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Glycolysis for the Microbiome Generation

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Summary

For a generation of microbiologists who study pathogenesis in the context of the human microbiome, understanding the diversity of bacterial metabolism will be essential. In this opening chapter, I will briefly describe how and why I became, and remain, interested in metabolism. As one example of metabolic diversity, I then will describe and compare some of the strategies used by bacteria to consume sugars. I will end with a plea to embrace metabolism in the endeavor to understand pathogenesis.

Keywords

Metabolism; glycolysis; bacteria; microbiome; acetylation; phosphorylation

Beneath behavior lies metabolism

There is no life without metabolism. There is nothing surprising about this statement; it is blatantly obvious and true for both host and bacteria, whether commensal or pathogen. What is surprising is the delayed general recognition that metabolism plays a, or perhaps, the central role in pathogenesis, which is simply a manifestation of the need for certain ‘bad’ bacteria to grow and divide on or in a host. Perhaps this delay is natural, as researchers tend to focus on particularities, in this case, cellular processes unique to pathogenesis. Another reason for this delay is likely the aversion of late 20th century microbiologists, who came to science after the heyday of bacterial metabolic research, and who were forced to memorize whole swaths of the metabolic chart, usually out of context and with little understanding of the intricate linkages between metabolic pathways and their connections to other cellular processes.

This certainly had been my experience, at least until the day Pat Conley and I added acetate to *Escherichia coli* cells ‘guttled’ for all but one of the chemotaxis proteins (CheY) and unexpectedly observed flagellar motors intermittently rotate clockwise instead of incessant counterclockwise rotation [1]. Although we strongly suspected that this behavior required that the acetate be metabolized, we had no idea how. So, in the days before the Internet, we went looking for the metabolic chart, which we quickly discovered was pristine, still in its

plastic wrapper within its cylinder, behind one of the lab doors. Apparently, this biophysics lab (headed by Howard Berg) had had no prior need for metabolism. The lab was studying bacterial behavior - chemotaxis and motility - not metabolism. But, on that day, I began to investigate the metabolism that underlay that bacterial behavior.

From the metabolic chart, we learned that *E. coli* cells convert acetate into acetyl coenzyme A (acCoA) by means of the reversible acetate kinase (AckA) -phosphotransacetylase (Pta) pathway, whose intermediate is acetyl phosphate (acP) or through the irreversible acetyl CoA synthetase (Acs), whose intermediate is acetyladenylate (acAMP) (Figure 1). From my subsequent reading of the 'ancient' literature, typically JBC volumes stored horizontally on the top shelf in Harvard's Biolabs library and covered in years of dust, I 'discovered' Fritz Lipmann, Feodor Lynen, Hans Krebs and others who had sought the 'activated acetate' that we now know to be acCoA [2, 3]. Whether derived from glucose via glycolysis or from acetate via Acs or the AckA-Pta pathway, the resultant acCoA replenishes the tricarboxylic acid (TCA) cycle and the glyoxylate shunt to generate energy and provide building blocks for the synthesis of amino acids, nucleotides, and other essential compounds. AcCoA also plays direct roles in the synthesis of fatty acids, amino acids, and most secondary metabolites, including many antibiotics. As such, acCoA could be considered the keystone molecule of central metabolism.

In our 1988 report, we provided evidence that an activated acetate molecule was responsible for our acetate effect on flagellar rotation. We thought that it was acetyladenylate (acAMP), the intermediate of Acs pathway [1]. Subsequently, others determined that multiple mechanisms were at work, that Acs could acetylate the two-component response regulator CheY, presumably using acAMP as the acetyl donor, that acP could donate either its phosphoryl or acetyl group to CheY, or that acCoA could donate its acetyl group. Each post-translational modification (phosphorylation and acetylation) inhibits the other, but both independently increase the probability that CheY will bind the flagellar motor and induce clockwise rotation [1, 4-17]. We now know that, under physiologically relevant conditions, acP can donate its phosphoryl group to and activate other response regulators, including NtrC, OmpR, RcsB, CpxR, RssB, SirA/UvrY, Rpr2, DegU and FlgR from *E. coli*, *Salmonella enterica*, *Yersinia pestis*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Borrelia burgdorferi* [18-35]. For reviews, see [36, 37].

Following the initial reports that CheY could be acetylated [4, 6], Jorge Escalante-Semerena and his student Vincent Starai reported that a protein acetyltransferase (known as Pat in *S. enterica* and YfiQ, Pka or PatZ in *E. coli*) catalyzes the N ϵ -lysine acetylation of Acs using acCoA as the acetyl donor [38]. They had earlier linked acetylation to central metabolism by showing that the reversal of Acs acetylation required CobB [39], a member of the NAD⁺-dependent sirtuin family of lysine deacetylases [40]. It is now known that lysine acetyltransferases and deacetylases are ubiquitous in bacteria [41-44].

More recently, Choudhary's group and ours reported that acP can donate its acetyl group to thousands of lysines on hundreds of proteins, many of which are central to pathogenesis. Amazingly, this process does not require an enzyme [45,46], but does require that the molecular environment of the lysine residue permit binding of the phosphoryl group and the

activation (deprotonation) of the lysine [45]. The full impact of protein acetylation remains to be investigated, but several studies have hinted that it could impact pathogenesis [20-22, 47, 48].

A glycolysis primer

Having made an argument that one small but central part of metabolism likely plays a role in bacterial pathogenesis, I will attempt to make that metabolism a bit more accessible. I will specifically devote the rest of this chapter to glycolysis. In this context, you should notice that NAD^+ , acCoA and acP are mentioned often.

Metabolism refers to those biochemical pathways that either generate biologically usable energy (catabolism) or consume that energy to permit growth (anabolism). Catabolism converts chemical or electromagnetic energy into the high-energy bonds of ATP. Cells generate ATP via two distinctly different mechanisms: substrate level phosphorylation and oxidative phosphorylation. Substrate level phosphorylation is a process that synthesizes ATP by converting an organic molecule from one form to another (Figure 2). In contrast, oxidative phosphorylation generates ATP via an ATP synthetase that uses a proton motive force established by driving electrons through a membrane-bound electron transport system associated with respiration, photosynthesis, or some other type of bacterial metabolism. Anabolism uses the energy of ATP to synthesize cellular components. Some pathways are strictly catabolic, while some are strictly anabolic. The central metabolic pathways, however, tend to be amphibolic; they contribute both energy (catabolism) and biosynthetic precursors (anabolism). Glycolysis is amphibolic.

Given the knowledge that the human microbiome consists of thousands of different species [49-51] that are mostly uncharacterized, it is important to remember that different metabolic programs exist. Some bacteria are strict anaerobes, others strict aerobes, while facultative anaerobes can do both. Some are strict fermenters, others strict non-fermenters (i.e., they rely on respiration), while some can do both. In this context, note that diverse glycolytic strategies are available. These include (but are not limited to) the Embden-Meyerhof-Parnas, the Pentose Phosphate, and the Entner-Doudoroff pathways, which are commonly used by pathogens of the family Enterobacteriaceae, such as *E. coli* and *S. enterica*. Other strategies include the homolactic acid and heterolactic acid pathways and the Bifidobacterium shunt, strategies used by species of the genera *Lactobacillus*, *Bifidobacterium*, and *Gardnerella*, which have been found in diverse niches of the human body including the gut [52-54], vagina [55-59] and bladder [60-64].

By convention, glycolytic pathways are depicted with glucose as the substrate, because this simple sugar requires the fewest catalytic steps to enter central metabolism via glycolysis. However, glycolysis can metabolize other carbon sources. For example, many hexoses can enter glycolysis after being isomerized to the activated (phosphorylated) forms of glucose or fructose, while pentoses must be converted to the activated form of xylulose. Whereas acid sugars first must be reduced, sugar alcohols must be oxidized. Similar scenarios hold true for pathway intermediates and their derivatives.

The Embden-Meyerhof-Parnas (EMP) Pathway

The EMP pathway is the most recognizable glycolytic pathway, primarily because it is the pathway taught in biochemistry and cell biology classes. The reason for this choice is simple. Eukaryotes use it to produce the pyruvate that mitochondria convert to the acCoA that replenishes the TCA cycle. Some yeasts and bacteria use the EMP pathway to synthesize ethanol and CO₂, while tumor cells, lymphocytes, neuroblasts, and lactic acid bacteria use it to produce lactic acid. Other bacteria use the EMP pathway to produce diverse gases, fatty acids, and alcohols.

The upper portion of the EMP pathway invests two ATP molecules to activate (phosphorylate) the hexose sugar glucose, and rearrange it for cleavage into two triose phosphate molecules (Figure 3). The key enzyme is 6-phosphofructokinase (PFK). In the lower portion of the EMP pathway, each triose phosphate molecule is phosphorylated and oxidized (via NAD⁺) in a reaction that is catalyzed by glyceraldehyde dehydrogenase (GAPDH). Two subsequent substrate level phosphorylations yield 4 ATP and 2 pyruvate molecules [65, 66].

The aforementioned description is a bit simplistic, however. For example, in many bacteria, including *E. coli* and its relatives, glucose and other hexoses are transported and phosphorylated simultaneously using PEP as the phosphoryl donor instead of ATP. Thus, one of the two PEP molecules generated from glucose by the EMP pathway is used to transport and phosphorylate another glucose molecule [65]. Also, the yield per glucose is always smaller because intermediates are extracted from the EMP for entry into anabolic pathways. For example, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, and pyruvate are precursors for the biosynthesis of lipids, vitamin B₆, and certain amino acids, respectively. To supply these and other precursors for biosynthesis, flux through the EMP pathway must be maintained. The NADH generated by the EMP pathway also plays an anabolic role, as it can reduce NADP⁺ to NADPH, which is the primary reducing agent for biosynthesis.

The Pentose Phosphate (PP) Pathway

The PP pathway (also known as the phosphogluconate or hexose monophosphate pathway) oxidizes glucose-6-phosphate to pentose phosphates (Figure 3). It is distinctive for several reasons. First, it uses a different set of reactions than the EMP pathway. Second, it oxidizes sugars with NADP⁺ rather than NAD⁺. As mentioned above, the resultant NADPH is a major source of electrons for diverse biosynthetic (anabolic) processes. Third, it produces D-ribose-5-phosphate, sedoheptulose-7-phosphate, and erythrose-4-phosphate, which function as precursors for the biosynthesis of amino acids, nucleic acids, and other macromolecules, including ATP, coenzyme A, NADH and FADH₂. Thus, the PP pathway is an essential central metabolic pathway.

The PP pathway begins with three molecules of one glycolytic intermediate, β-D-glucose 6-phosphate, and ends with formation of three others, two molecules of β-D-fructose 6-phosphate and one of D-glyceraldehyde 3-phosphate. The PP pathway is often divided into its preliminary oxidative and subsequent non-oxidative portions. In the former, β-D-glucose

6-phosphate is oxidized by NADP^+ to D-ribulose 5-phosphate with the evolution of CO_2 ; in the latter, a series of transaldolase and transketolase reactions convert the D-ribulose 5-phosphate to β -D-fructose 6-phosphate and D-glyceraldehyde 3-phosphate. EMP pathway enzymes complete the metabolism of both. Because 1/6 of the carbon is released as CO_2 , the net yield per glucose is 1.8 ATP [66].

The Entner-Doudoroff (ED) Pathway

Two enzymes form the core of the ED pathway: 6-phosphogluconate dehydratase (EDD) and 2-keto 3-deoxy-D-gluconate 6-phosphate (KDPG) aldolase (EDA). The former enzyme oxidizes D-gluconate 6-phosphate (from the PP pathway or by phosphorylation of gluconate) to KDPG, which is cleaved by the latter enzyme to pyruvate and D-glyceraldehyde 3-phosphate (Figure 3). The D-glyceraldehyde 3-phosphate is oxidized to pyruvate by the enzymes of the EMP pathway, with 1 net ATP produced per glucose [66, 67]. As with the EMP pathway, intermediates are extracted for anabolic processes.

The ED pathway is critical to many pseudomonads. These bacteria generally lack PFK and thus cannot use the EMP pathway. Instead, the core of central metabolism is formed by the ED pathway, which operates in a cyclic manner. By means of certain enzymes of gluconeogenesis, the D-glyceraldehyde 3-phosphate is recycled to D-gluconate 6-phosphate [68]. In *E. coli* and other bacteria, the ED pathway plays a more peripheral role. In these organisms, the EMP and PP pathways form the core of central metabolism. However, when gluconate or other acid sugars are present, the ED pathway is induced along with the gluconate transporter and gluconokinase, which activates the gluconate to D-gluconate 6-phosphate [67, 69].

Fermentation

Pyruvate is the end result of the EMP, PP and ED pathways. In the absence of oxygen, this pyruvate (or its derivatives) is further metabolized by fermentation, which uses substrate level phosphorylation to synthesize energy during the partial oxidation of an organic compound. To perform this partial oxidation, pathway intermediates act as electron donors and electron acceptors. The fundamental fermentation logic is as follows: activate a substrate, use an electron acceptor to partially oxidize that activated substrate, use some of the energy released by oxidation to generate ATP, and recycle the electron acceptor by reducing the oxidized substrate (Figure 4A).

An advantage of fermentation is that it is fast. Although fermentation generates only 3 to 7% of the 38 ATPs that oxidative phosphorylation can potentially produce, fermentation produces ATP at about 100 times the rate of oxidative phosphorylation. The faster rate is such an advantage that many cells ferment in the presence of glucose instead of respire, even in the presence of oxygen. This behavior, called the Crabtree effect, aerobic fermentation, or overflow metabolism, was first described in tumor cells that performed lactic acid fermentation instead of aerobic respiration. It also powers fast growing eukaryotic cells, such as neuroblasts and lymphocytes [70-73]. And, it occurs in many bacteria [74-76]. For example, when presented with high concentrations of glucose, *E. coli* ferments even when oxygen is present [77, 78]. The opposite behavior is called the Pasteur effect [79]. When

growth conditions favor a shift from fermentation to aerobic respiration, cells lower their rate of catabolism. This occurs because aerobic respiration is more efficient and generates greater energy per glucose molecule. The mechanisms that regulate the 'choice' to ferment or respire remain controversial [36, 80, 81].

The strategy for reducing the pyruvate produced by glycolysis determines the fermentation product and the fermentation pathway name. More importantly, this 'choice' of fermentation end product determines the balance between the net ATP and net recycled electron acceptors (i.e., NAD^+ and NADP^+). A familiar fermentation strategy produces lactic acid. This strategy, called homolactic acid fermentation [82-84], is used by many of the so-called lactic acid bacteria, such as *Lactobacillus*, *Lactococcus* and many Streptococci. It nets 2 lactate molecules and 2 ATP per glucose (Figure 4B). It uses the EMP pathway to oxidize one glucose molecule to 2 pyruvates, 2 ATP, and 2 NADH. Both pyruvates are reduced to lactate by NADH, which is oxidized to NAD^+ . The lactate is excreted into the surrounding environment. Another familiar pathway is ethanol fermentation by *Saccharomyces* [85]. In this strategy (Figure 4C), each pyruvate molecule is reduced to ethanol and CO_2 via acCoA. Per glucose, the net products are 2 ethanol, 2 CO_2 , 2 ATP, and 2 NAD^+ .

Members of the family Enterobacteriaceae tend to perform mixed acid fermentations [77, 78, 86]. These fermentation products can include lactate and ethanol but also acetate and succinate, and CO_2 (Figure 5). The major advantage of mixed acid fermentation is its ability to generate additional ATP while recycling NAD^+ . ATP generation occurs by channeling one pyruvate through acCoA to acP, which is converted by AckA to acetate and ATP. This strategy avoids excess NADH production with the added advantage of generating another ATP [87]. NAD^+ recycling happens when pyruvate is converted to products such as lactate, ethanol or succinate. Like ethanol fermentation, succinate fermentation recycles two NAD^+ per three-carbon intermediate, but at the cost of an extra ATP because PEP is not converted to pyruvate.

Another mixed product fermentation involves acetoin, butanediol and ethanol. This strategy is common to *Enterobacter*, *Serratia*, *Erwinia*, and some *Bacillus* species. Much of the pyruvate is converted to acetoin, which is reduced by NADH to 2,3-butanediol. Ethanol is also produced, as are small amounts of acids. Most fermentation products are organic acids, which acidify the environment, often to the detriment of the fermenting organism. Thus, the advantage of fermenting to the neutral end products acetoin, butanediol, and ethanol is that the organism avoids acidification of its environment [88, 89]. Other fermentation strategies exist. For example, *Corynebacteria*, *Propionibacterium* and *Bifidobacterium* convert pyruvate to propionate via succinate [90, 91], while *Clostridia* convert it to butyrate or to butanol and acetone [89].

Some lactic acid bacteria (*Lactobacillus* and *Bifidobacterium*) possess the heterolactic acid pathway (Figure 6), which uses a unique enzyme (phosphoketolase, EC 4.1.2.9) to cleave a pentose sugar to a 3-carbon phosphate and a 2-carbon phosphate [84, 92]. As in the EMP and PP pathways, the first step activates glucose. Unlike the EMP, 2 NAD^+ -mediated oxidations occur before the cleavage. β -D-glucose-6-phosphate is oxidized to D-gluconate 6-phosphate, which is then oxidized and decarboxylated. The resultant xylulose-5-phosphate

is cleaved to D-glyceraldehyde 3-phosphate and acP. As in the EMP pathway, the D-glyceraldehyde 3-phosphate is converted to lactate, producing 2 ATP by substrate level phosphorylation. The acP is converted to ethanol by 2 NADH-mediated reductions that balance the 2 pre-cleavage oxidations. Per glucose, the net products are 1 lactate, 1 ethanol, 1 CO₂ and 1 ATP. Thus, the efficiency is about half that of the EMP pathway.

Two types of phosphoketolases exist: the single-specificity enzyme that catalyzes the key reaction of the heterolactic pathway (EC 4.1.2.9) and a dual-specificity enzyme (XFP) that also can hydrolyze β -D-fructose 6-phosphate to D-erythrose 4-phosphate and acP (EC 4.1.2.22). The dual function enzyme functions in the *Bifidobacterium* shunt [93-95]. This shunt is used by several *Bifidobacterium* species and *Gardnerella vaginalis* (Figure 7). Glucose is first activated and isomerized to β -D-fructose-6-phosphate, which is cleaved by the dual specificity phosphoketolase to acP and D-erythrose 4-phosphate (EC 4.1.2.22). AckA converts the former to acetate and ATP. The latter reacts with another β -D-fructose-6-phosphate to ultimately form two molecules of xylulose-5-phosphate, which are cleaved by XFP to acP and D-glyceraldehyde-3-phosphate (EC 4.1.2.9) [96-98]. The former is converted to acetate and ATP, while the latter is converted to lactate, as described for the EMP pathway. Thus, the result is a mixed acid fermentation (acetate and lactate). Since 2 glucose molecules (in the form of 2 molecules of fructose 6-phosphate) are required, the net products per glucose come to 1 acetate, 1/2 lactate, and 2 ATP.

Concluding remarks

I tell my medical students that they are walking talking incubators; we cannot survive without our bacteria. I tell my graduate students that the metabolic chart is just a map. If one understands the symbols, then one can visit intriguing places.

For example, knowledge of mixed acid fermentation helps explain why short chain fatty acids (SCFA) constitute approximately two-thirds of the colonic anion concentration, primarily as acetate, propionate, and butyrate. These SCFA are produced by diverse bacteria, are rapidly absorbed by the colonic mucosa, and provide the primary energy source for colonocytes, hepatic cells, fat cells, and muscle cells [90, 99-102]. SCFA also perform functions of considerable significance to the health of the host, including protecting colonocytes against colitis ulcerosa, diverticulosis and colorectal cancer [90, 91, 103-107].

As microbiologists in a microbiome world, we must embrace metabolism. It is the gateway to a deeper understanding of pathogenesis.

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Figure 1. Acetyl-coenzyme A (AcCoA) is the keystone molecule of central metabolism

Glucose is metabolized via the EMP pathway to AcCoA in an NAD⁺-dependent manner. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDH, pyruvate dehydrogenase. The AcCoA interconverted with amino acids and fatty acids. It replenishes the NAD⁺-dependent tricarboxylic acid (TCA) cycle. It is the substrate for most secondary metabolites and it is the acetyl donor for some lysine acetylations, such as the PAT-dependent acetylation of ACS (acCoA synthetase). Acetate dissimilation requires the Pta-AckA pathway. The enzyme PTA (phosphotransacetylase) converts AcCoA and inorganic phosphate (P_i) into coenzyme A (CoA) and the high-energy pathway intermediate AcP. AcP can donate its phosphoryl group to certain two-component response regulators (RR). AcP also can donate its acetyl group to hundreds of proteins. The enzyme ACKA (acetate kinase) converts AcP and ADP to acetate and ATP. The acetate freely diffuses across the cell envelope into the environment. Acetate assimilation requires the high-affinity enzyme ACS. In a two-step process that involves an enzyme-bound intermediate (acAMP), ACS converts acetate, ATP, and CoA into AMP, pyrophosphate (PP_i), and acCoA. ACS activity is inhibited by acetylation of a conserved lysine catalyzed by the lysine acetyltransferase (PAT, also known as YfiQ and Pka). Reactivation is catalyzed by the NAD⁺-dependent deacetylase CobB. Adapted from (Hu et al., 2010).

Figure 2. Three substrate level phosphorylations

The first two examples are steps in the lower half of the EMP pathway. The third is a step in acetate fermentation.

Figure 3. Glycolysis in *E. coli* and related bacteria

The Embden-Meyerhoff-Parnas (EMP), the Pentose Phosphate (PP) and Entner-Doudoroff (ED) pathways. The boxes highlight reactions unique to the PP and ED pathways. PEP, phosphoenol pyruvate; PFK, phosphofructokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EDD, 6-phosphogluconate dehydratase; EDA, 2-keto 3-deoxy-D-gluconate 6-phosphate aldolase. When glucose is metabolized by the EMP, the lower half of the pathway is repeated twice. In *E. coli* and related bacteria, glucose is transported and phosphorylated using PEP as the phosphoryl donor. Thus, one of the two PEP molecules generated by the EMP pathway is used to transport and phosphorylate another glucose molecule. For the PP pathway to function, two glucose molecules must be metabolized. Pentose sugars and sugar acids can be metabolized via the PP and ED pathways, respectively.

Figure 4. Fermentation

A) General strategy, B) Homolactic acid fermentation, and C) Ethanol fermentation.

Figure 5. A mixed acid fermentation

Blue steps consume NAD^+ , whereas red steps recycle NAD^+ . Green compounds are excreted. GAP, glyceraldehyde 3-phosphate;; PEP, phosphoenol pyruvate; HSCoA, coenzyme A; P_i , inorganic phosphate.

Figure 6. The heterolactic pathway

EC 4.1.29 is a phosphoketolase that cleaves a 5 carbon phosphosugar (xylulose 5-phosphate) into a 3 carbon phosphate (glyceraldehyde 3-phosphate) and the 2 carbon phosphate (acetyl phosphate). Note that all the NAD^+ -consuming steps are balanced by NAD^+ -producing steps. Because acetyl phosphate is used to recycle NAD^+ , it is not used to generate ATP.

Figure 7. The Bifidobacterium shunt

Xfp is a bifunctional phosphoketolase. One activity (EC 4.1.2.22) cleaves a 6 carbon phosphosugar (fructose 6-phosphate) into a 4 carbon phosphate (erythrose 4-phosphate) and the 2 carbon phosphate (acetyl phosphate). A second activity (EC 4.1.29) cleaves a 5 carbon phosphosugar (xylulose 5-phosphate) into a 3 carbon phosphate (glyceraldehyde 3-phosphate) and the 2 carbon phosphate (acetyl phosphate). Note that all the NAD^+ -consuming steps are balanced by NAD^+ -producing steps. Acetyl phosphate is used to generate ATP.