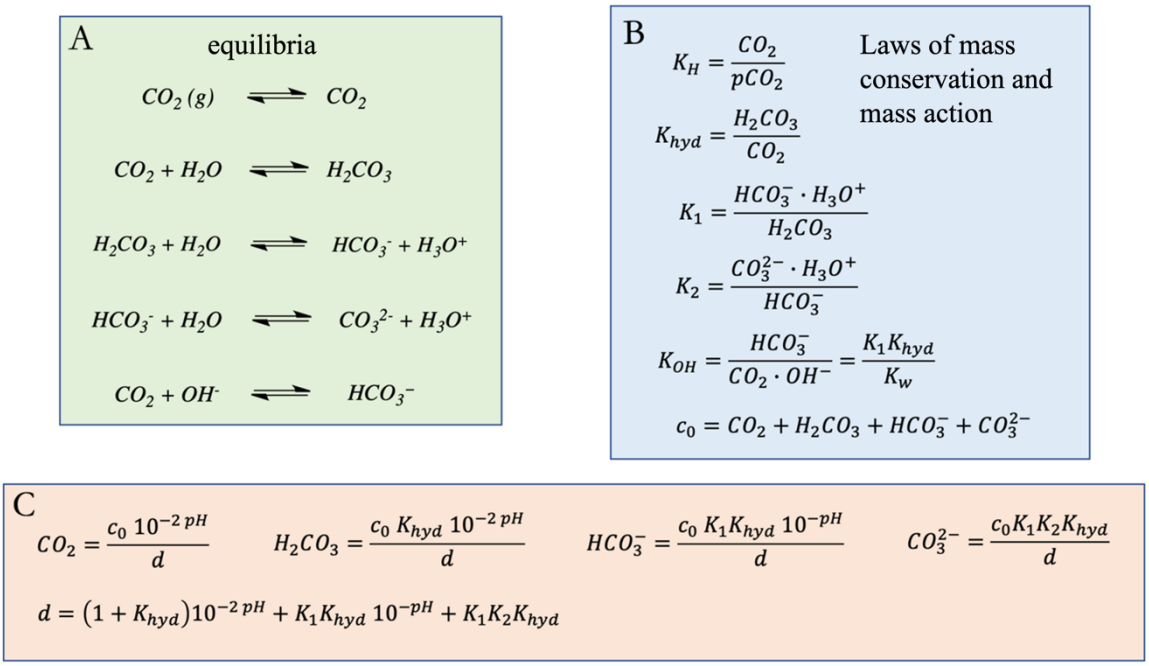

Figure 3. Calculation of the concentrations of CO_2 and its species obtained following the hydration reaction and subsequent ionization reactions. A. Involved equilibria. B. Corresponding mass action laws. The total carbon concentration c_0 is obtained from Henry's law using the expression $CO_2 = K_H \cdot pCO_2$, where K_H e pCO_2 represent Henry's constant and the partial pressure of CO_2, respectively. C. Solution of the equation system.

Figure 3B shows the corresponding equilibrium constants and the law of conservation of mass, where c_0 indicates the total amount of carbon present and distributed among the various forms. The solution to the system of equations in

Figure 3B is given by the equations shown in Figure 3C. The graph resulting from these equations is illustrated in Figure 4. The objective is to determine the concentration of CO_2 in the mitochondrion at the pH of the matrix and at 37 °C. To achieve this goal, two main issues need to be addressed: a) estimating the pH of the mitochondrial matrix, and b) estimating the partial pressure of CO_2 (pCO_2) inside the mitochondrion.

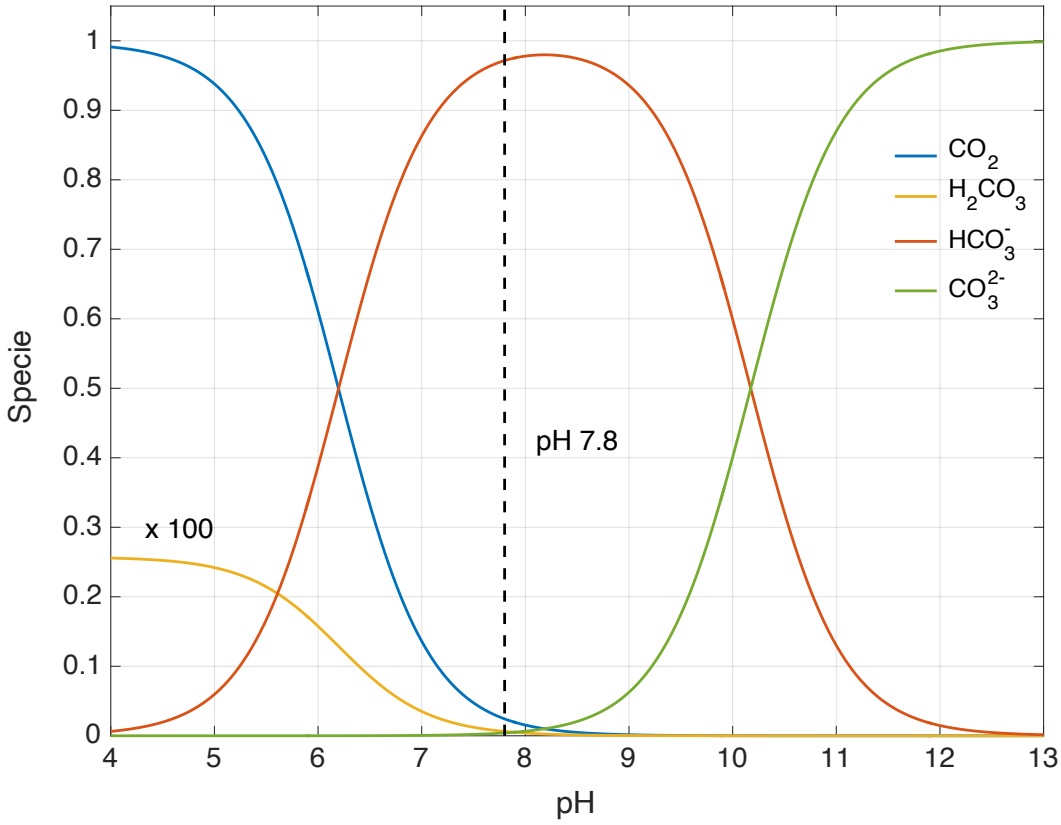

Figure 4. pH dependence of the relative concentration of CO2 and its derivatives with water at 37°C. The equilibrium constant values used are: K_H = 0.0239 M atm^{-1}, K_{hyd} = 2.58·10-3, K_1 = $2.46 \cdot 10^{-4}$ M, K_2 = $6.69 \cdot 10^{-11}$ M, K_w = $2.34 \cdot 10^{-14}$ M^2. pCO_2 = 69 torr, which corresponds to a total carbon concentration of 2.17 mM. The concentrations of all species shown in the figure have been normalized to this value. The equilibrium constant values are taken from references (3, 4). The dashed line shows the estimated intramitochondrial pH.

a) The intramitochondrial pH has been determined to be close to 7.8 through several studies using various measurement methods (5-8). These studies employed techniques such as genetically encoded pH sensors, pH-sensitive variants of fluorescent proteins, and direct measurements in living cells. The research has consistently reported mitochondrial matrix pH values ranging from about 7.5 to 8.0, with many studies converging around the slightly alkaline value

of 7.8. Measurements were performed in both resting cells and under various conditions, and the results have been consistent over time, as the studies span a period of more than a decade. The convergence of these results, obtained through different methods and at different times, provides a scientific basis for asserting that the pH inside mitochondria is indeed close to 7.8.

b) Determining the mitochondrial pCO_2 at 37°C and pH 7.8 requires careful analysis of several factors and a critical review of the relevant scientific literature. Mitochondria, being the main sites of cellular energy production, generally have a higher pCO_2 compared to the cytosol, due to their intense metabolic activity and consequent CO_2 production. Some estimates suggest that intramitochondrial pCO_2 may be 1.5 to 2 times higher than cytosolic pCO_2 (9). Considering that cytosolic pCO_2 in resting mammalian cells has been reported to be around 46 mmHg, one could hypothesize a mitochondrial pCO_2 between 69 and 92 mmHg. However, it is crucial to emphasize that these values are approximate and can vary significantly depending on cell type, metabolic state, and other physiological factors. Based on these data, Figure 4 has been developed, which illustrates that at the physiological pH of 7.8, the percentage of free CO_2 is slightly above 2.4%. This percentage corresponds to a concentration of approximately 53 μM of dissolved CO_2.

Conclusions.

This brief investigation has demonstrated that pyruvate carboxylase uses bicarbonate as its primary substrate, rather than free CO_2. This mechanism has evolved in response to the specific biochemical conditions of the mitochondrial matrix, where the alkaline pH favors the presence of bicarbonate over dissolved CO_2. The very nature of scientific research, in its ceaseless yearning for an ever more refined understanding of natural phenomena, draws vital nourishment from constructive dialogue and the fruitful exchange of ideas among curious minds well-versed in the subject.

Any Reader who has further perplexities or is animated by a desire to contribute to the refinement of any aspect of this succinct but thoughtful survey is invited to make his or her valuable contribution by e-mail. This work was originally published in Italian in *The Flying Biochemist***:** a new recurring web feature of the SIB Education Group.

https://sib-biochemistry.it/services/the-flying-biochemist/

Acknowledgments. Heartfelt praise must be directed to the Students, whose curious minds and penetrating questions serve as intellectual catalysts, stimulating not only their own learning but also that of their mentors!