Review Article

Mechanisms of Glycolysis and Fermentation: A Non-Equilibrium Thermodynamics Perspective

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Every single chemical formula of modern models of glycolysis violates the scientific rules formulated by Newton (Physics) and Lavoisier (Chemistry). Yet, the formulae of the pioneers who investigated metabolism did not violate the laws of nature. Recently, the well-established models of metabolism have collapsed by re-introducing hydrogen (Chemistry, Physics) as the energy entity driving the Krebs-cycle (Biochemistry). This review builds on a scientific concept of metabolism by introducing that glycolytically generated energy is either transformed into ATP or drives a biological process. The dynamic production and utilization of lactate (lactate flow non-equilibrium) is introduced as a central ATP-driven process and the first step of biosynthesis. A metabolism model based on non-equilibrium thermodynamics replaces the current understanding that one end product of glycolysis is consumed by mitochondria with two intermediates of the two-cell model of metabolism that are consumed by mitochondria. The pyruvate dehydrogenase complex, consuming pyruvic acid, saves one redox unit (2H) for storage as lipid or glycogen, whereas mitochondrial consumption of lactic acid enhances ATP recovery. An uncounted number of signalling pathways temporarily regulate the distribution of this single redox unit. Glycogenolysis massively impacts the flow non-equilibrium, an event permanently memorized by cells. The two-cell model of metabolism starts to functionally unite fields such as memory formation, obesity, exercise, schizophrenia, cancer, and inflammation by the common denominator: metabolism.

Introduction (Physics)

Equilibrium thermodynamics, the 2nd law of thermodynamics, provides the understanding that an apple falls on Earth and Earth falls on an apple[1]. Non-equilibrium thermodynamics, the 4th law of

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thermodynamics, has to provide an understanding of how the apple has reached flow non-equilibrium with Earth in order to fall down. Non-equilibrium thermodynamics is the Physics of Biology.

Maxwell's observation of the dissipation of energy in nature guided Thomson to the formulation of the thermodynamic demon hypothesis in 1879[2]. The hypothesis implies that thermodynamic demons carry apples on trees to let them drop on physicists' heat. Physicists have hunted the demons for more than a century, in vain. Recently, the demon hypothesis was updated to the zombie-hypothesis: Maxwell's zombies mulling and mauling the 2nd law of thermodynamics[3]. The undead are open systems; they eat and drink. Open systems do not fall under the jurisdiction of the 2nd law of thermodynamics. The undead metabolize and temporarily retain the gained energy by transferring the energy to a more stable energy particle. A living organism is an open system in steady state flow non-equilibrium with the environment and falls under the jurisdiction of the 4th law of thermodynamics.

Physicists have developed a comprehensive working plan to understand life. The aim of biochemistry is to provide physicists with the name of the energy particle (demon, zombie).

Today, in Biology, it is known that apple trees transform sunshine into the fuel glucose and glucose is burned to water, carbon dioxide, and apple. The chemistry of the process burning glucose to water, carbon dioxide, and ~ -ΔG 2800 kJ/mol is known. Apple trees do not emit the energy of ~ -ΔG 2800 kJ/mol. The biochemistry of metabolic burning of glucose to water, carbon dioxide, and apple is unknown. The historical name of the thermodynamic demon is *Phlogiston*, which is emitted when substances are burned[4]. The *Phlogiston* cult was replaced by the formulation of the law of the conservation of mass. Thus, if the demon has a molecular mass, then *Phlogiston* is hidden in the chemical formulae of glycolysis and fermentation. In open systems, *Phlogiston* turns into a zombie. In vivo, *Phlogiston* is generated during the metabolic burning or oxidation ($NAD^+ \rightarrow NADH\text{-}H^+$-system) of glucose and moves to a defined coupled biological process. The coupled biologic process retained energy from emitting as heat by transforming energy to a temporarily more stable energy particle. The thermodynamic zombie vanishes from chemical formulae during transformation.

Chemical formulae of the metabolic burning of glucose show carboxylic acids (R-COOH), even if it is known that acids promptly dissociate in water to anion ($R\text{-}COO^-$), proton (H^+), and heat[5]. Now consider the kinetics of a proton-linked Monocarboxylate Transporter (proton-linked MCT): 1st, a proton (H^+) binds; 2nd, an anion (R-COO-) binds; and the charge-neutral acid (R-COOH) moves through the membrane[6]. The kinetics is in line with the 3rd law of thermodynamics, the law of

motion. First energy, second reaction. Thus, H^+ is the energy particle freed during burning ($NADH-H^+$), the energy particle that freely dissociates (emits) in water, and the energy particle that initiates and guides unidirectional movement in biological processes.

Enzymes catalysing the burning of glucose are organized in complexes or metabolons[7][8][9]. Enzyme complexes, such as the pyruvic acid dehydrogenase complex (PADHc), lactic acid consuming Citric Acid Cycle complex, and the mitochondrial ATP-synthase complex, have in common that the intermediates are acidic (figure 1).

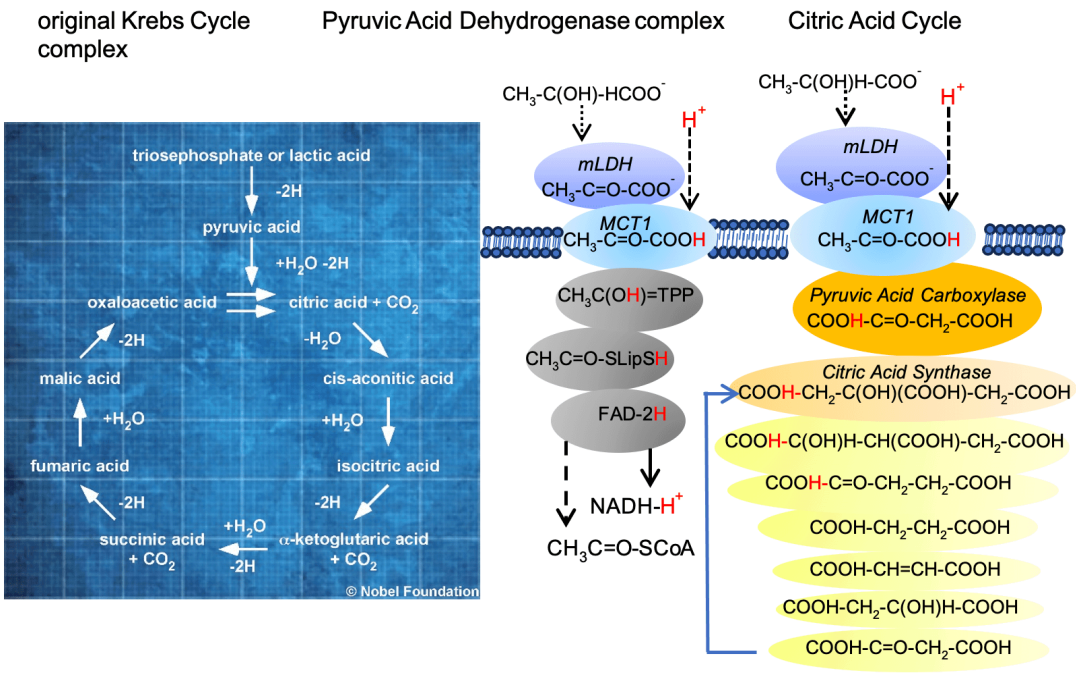

Figure 1. Mitochondrial consumption of lactic acid. The blueprint of the original concept of the Citric Acid Cycle encrypts the biochemical path of metabolic burning of lactic acid (Pyruvic Acid Dehydrogenase complex) and biosynthesis of di- and tri-carboxylic acids (Citric Acid Cycle complex)[10]. Mitochondrial lactate dehydrogenase metabolically burns lactate ($CH_3-C(OH)H\ -COO^-$) to pyruvate ($CH_3-C=O-COO^-$)[11]. Proton-linked Monocarboxylate Transporter 1 (MCT1) transfers pyruvic acid ($CH_3-C=O-COOH$) to (left) Pyruvic Acid Dehydrogenase complex or (right) Citric Acid Cycle complex 1. Pyruvic Acid Dehydrogenase complex metabolically burns pyruvic acid to acetyl-SCoA and $NADH-H^+$[12][13]. Pyruvic Acid Carboxylase refiles the Citric Acid Cycle complex with oxaloacetic acid ($COOH-C=O-CH_2-COOH$). Pyruvic Acid Carboxylase catalyses ATP driven synthesis of oxaloacetic acid. Citric Acid Synthase catalyses biosynthesis of citric acid[14][15][16][7].

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This review introduces that the first intermediates of the glycolytic metabolon are acids. The Proton Transport Chain hypothesis states that intermediates are directly or water-free transferred within complexes to save dissociation heat for the coupled biological process[17]. First, the proton is transferred to the coupled enzymatic process; 2nd, the anion follows. In 1976, the flow of the energy particle proton (H^+) through mitochondrial ATP synthase was named proticity[18]. The biochemistry of enzyme complexes indicates that enzyme complexes are bio-wires (figure 2).

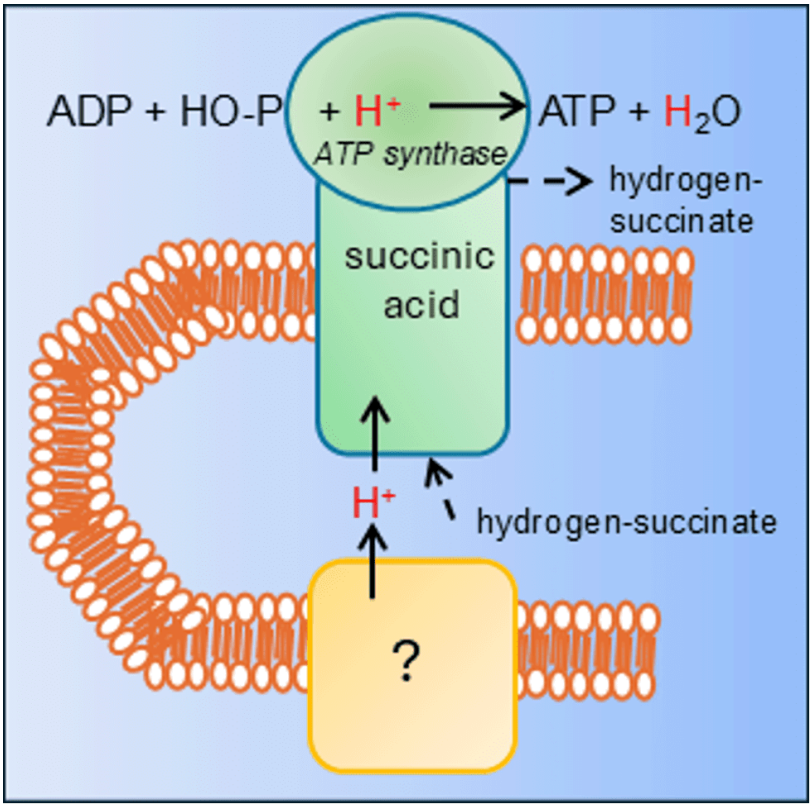


Figure 2. Mitochondrial ATP synthase. Proton (H^+) and hydrogen-succinate form charge-neutral succinic acid. Succinic acid is wired through the ATP-synthase complex. The complex catalyses the transfer of energy from unstable succinic acid to the temporarily more stable *tri*-phosphoric acid anhydride group of ATP. The proton (H^+) is discharged to water[19][20].

Enzymes stabilize their substrate water-free within the active site. The substrate is solved by the enzyme complex, not by water. The physical quantity [mol/L] is applied to calculate an enzymatic

catalysed reaction of a thermodynamic equilibrium. Direct or water-free transfer of an intermediate within an enzyme complex is mathematically one molecule in zero Litre of water. 1 molecule of acid divided by 0 L of water is an infinite molarity. An infinite molarity of one product changes the understanding (Physics and Mathematics) of a reversible chemical process into the understanding of the biochemical process of an irreversible metabolic flow.

One enzyme catalyses 1 molecule at a time. Molecule/time is the physical quantity of a flow. Acid/time is the physical quantity of the flow of the energy entity H^+ and material (anion) through enzyme complexes.

In ecology, the tentative 4^{th} law of thermodynamics states: a flow of energy and material is sufficient to form ordered structures[21]. In this case, we suggest calling the thermodynamic zombie, proton (H^+).

The biochemistry of the proton-linked MCT_1-Carbonic Anhydrase II complex, located at the cell membrane, is best visualized as an 'ordered structure' in a state of flow non-equilibrium. Carbonic Anhydrase II activity is located outside the cell (Figure 3). At time point 0, the environment and the cell are in lactate equilibrium. Carbonic Anhydrases contain zinc (Zn^{2+}), which is a Lewis acid in their active sites. Zn^{2+} reacts with environmental water to initiate the Proton Transport Chain: $Zn^{2+} + H_2O \rightarrow Zn^{2+}[OH^-] + H^+$[22].

Thus, firstly, the energy particle H^+ taken from water is water-free transferred to the proton-linked MCT_1; secondly, lactate binds, and lactic acid moves into the cell.

The cell continuously emits carbon dioxide (the acid anhydride of carbonic acid). This emission restores the activity of Carbonic Anhydrase II, following the reaction: $Zn^{2+}[OH^-] + CO_2 \rightarrow Zn^{2+} + HCO_3^-$. The flow of CO_2 permanently restores Carbonic Anhydrase II activity. This process generates a proticity that pumps lactic acid from the environment into the cell, causing the cell to enter a state of lactate flow non-equilibrium with its surroundings.

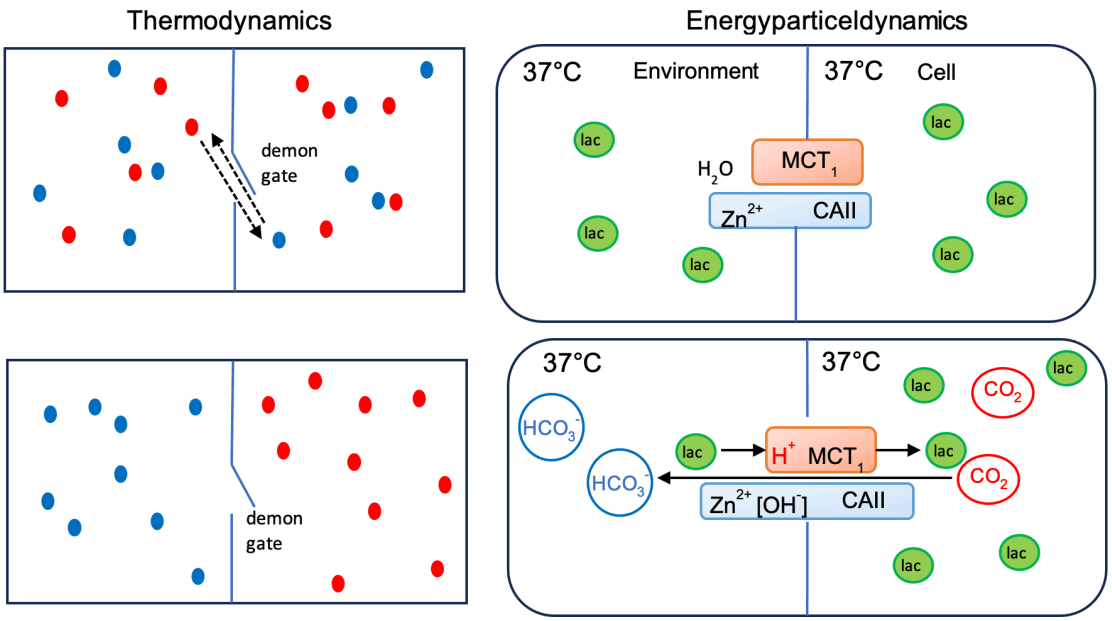


Figure 3. Thermodynamic demons and zombies. (left) A thermodynamic demon guards the demon gate and sorts blue and red circles. (right) Cells and environment are in lactate (lac) equilibrium. Carbonic anhydrase II (CAII) carries the acid zinc^{2+}[23]. Zn^{2+} reacts with water. Freed H^+ is transferred to proton-linked MCT_1 (MCT_1). Emitting CO_2 (red) recovers the activity of the CAII-proton-linked MCT_1 complex. A lactate flow non-equilibrium is generated. End product of the reaction is bicarbonate (HCO_3^-, blue).

The path the authors have taken to this review started with the formulation of the Proton Transport Chain hypothesis and is documented in five reviews[24][7][25][26][17]. This review discusses the chemical formulae of glycolysis and fermentation published by Meyerhof[5]. Meyerhof's review is the most recently published concept of glycolysis and fermentation not violating the law of the conservation of mass that we have found.

The understanding of Chemistry, the 2nd law of thermodynamics, guided scientists to define lactic acid as the exported end product of fermentation and lactate as the end product of glycolysis[27][28]. This review updates the chemistry of glycolysis and fermentation to the biochemistry of glycolysis and fermentation. In line with the 4th law of thermodynamics, the path of the energy entity proton within the glycolytic metabolon was followed.

We assume this review presents the first scientific (in line with the laws of nature and published pioneer work) integration of the NAD^+/NADH-H^+ system and ATP in glycolysis and fermentation[5].

Understanding the *tri*-phosphoric acid anhydride group of ATP as the storage of the energy of a proton allowed us to introduce lactate as the first ATP-driven biosynthesis product. The glycolytic flow of energy and material generates a cytosolic lactate flow non-equilibrium. The generation of this cytosolic lactate concentration gradient enables the mitochondrial lactate dehydrogenase-proton-linked MCT_1 complex (figure 1) to catalyse the metabolic burning of lactate to pyruvate and $NADH\text{-}H^+$ and the membrane transfer of charge-free pyruvic acid to the membrane-associated PADHc and Citric Acid Cycle complex.

Finally, the first part of the apple is on the tree. The energy particle (H^+) freed during the hydrolysis of ATP: $ATP + H_2O \rightarrow ADP\text{-}H^+ + HO\text{-}P$ does not chaotically emit, heat up, and acidify the cell but is wired from hexokinase to Pyruvate Hydrogenase (PH) to generate a cytosolic lactate flow non-equilibrium. Lactate, understood as the first biosynthesis product, falls into mitochondrial degradation to be metabolically burned to pyruvic acid and $NADH\text{-}H^+$. Thus, 85 years after the formulation of the lactic acid-consuming Citric Acid Cycle, Krebs' work is rationally linked with glycolysis[14][15][29]. A century after the discovery of the $NAD^+/NADH\text{-}H^+$ system and ATP, the co-enzymes are finally scientifically (in line with the laws of nature) integrated into glycolysis and fermentation.

Introduction II (Biochemistry)

F. Cori: “Some 50 years ago - in 1929, to be exact - we proposed a cycle of the glucose molecule which could be in turn liver glycogen, blood glucose, muscle glycogen. The conversion of lactic acid to liver glycogen would complete the cycle”[30][31]. The Cori cycle was formulated in the year K. Lohmann discovered ATP[32]. At this time, the citric acid cycles and thereby mitochondrial participation in biosynthesis and ATP recovery was undiscovered. F. and G. Cori determined that insulin accelerates the Cori cycle from liver to muscle glycogen, while epinephrine accelerates the cycle in the opposite direction[30]. Today, it is known that other organs such as blood (thrombocytes, macrophages, T-cells) or brain (astrocytes) also contain glycogen. The Cori cycle developed into the two-cell model of metabolism[25].

Cells memorize the event of glycogenolysis[33][34][35]. Astrocytic glycogenolysis, or more specifically the astrocyte neuron lactate shuttle hypothesis (ANLS), was introduced as energy on demand. Briefly, memory formation is triggered by a boost of fuels directed from astrocytes to neurons. More recently, the ANLS hypothesis was interpreted as astrocyte neuron communication (ANC)[24]. ANC shifted the

biological function of glycogenolysis from fuel to a signalling pathway and added pyruvic acid as a metabolic messenger to the two-cell model of metabolism. Glycogen was introduced as storage of the metabolic signalling molecules glucose, lactic acid, and pyruvic acid. Transmitters, such as insulin and epinephrine, temporarily adapt the steady-state flow non-equilibrium between two cells according to environmental changes[30][31]. The steady-state lactate/pyruvate flow ratio in the liver is approximately 7/1, whereas the steady-state lactate/pyruvate flow ratio in resting muscle is approximately 12/1 and 159/1 in working muscles[36][37]. Transmitters triggering glycogenolysis massively impact the cytosolic lactate/pyruvate flow ratio. The temporary imbalance is memorized by changes in expression levels of enzyme isoforms and enzyme subtypes in a way leading to a permanent acceleration of the flow of metabolic messengers[38].

The two-cell model of metabolism also comprises cell adhesion proteins physically stabilizing cell-cell communication[25]. The two-cell model of metabolism has given D.O. Hebb`s `reverberatory activity´ the molecular background of glucose metabolism[39]. Our approach has opened avenues by linking the scientific fields of memory formation and schizophrenia via a functional concept of metabolism. Genetic and environmental factors statistically associated with schizophrenia are candidates to be sorted into the two-cell model of metabolism. Predisposition to schizophrenia was functionally linked to instability of the physical interaction between astrocytes and neurons. Pronounced leakage of fuels into the interstitial fluid during stress-triggered astrocytic glycogenolysis was linked to acute schizophrenia[40]. Leaked glucose is a signalling molecule of inflammation, an activator of microglia cells, and triggers the release of inflammatory messengers[25]. Thus, glucose metabolism is the common denominator of all biological processes.

Unfortunately, all of the above cannot be rationally followed on a molecular level. As already stated at the beginning of the review, the synthesis, export, import, and mitochondrial consumption of lactic acid have been under investigation for more than a century. Today, however, the well-established chemical formulae of glucose metabolism violate the law of the conservation of mass, as well as published pioneer work. The chemical formulae no longer provide the obligatory proton (H^+) to synthesize lactate and the obligatory proton (H^+) to export lactic acid (lactate-H^+). Whereas the synthesis, export, and import of lactic acid have been under investigation for more than a century[41][42], the molecular mechanisms behind "energy for storage as glycogen" and "energy on demand" lie far outside common understanding. *In vivo*, a single cell simultaneously synthesizes lactate from

pyruvate and metabolically burns lactate to pyruvate. Mitochondrial consumption of lactate entails that the mitochondrial LDH-proton-linked MCT_1 complex unidirectionally catalyses the reaction aiming to equalize a cytosolic lactate flow non-equilibrium[43]. Whereas Pyruvate Hydrogenase (LDH isoform) anchored with the glycolytic metabolon must unidirectionally generate a cytosolic lactate flow non-equilibrium (biosynthesis) to enable mitochondrial consumption of lactate.

Two LDH isoforms simultaneously acting in opposite directions are introduced in the context of memory formation and formulated in the ANLS hypothesis. Nevertheless, the understanding behind the ANLS hypotheses/*in vivo* metabolism lies far outside common understanding because it is well-established that the isoforms of LDH catalyse the identical thermodynamic equilibrium[44]. Therefore, it is well-established that we do not understand biosynthesis or the generation of a cytosolic lactate flow non-equilibrium on the basis of equilibrium thermodynamics (enzyme kinetics). Nevertheless, *in vivo*, lactate is simultaneously synthesized, exported, imported, and degraded[42][28]. Biology is obsessed with thermodynamic demons. These demons are known to have the ability to stop, strike, push, or pull any lactate molecule at will, thereby altering its natural course of motion[2].

This review continues to challenge an understanding based on equilibrium thermodynamics and enzyme kinetics by developing a flow non-equilibrium thermodynamics-based concept of metabolism. "Equilibrium is an inert state of death; here the flow of matter and energy through a biological system stops, and the system reaches a lifeless state of thermodynamic equilibrium"[45]. In other words, equilibrium thermodynamics and enzyme kinetics are the path to understand lifeless material. The mathematics of equilibration thermodynamics is well-established from basic enzyme kinetics; isolated enzymes in homogenous and closed systems catalysing the identical lifeless state of a thermodynamic equilibrium. Isolated enzymes are dead material acting in line with the 2^{nd} law of thermodynamics.

Our approach to beginning to understand the dissipation of the energy particles in nature[2] is to open the reference of glycolysis/fermentation, published by O.F. Meyerhof in 1951[5].

Methods

Meyerhof's Scheme 1 is the peak of knowledge and understanding of the chemistry of glycolysis and fermentation and the scientific reference for glycolysis and fermentation[5]. Meyerhof understood that the 2^{nd} law of thermodynamics excludes the storage of energy as ATP; he also understood that the

law of the conservation of mass excludes that 1 H^+ is both used to generate ATP ($(ADP + P\text{-}OH)^- + H^+ \rightarrow ATP + H_2O$) and exported as lactic acid ($lactate\text{-}H^+$). Meyerhof's understanding of science prevented him from integrating ATP (discovered 1929) and NAD^+ (discovered 1906) in Scheme 1[46][32][19]. Neither ATP nor the $NAD^+/NADH\text{-}H^+$ system can be scientifically integrated into a concept based on equilibrium thermodynamics. Consequently, all models of glycolysis showing ATP are alchemistic inventions not supported by the law of the conservation of mass, the 2nd law of thermodynamics, and published pioneer work on glycolysis and fermentation.

Set theory: the one and only common intersection of the chemical formulae presented by Meyerhof and actual glycolysis inventions is glucose. Subduction of the atoms of glycolytic intermediates from the scientific reference indicates the atoms not currently accounted for or recognized in the current models of glycolysis and fermentation. In modern models of glycolysis, all protons (H^+) have been deleted from chemical formulae

Changes to the original concept, such as replacing phosphoric acid[5] with ATP, entail that 1 glycolytically generated H^+ must be removed from the chemical formula of the metabolic pathway. Meyerhof understood that the integration of ATP necessitates changing the end product from lactic acid to lactate. Stoichiometry dictates that 1 H^+ is either exported as lactic acid or stored as ATP. Pioneers were aware that the chemical mechanisms of fermentation are nearly completely understood and just the fate of some protons remained unexplainable[47].

Notably, pioneers knew that acids rapidly dissociate in water, but Meyerhof's Scheme 1 shows a Proton Transport Chain starting with undissociated 3-(dihydrogen)-phosphoglyceric acid and ending with the export of lactic acid[5]. The law of the conservation of mass dictates that the hydrolysis of the acid anhydride 1,3-diphosphoglyceric acid provides the acid 3-(dihydrogen)-phosphoglyceric acid. Pioneers experimentally demonstrated that the glycolytic metabolon protects the acidic intermediates from dissociation. Kennedy and Lehninger:

> "*Fluoride was added to inhibit enolase and the endpoint measured manometrically indicated the formation of 3-phosphoglyceric acid, which causes CO_2 liberation from a bicarbonate buffer.*"[48].

Let us consider the state of knowledge and understanding in 1951[5] and the current state of knowledge and understanding[49].

Meyerhof understood:

H_2PO_3-O-CO-C(OH)H-CH_2-O-PO_3H_2 + H_2O $\rightleftarrows$ H_3PO_4 + COOH-C(OH)H-CH_2- PO_3H_2

1,3-Diphosphoglyceric acid and Water are in equilibrium with Phosphoric acid and 3-Phosphoglyceric acid.

Today:

P-O-CO-C(OH)H-CH_2-O-P + ADP $\rightleftarrows$ COO^--C(OH)H-CH_2-O-P + ATP

1,3-bisPhosphoglycerate and ADP are in equilibrium with 3-Phosphoglycerate and ATP.

Our approach glycolysis:

$^-HPO_3$-O-CO-C(OH)H-CH_2-O-PO_3H_2 + ADP $\longrightarrow$ ATP + COOH-C(OH)H-CH_2-PO_3H_2

1-(monohydrogen), 3-(dihydrogen)-Diphosphoglyceric acid and ADP are unidirectionally metabolized to ATP and 3-(monohydrogen)-Phosphoglyceric acid

Our approach biosynthesis:

$^-HPO_3$-O-CO-C(OH)H-CH_2-O-PO_3H^- + ADP $\longleftarrow$ ATP + COO^--C(OH)H-CH_2- PO_3H^-

1,3-(monohydrogen)-Diphosphoglyceric and ADP are unidirectionally synthesised from ATP and 3-(monohydrogen)-Phosphoglycerate.

Depending on the individual point of view, the modern formula is either illustrated as an equilibrium or a unidirectional process. The phospho-groups are more commonly illustrated as two-times negatively charged (all protons are deleted) than yellow dots. A common feature of modern models is the change in nomenclature of 1,3-Diphosphoglyceric acid 1,3-bisPhosphoglycerate.

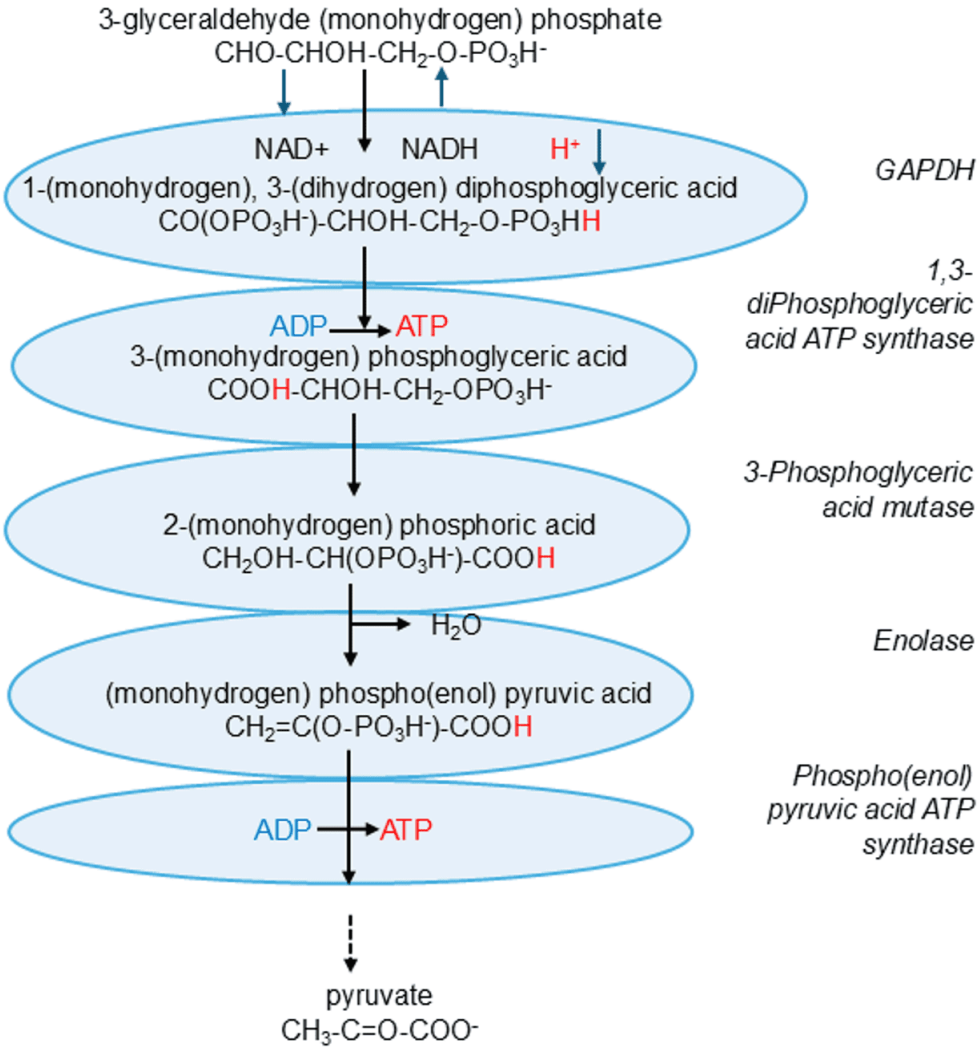
Our approach physically separates the pathways of glycolysis from cytosolic biosynthesis and chemically differentiates between the acidic glycolytic intermediates and the pH-neutral cytosolic pool of carboxylates.

The aims of this review are to (i) determine the glycolytic reactions generating the energy particle proton (H^+), (ii) trace the flow of protons driving the reduction of pyruvate to generate a lactate flow non-equilibrium, (iii) trace the proton flow initiating monocarboxylic acid export (fermentation), and (iv) trace the flow of energy and material storing the energy of the particle (H^+) as a temporarily stable glycolytic steady state ATP flow non-equilibrium (glycolysis).

The amalgamation of the law of the conservation of mass and the 4^{th} law of thermodynamics constitutes the principles guiding the scientific domain of biochemistry. The authors' interpretation and nature's application of natural laws may vary. Hence, we recommend seizing the opportunity to align the laws of nature with the individual well-established model of metabolism. Do all chemical formulas of well-established glycolysis genuinely fail to adhere to stoichiometry? Does glycolysis generate 2 protons twice, aiming to store the energy as a steady state ATP flow non-equilibrium?

Integration of ATP generation into the original Embden-Meyerhof-Parnas pathway

Glycolysis produces 2 H^+ twice for storage as two sets of 2 ATP. Meyerhof depicted 3-glyceraldehyde-(dihydrogen)-phosphate as the substrate of oxidative phosphorylation. We suggest cytosolic 3-glyceraldehyde-(monohydrogen)-phosphate as the primary substrate of the oxidative branch of the glycolytic metabolon and to replace -2H by integrating NADH-H^+ into glycolysis (pathway 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyses the oxidative phosphorylation (metabolic burning) of 3-glyceraldehyde-(monohydrogen)-phosphate to 1-(monohydrogen), 3-(dihydrogen)-diphosphoglyceric acid. The glycolytically formed energy particle (H^+) is suggested to be attached at the 3-(monohydrogen)-phosphate group. 1-(monohydrogen), 3-(dihydrogen)-diphosphoglyceric acid moves to the coupled enzymatic process. 1,3-Diphosphoglyceric acid ATP synthase (PGK isoform) catalyses energy transfer from H^+ (dihydrogen-phosphate group) to the more stable adenosine *tri*-phosphoric acid anhydride (ATP) and the hydrolysis of 1,3-diphosphoglyceric acid to 3-(monohydrogen)-phoshoglyceric acid. The second energy particle is hidden in the acid anhydride group of 1,3-diphosphoglyceric acid.



Pathway 1. Oxidative branch of the glycolytic flow. Meyerhof's Scheme 1 presents the chemistry of glycolysis and fermentation[5]. We divided the glycolytic metabolon into oxidative and reductive branches. Here, all reversible reactions have been changed into a unidirectional flow. The proton (H^+) of the acidic intermediates protected from dissociation by the metabolon (blue) is illustrated in red. Phosphoric acid is represented by ATP, and the +/-2H transfer is depicted as the NAD^+/NADH-H^+system. The metabolic burning of 3-glyceraldehyde (monohydrogen)-phosphate generates two H^+ to be stored as two ATP. The first H^+ (NADH-H^+) attaches at the (monohydrogen)-phosphate group to form the acidic (dihydrogen)-phosphate group and initiate the Proton Transport Chain. The next catalytic reaction stores one glycolytically generated H^+ as ATP and hydrolyses of the acid anhydride group of 1,3-diphosphoglyceric acid. Hydrolysis frees the second H^+ as 3-phosphoglyceric acid. Phospho(enol)p yruvic acid ATP

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synthase transfers the energy of the carboxylic acid to ATP, and pyruvate is given into the cytosol of carboxylates.

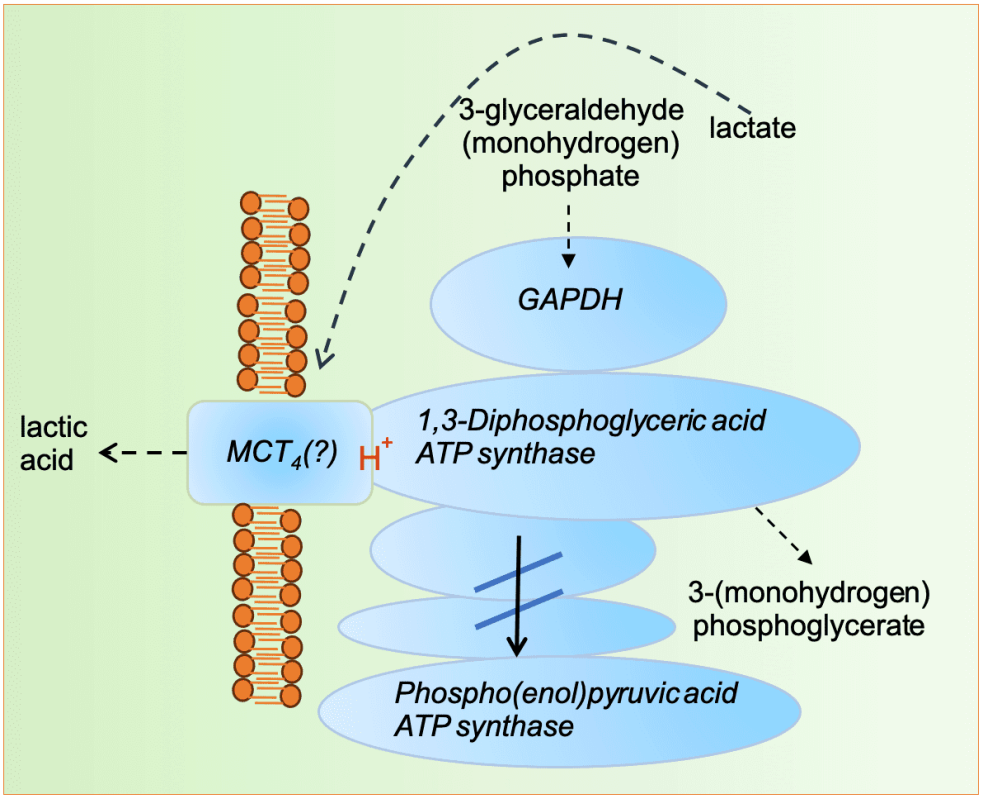
The glycolytic flow of the proton (H^+) and material (glycolytic intermediates) is unidirectional. Therefore, all reaction arrows Meyerhof indicated as reversible processes (2^{nd} law of thermodynamic dynamics) are changed into a unidirectional or irreversible flow (4^{th} law of thermodynamics). Flow-generated energy is stored as cytosolic ATP flow non-equilibrium. *In vivo*, energy does not chaotically emit; energy flow has formed the glycolytic metabolon. The glycolytic flow of energy particle H^+ ends with the storage of energy as temporarily more stable cytosolic ATP flow non-equilibrium catalysed by Phospho (enol)p yruvic acid ATP synthase (or a specific isoform of pyruvate kinase)[50]. Pyruvate, the end product of the oxidative branch, is given into the cytosolic pool of carboxylates.

The integration of ATP and NADH-H^+ into the chemistry of glycolysis and fermentation challenges stoichiometry by opening two questions: firstly, fermentation: how can lactic acid (lactate-H^+) be exported when the proton of lactic acid already vanished from the chemical formula during discharging to water and storage of the energy as ATP? Secondly, regarding the recovery of the co-enzyme NADH or the cytosolic lactate flow concentration gradient; what is the origin of the obligatory proton (H^+) driving biosynthesis of lactate when glycolytically generated energy (NADH-H^+) is stored as cytosolic ATP flow non-equilibrium?

The fermentation pathway

Experimental work suggests that the association of GAPDH and 1,3-Diphosphoglyceric acid ATP synthase (isoform of PGK) at the plasma membrane is a regulated process[51]. An acid, such as 3-(monohydrogen)-phosphoglyceric acid, released in the immediate proximity of proton-linked MCT initiates the export of monocarboxylic acid[6]. Close proximity can be interpreted as creating an acidic micro-environment, encouraging the transporter to equalize the generated membrane pH gradient. In this scenario, 3-(monohydrogen)-phosphoglyceric acid reacts with the bicarbonate-buffered micro-environment of the glycolytic metabolon, and hydration energy is emitted as heat and carbon dioxide. Nevertheless, an acidic micro-environment is sufficient to explain the characterization of proton-linked MCT_4 as an exporting carboxylic acid transporter[52][53].

Evolution surely optimizes the efficiency of proton (H^+) flow by transitioning from the creation of an acidic flow micro-environment (pH) to the direct transfer of the energy particle H^+ from unstable 3-(monohydrogen)-phosphoglyceric acid to proton-linked MCT. It is rational to postulate that the glycolytic metabolon releases 3-(monohydrogen)-phosphoglyceric acid if the flow of energy and material is blocked[48]. The flow can be blocked by low cytosolic ADP. Thus, fermentation is introduced as regulated incomplete glycolysis (Pathway 2).



Pathway 2. Fermentation. The oxidative branch of the glycolytic metabolon (blue) is attached at the plasma membrane. Quenched glycolytic flow triggers the release of 3-phosphoglyceric acid[48]. 3-phosphoglyceric acid transfers the proton (H^+) to proton-linked MCT, initiates binding of lactate and export of lactic acid. The end product 3-phosphoglycerate is given into the cytosolic pool of carboxylates.

Proton-linked MCT-glycolytic metabolon unidirectionally catalyses:

3-(monohydrogen)-phosphoglyceric acid (glycolytic metabolon) + lactate (cytosolic pool) → lactic acid (environment) + 3-(monohydrogen)-phosphoglycerate (cytosol)

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Altering the historical perspective from the gradual degradation of the carbon backbone to the flow of the energy particle can be a somewhat perplexing concept. Notably, the exported monocarboxylate is not synthesized during fermentation but rather sourced from the cytosolic pool. The ATP balance of fermentation is +/- 0. These findings prompt the question: What purpose does fermentation serve?

Fermentation is the biosynthesis of 3-(monohydrogen)-phosphoglycerate (3-PG). For the price of one monocarboxylate taken from the cytosolic pool and one H^+ not given into storage as ATP, a cytosolic 3-PG non-equilibrium is generated. Similar to the recently reintroduced term `incomplete burning´, fermentation can be understood as incomplete glycolysis[54].

Equilibrium thermodynamics and enzyme kinetics guided the understanding of synthesis as the reverse reaction of degradation. Non-equilibrium thermodynamics allows us to understand synthesis as incomplete degradation. The mechanism of incomplete burning was introduced in the context of the regulation of the Citric Acid Cycle complexes: an imbalance of the fuel/oxygen ratio in favour of the fuel quenches the recovery of the co-enzymes. Quenched recovery of the activity of mitochondrial malic acid dehydrogenase (NAD^+) blocks metabolic burning, and malic acid is NAD^+-regulated pushed into the pool of carboxylates. Generation of a cytosolic (monohydrogen)-malate flow non-equilibrium opens biosynthesis pathways consuming (monohydrogen)-malate.

Generating a 3-PG flow non-equilibrium is a rational prerequisite for serine biosynthesis. Proliferation of some cancer cells depends on serine biosynthesis; 3-phosphoglycerate dehydrogenase and proton-linked MCT_4 are targets for anti-cancer treatment. 3-PG levels seem to be directly involved in p53 activation to control cell fate[55][56][57][58]. Thus, we suggest that obligate aerobe cells can regulate the switch to (aerobe) fermentation at a high ATP/ADP ratio to open biosynthesis pathways consuming 3-PG.

We generalized the observation that enzyme complexes protect acidic intermediates from dissociation. Dissociation is the exothermic reaction of an acid with water. Pioneers experimentally demonstrated that the glycolytic metabolon physically separates intrinsically formed 3-phosphoglyceric acid from the cytosolic pool of 3-PG[48]. In 1976, the flow of protons through enzyme complexes was named proticity. Mitchell considered the direct chemical involvement of the proton in ATP recovery: $(ADP + HO\text{-}P)^- + H^+ \rightarrow ATP + H_2O$, whereas Walker determined that 3-5 protons are necessary to mechanically drive the recovery of 1 ATP[59][19]. Mitchell's and Walker's concepts based on an osmotic pH gradient.

A pH gradient is an experimental tool also used to characterise proton-linked MCTs. An artificial acidic environment urges all cell membrane-located proton-linked MCTs to import monocarboxylic acids. A pH gradient overwrites the CO_2-driven activity of proton-linked MCT_1-CAII (figure 3) and reverses the catalytic direction of proton-linked MCT_4 (pathway 2) The introduced water-free or direct transfer of nascent protons (figure 3) skips the exothermic reaction of the acid; thereby, the environmental pH turns into a non-factor.

Nath further developed Mitchell's and Walker's theory by firstly postulating and later experimentally demonstrating that charge-free succinic acid is wired through mitochondrial ATP synthase[60][61].

The reintroduced intrinsic formation of 3-phosphoglyceric acid strongly supports the stoichiometry of ATP recovery presented by Mitchell and challenges the well-established model based on the pH gradient (figure 2).

The cytosolic lactate flow non-equilibrium

Glycolysis generates 2 H^+ twice to be stored as two sets of 2 ATP. The net chemical formula of the glycolytic flow of protons (H^+) leads to pyruvate:

Glucose → 4 ATP + 2 $pyruvate^-$ + 2 NADH

$C_6H_{12}O_6 = 4\ H^+ + 2\ C_3H_3O_3^- + 2\ H^-$

The glycolytic flow has the physical quantity (acid/time). An enzyme complex catalyses one metabolite at a time, not two metabolites as the net chemical formula suggests. Therefore, a flow is accomplished when the reduced co-enzyme (NADH) is recovered twice to NAD^+ and the process can start again. The GAPDH-PH complex arrests the co-enzyme (NAD^+/NADH) and thereby determines that the redox unit (2H) is transferred to pyruvate[62]. The biochemical formula of Pyruvate Hydrogenase (PH)-catalysed recovery of NAD^+ is:

Pyruvate (cytosolic pool) + NADH (glycolytic metabolon) + H^+ (proticity) → (cytosolic) lactate (flow non-equilibrium) + NAD^+ (glycolytic metabolon)

$2\ C_3H_3O_3^- + 2H = 2\ C_3H_5O_3^-$

Pyruvate is the end product of the oxidative branch of glycolysis, intermediate of glycolysis, and substrate of the reductive branch (biosynthesis). The hydrolysis of the acid anhydride ATP is a well-established source of energy for driving biosynthesis.

Providing an acid to the GAPDH-PH complex provides a mathematically infinite concentration of H^+, driving a lactate flow non-equilibrium. Lactate stores energy by discharging the energy entity and the covalent binding of H. Therefore, the net chemical formula of glycolytic flow ends in the first ATP-driven biosynthesis product:

Glucose → - 2 ATP (consuming) + 4 ATP (non-equilibrium) + 2 lactate (non-equilibrium)

$C_6H_{12}O_6 = -2\ H^+ + 4\ H^+ + 2\ C_3H_5O_3^-$

The original EMP pathway was presented in line with the law of the conservation of mass and equilibrium thermodynamics. One molecule of glucose has to be understood to be in equilibrium with two molecules of lactic acid (2 H^+ + 2 lactate)[5]. Chemistry is based on a homogenous pool of all components. In a pool, -2 H^+ and +4 H^+ represent identical mathematical parameters to be equalized by the reactions (basic enzyme kinetics) to +2 H^+. To navigate the review more effectively, the established notion of a pool (closed system) needs to be broadened to include an understanding of a flow (open system). A flow of protons operates along a timeline. E. Rutherford might elucidate the physics of a flow in this manner: "No man ever drinks the same draft (beer) twice, for it is not the same beer and not the same man." Similarly, successful bartenders comprehend that two empty glasses and four beers differ from two beers.

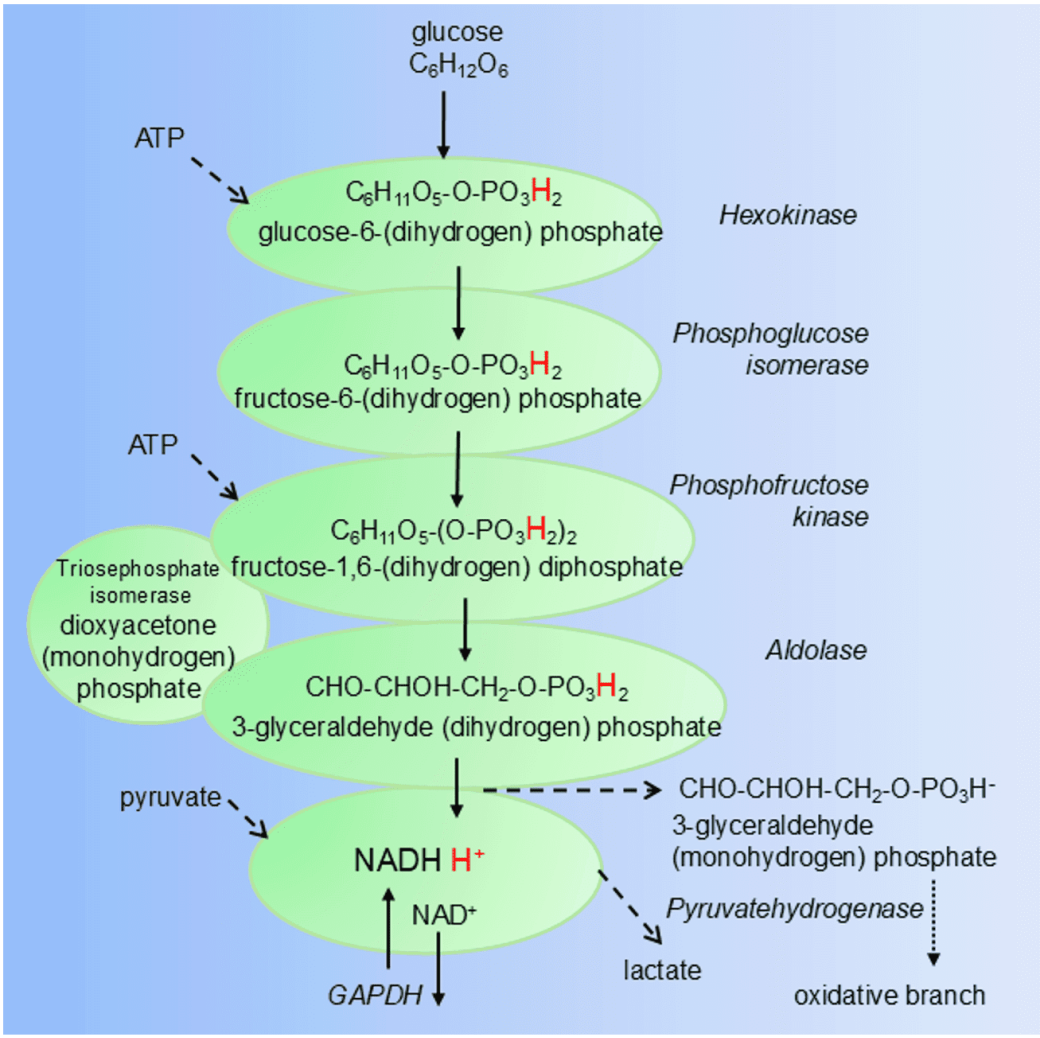
By incorporating the cytosolic lactate flow non-equilibrium as the initial ATP-driven synthesis product, we are on the verge of taking the first step towards bringing Newton's apple back up to the tree. What is lacking, however, is the beginning of the knot: the reductive branch of the glycolytic metabolon.

The first glycolytic metabolon

Although Kurganov and co-workers did not consider hexokinase (HK) as part of the first glycolytic multienzyme complex[8], we have integrated HK in the first glycolytic Proton Transport Chain as follows (Pathway 3):

HK catalysed phosphorylation of glucose and freed 1 proton (H^+). This proton can either associate with glucose-6-(monohydrogen)-phosphate or chaotically emit into the environment. Attaching the energy particle H^+ to glucose-6-(monohydrogen)-phosphate forms the acid glucose-6-(dihydrogen)-phosphate and initiates a Proton Transport Chain. This flow can be directed, with HK forming complexes with either phospho-glucose isomerase (glycolysis) or glucose-6-phosphate

dehydrogenase (Pentose Phosphate Pathway). The pH sensitivity of the phospho-glucose isomerase-catalysed reaction supports that the proton (H^+) stays attached as glucose-6-(dihydrogen)-phosphate[63]. The coenzyme of glucose-6-phosphate dehydrogenase ($NADP^+$) is an ideal target to double the energy particle H^+ ($NADPH\text{-}2H^+$) for forcing anabolic processes[64]. Therefore, we count HK as the first enzyme of a Proton Transport Chain ending at the GAPDH-PH complex, generating the cytosolic lactate flow non-equilibrium (Pathway 3).



Pathway 3. Reductive branch of the glycolytic flow of protons. We divided the glycolytic metabolon into oxidative and reductive branches. We have replaced reversible reactions with a unidirectional flow, with phosphoric acid substituted by the acid anhydride ATP, and +/-2H replaced by the NAD^+/NADH-H^+ system. The adjustments have been made in line with the law of the conservation of mass. Phosphorylation of glucose forms the acidic intermediate glucose-6-(dihydrogen)-phosphate and initiates the Proton Transport Chain. The acidic intermediate 3-glyceraldehyde (dihydrogen)-phosphate provides the proton to drive the Pyruvate Hydrogenase catalysed reduction of pyruvate to lactate. Recovered NAD^+ returns to GAPDH (oxidative branch). The introduced substrate of the oxidative branch, 3-phosphoglycerate (monohydrogen)-phosphate, is given into the cytosolic pool of carboxylates.

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This review started with the sentence “Every single chemical formula of modern models of glycolysis violates two laws of nature.”. The reaction catalysed by phosphofructokinase (PFK) liberates the second energy entity (H^+), theorized to combine with pyruvate for lactate biosynthesis. We propose triosephosphate isomerase as the partnering molecule of PFK. The transfer of one H^+ to triosephosphate isomerase is expected to drive the isomerase-catalysed reaction in a unidirectional manner to form 3-glyceraldehyde-(dihydrogen)-phosphate:

Dioxyacetone-(monohydrogen)-phosphate (cytosol) $CH_2OH\text{-}C{=}O\text{-}CH_2\text{-}O\text{-}PO_3H^- + H^+ \rightarrow$

3-Glyceraldehyde-(dihydrogen)-phosphate (reductive branch) $CHO\text{-}CHOH\text{-}CH_2\text{-}O\text{-}PO_3H_2$

The transferred energy particle H^+ is further given to the GAPDH-(NADH)-PH complex to generate the cytosolic lactate flow non-equilibrium (biosynthesis). 3-Glyceraldehyde-(monohydroxgen)-phosphate ($CHO\text{-}CHOH\text{-}CH_2\text{-}O\text{-}PO_3H^-$) is the end product of the reductive branch and is given into the cytosolic pool of carboxylates. The cytosolic pool of 3-glyceraldehyde-(monohydrogen)-phosphate was introduced as the primary substrate of the oxidative branch of the glycolytic metabolon.

Therefore, we suggest a PFK-triosephosphate isomerase complex pumps cytosolic dioxyacetone-(monohydrogen)-phosphate into the reductive branch of the glycolytic metabolon. The energy particle finally fuses with pyruvate to produce the cytosolic lactate flow non-equilibrium.

This represents a provisional framework, quite possibly the initial instance where the distinct pathways of fermentation and glycolysis, cytosolic lactate flow non-equilibrium, and a scientific incorporation of ATP and NADH-H^+ in metabolism are presented. Should we reconsider our assertion that lactate is the primary step in returning Newton's apple to the tree? It is plausible that the initial step in creating an "apple" non-equilibrium with Earth is, perhaps, the introduction of metabolic pumping cytosolic dioxyacetone-(monohydrogen)-phosphate into biosynthesis.

Discussion

The chemistry (Lavoisier) of glycolysis and Citric Acid Cycle was ascertained before World War II has started: Glucose is glycolytically split into lactic acid[65]. The Citric Acid Cycle metabolically burns lactic acid to water and carbon dioxide[66][67][68]. Physics (Newton) does not allow to understand the state of knowledge pioneers reached. In order that the Citric Acid Cycle metabolically burns lactic acid to pyruvic acid (figure 1), glucose must be split to pyruvate and pyruvate must be substrate of

glycolytic biosynthesis of lactate. Physics did not open the door to understand biosynthesis[69]. Physics defined lactate/lactic acid as end product of glycolysis/fermentation (waste) and not first product of biosynthesis.

Since WW II at least two fatal mistakes guided the development of understanding metabolism. Firstly, Lardy and Ziegler investigated ex vivo synthesis of phosphopyruvate from pyruvate[70]. The authors presented biosynthesis as reverse reaction of degradation by sorting the finding of phosphopyruvate synthesis into the scientific concept of glycolysis. Doing so, phospho(enol)p yruvic acid (glycolysis) was replaced by phosphopyruvate (synthesis). Deleting of one H^+ from Scheme 1 violates the rule A. and M. Lavoisier have formulated and provoked Meyerhof to comment the article[71]. The law of the conservation of mass as well as published pioneer work dictate to open a second, an independent pathway for carboxylates. We separated carboxylic acids (glycolysis, figure 5) from carboxylates (synthesis).

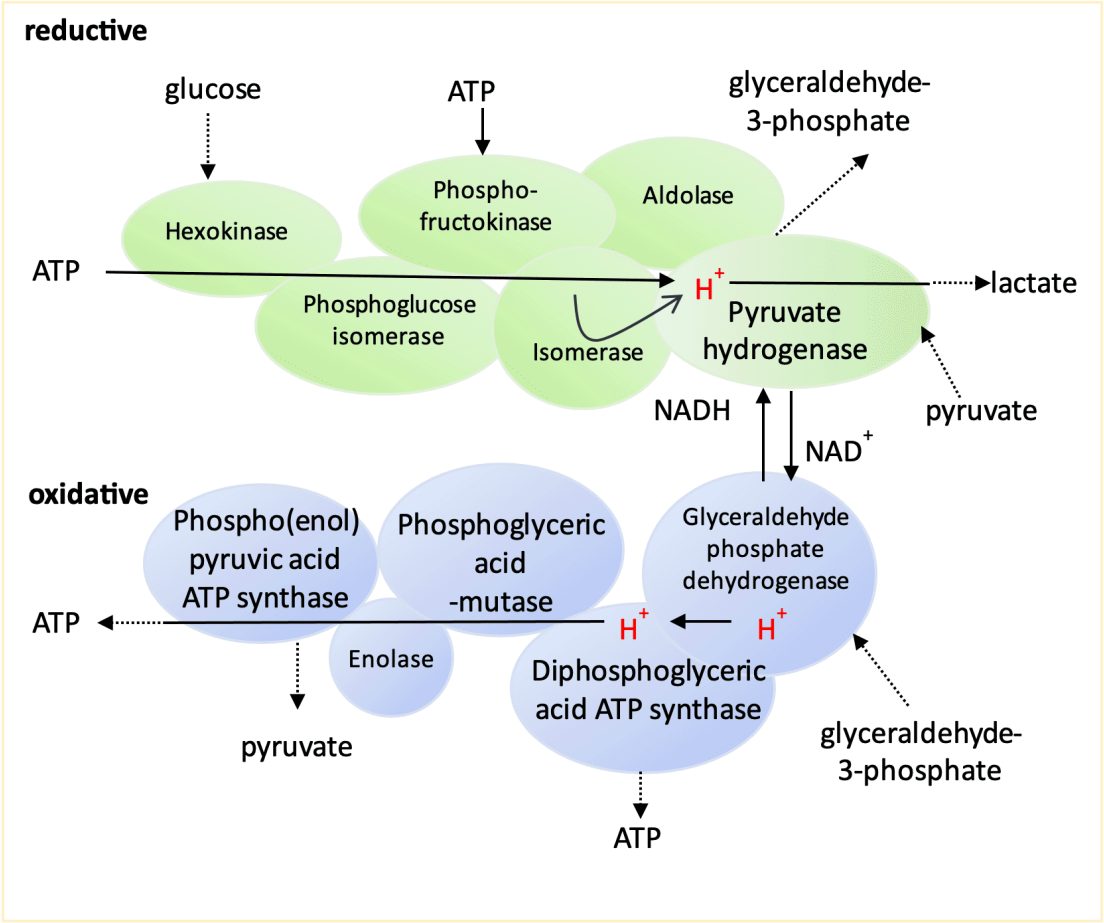

Figure 5. Glycolytic metabolon. The reductive branch (green) of the glycolytic metabolon wires the proton to lactate synthesis. Hexokinase catalysed reaction starts the proton transport chain by transferring the proton of (ADP-H^+) to form glucose-6-(dihydrogen)-phosphate. The energy particle guides the intermediates through the reductive branch. Pyruvate hydrogenase (PH) catalyses ATP-driven biosynthesis of lactate from the glycolytic intermediate pyruvate. The oxidative branch (blue) starts with Glyceraldehyde phosphate dehydrogenase (GAPDH) catalysed metabolically burning of glyceraldehyde-3-(monohydrogen) phosphate to 1-(monohydrogen), 3-(dihydrogen)-phosphoglyceric acid. Burning of the aldehyde to a carboxylic acid group provides 2 H^+. Firstly, the carboxylic acid group of 3-(monohydrogen)-phosphoglyceric acid; secondly, NADH-H^+. The H^+ of NADH-H^+ is transferred to 3-(monohydrogen)-phosphoglyceric acid to form 3-(dihydrogen)-phosphoglyceric acid. The carboxylic acid group is capped by forming the acid anhydride 1-(monohydrogen), 3-(dihydrogen)-diphosphoglyceric acid. Diphosphoglyceric acid ATP synthase is suggested to catalyse two steps: firstly, storage of the free H^+ (dihydrogen-phospho-group) and secondly the hydrolysis of the cap. The energy particle of 3-(monohydrogen)-phosphoglyceric acid

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guides the intermediates through the complex. Phospho(enol)p yruvic acid ATP synthase catalyses the final reaction of the oxidative branch: transfer of wired H^+ to temporarily more stable ATP.

Not opening an independent pathway for carboxylates (synthesis), the scientific concept of glycolysis (Scheme 1) transformed into an alchemistic illustration[71].

Secondly, metabolism -a topic under investigation- has developed into a topic at school. Pupils learn the incorrect name of intermediates (carboxylates) and the incorrect name of enzymes like the alphabet, by rota. Consequently, glycolysis and Krebs cycle have developed into the Tower of Babel. For example, the scientific concept of the Citric Acid Cycle presenting unidirectionally burning of lactic acid to water and carbon dioxide (figure 1) has mutated into uncounted number of alchemistic illustrations. Carboxylates, such as citrate, oxaloacetate and succinate are illustrated under the title Citric Acid Cycle or TCA cycle. Wikipedia (German) propagates that H.A. Krebs discovered the citrate cycle and has understood synthesis as reverse reaction of burning. The nomenclature of basic molecules has turned into a matter of discussion.

Our way out of chaos is referring, citing, and working with the original publications. We learned this way during preparation of Re-thinking the Citric Acid Cycle. G.A. Brooks-laboratory characterized the functional mitochondrial LDH-proton-linked MCT_1 complex. The complex catalyses metabolic burning of lactic acid to pyruvic acid. The reaction provides $2H$[11]. The complex catalyses the first chemical formula of the original Citric Acid Cycle[66][67][68]. Karpusas and co-workers analysed the structure of citrate synthase and found "The structure resembles a proposed transition state of the condensation reaction and suggests that the condensation reaction proceeds through a neutral enol rather than an enolate intermediate"[72]. We understood the sentence: oxaloacetic acid is substrate of Citric Acid Synthase. The enzyme re-filling a citrate cycle was Pyruvate Carboxylase (PC). PC catalyses: $pyruvate^- + HCO_3^- + ATP \rightarrow oxaloacetate^{2-} + ADP + P$ (alchemy). We introduced the enzyme Pyruvic Acid Carboxylase (PAC). PAC catalyses: pyruvic acid + HCO_3^- + H^+ (ATP) → oxaloacetic acid + ADP + P (chemistry). Re-thinking the Citric Acid Cycle revealed the information that hydrolysis of ATP provides one H^+. Chemistry dictates that 1 H^+ has to be given into recovery of ATP: ADP + H^+ + HO-P → ATP + H_2O.

This manuscript reviewed glycolysis and fermentation. We found that glycolytic enzymes are organized in enzyme complexes. Enzyme complexes, such as the Citric Acid Cycle complexes and the

Pyruvic Acid Dehydrogenase complex, unidirectionally catalyse acidic intermediates. Reviewing pioneer work revealed names and chemical formula of the acidic intermediates of the glycolytic metabolon. Following the flow of H^+ through the glycolytic metabolon allowed us to integrating ATP (H^+) and NADH-H^+ in the pathway glycolysis and the pathway fermentation. By following the flow of energy particle we found the tentative 4^{th} law of thermodynamics[73]. Transferring the tentative 4^{th} law of thermodynamics to the subatomic level (H^+) of metabolism, this review changed the tentative law into the 4^{th} law of thermodynamics.

Our reviews are the references of glycolysis, fermentation and Citric Acid Cycles. Thus, scientists understanding carboxylates, such as 3-PG, 2-PG or phospho(enol)p yruvate as glycolytic intermediates violating the laws of nature, the original literature, will violate the most recently published literature and above of all the ethics of science. Publishing policy of journals does not tolerate scientific misconduct, i.e. the violation of ethical principles in performing and publishing scientific research. Kennedy and Lehninger have experimentally determined glycolytically formed 3-phosphoglyceric acid. Principle of Biochemistry (first edition) introduced 3-PG to explain glycolysis. Publishing policy of scientific journals cannot tolerate illustrations, discussions and evaluations based on these falsifications and fabrications anymore, or?

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Conflicts of interest

Dirk Roosterman and Graeme S. Cottrell declare that we have no conflict of interest.

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