

Introduction

CELLS IN CULTURE take up sugar, amino acids, lipids, and nucleosides from their growth medium. They metabolize these compounds to derive energy and use them to generate more cell mass, divide into more cells, and produce products. Proteins, which are polymers of amino acids connected by peptide bonds, constitute over 50% of the dry mass in a typical cell. Protein synthesis is an energetically costly process. Each peptide bond costs at least 3 ATP, which is nearly 1/10 of the energy generated by the oxidization of one glucose. A high-producing recombinant cell may produce over 40 pg per day of IgG protein. Each cell has about 400 pg of cell mass (or about 200 pg of cellular proteins) and can double its biomass in a day. The production of protein products is therefore a major energetic load for cells.

A classic cell culture medium contains 2–5 g/L of glucose, and somewhat lower levels of amino acids (about 0.8 mM, or 1 g/L). The consumption of these nutrients typically generates only about $2-5 \times 10^9$ cells/L, or approximately 0.2–0.5 g/L of cell dry mass. The efficiency of producing cell mass from glucose and other nutrients is rather low.

Glucose is the most important source of energy for most cells. Cultured cells also consume a significant quantity of glutamine, second only to glucose. The pathway for glucose metabolism (called glycolysis) is a key metabolic pathway and is virtually identical in all mammals. The same pathway, with a couple of additional entry reactions, is used when another sugar, such as galactose or fructose, is used as the carbohydrate source.

The complete oxidation of one glucose molecule consumes 6 O₂ and generates 6 H₂O and 6 CO₂ (Panel 3.1). The complete oxidation

of glutamine uses 4.5 O_2 and produces $2\text{ H}_2\text{O}$ and 5 CO_2 . For cells in culture, however, the majority of consumed glucose is not completely oxidized; rather, it is converted to lactate and excreted. By converting to lactate instead of completely oxidizing to CO_2 , much less energy is derived from each mole of glucose. This is the root cause of the low efficiency in the production of cell mass from glucose.

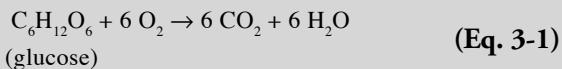
This type of “wasteful” metabolism is common to almost all vertebrate cells in culture. For bioprocessing, the accumulation of metabolic byproducts inhibits cell growth and impedes productivity.

Cells invariably produce lactate from glucose when growing rapidly. However, under some conditions (e.g., in the stationary phase of fed-batch culture), lactate may also be consumed. It is not unusual that under seemingly identical culture conditions using the same cell line and the same standard operating protocols, cells may have different metabolic behaviors. In some runs, lactate is produced throughout the fed-batch culture, from the rapid growth phase until the stationary phase. But in other runs, lactate production in the exponential growth phase switches to lactate consumption in the stationary phase.

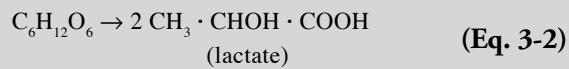
When the metabolic behavior of a cell culture process fluctuates, it is commonly seen that the high productivity runs switch from lactate production to lactate consumption, while low productivity runs remain in lactate production mode throughout the culture (Figure 3.1). Such observations attest to the importance of cell metabolism in influencing productivity. This chapter discusses the biochemical pathways that are of the utmost importance to cells in culture.

Panel 3.1. Reaction Stoichiometry of Breaking Up of Glucose and Glutamine

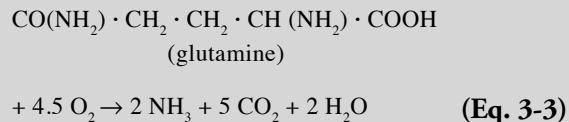
Glucose Oxidation



Glucose Anaerobic Metabolism



Glutamine Oxidation



Glucose and Energy Metabolism

GLUCOSE IS MAINLY CATABOLIZED through three pathways: glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle (Panel 3.2). In glycolysis, one mole of glucose is converted into two moles of pyruvate. In this segment of catabolism, only a small fraction of the chemical potential energy of glucose is converted into the “usable” form of chemical potential energy in the cell (i.e., ATP). Two moles each of ATP and NADH are generated from each mole of glucose.

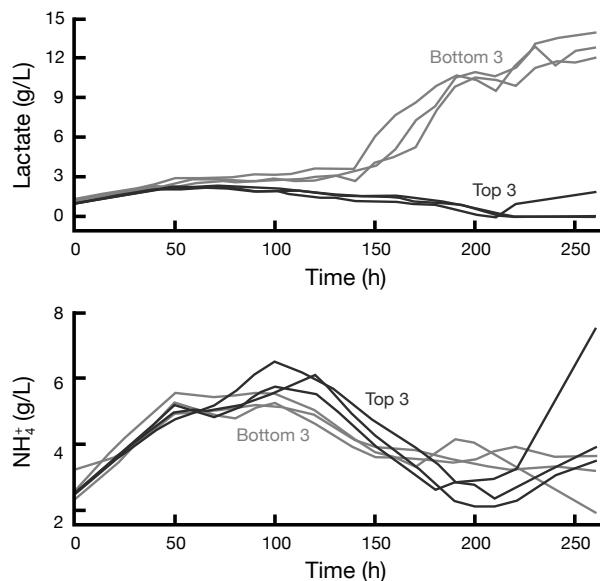


Figure 3.1. Lactate and ammonium profiles in manufacturing runs. The three runs with high product titers (dark gray) and low product titers (light gray) are shown. Lactate was produced and accumulated, and then either continued to increase or was consumed. The ammonium concentration profile was similar among all six runs. The lactate profile correlates to productivity, but not ammonium. Data from: Charaniya, S., et al., *Mining manufacturing data for discovery of high productivity process characteristics*. J Biotechnol, 2010. **147**(3-4): p. 186–97.

Panel 3.2. Metabolic Pathways of Glucose Oxidation

Main Metabolic Pathways of Energy Metabolism

- Glycolysis
 - May operate without oxygen
 - Low energy yield
- TCA cycle (tricarboxylic acid cycle, Krebs cycle)
 - Oxygen-dependent
 - Transfers chemical potential energy into NADH
- Pentose phosphate pathway
 - Generates five-carbon sugars and NADPH for reductive biosynthesis

Pyruvate may enter the TCA cycle for further oxidation, or it may become a shunted product as lactic acid (at a neutral pH it exists as lactate). During the TCA cycle, the carbon skeleton of glucose is finally broken down into CO_2 . PPP is a shunt from glycolysis. It generates five-carbon sugars for nucleoside synthesis and supplies NADPH for many biosynthesis reactions and to maintain a balanced redox state in the cell.

In eukaryotic cells, glycolysis and PPP take place in the cytosol, while the further oxidation of pyruvate to CO_2 occurs in the mitochondria. It is in the mitochondria that the majority of the chemical potential energy of glucose is converted to ATP for use in cellular synthesis and other energy-dependent cellular processes.

Oxidation of Glucose

Glycolysis

Glucose from the medium is imported into cells via the glucose transporter. During glycolysis, high-energy compounds (ATP and NADH) are generated. However, the first segment of glycolysis actually consumes two ATP for each glucose (Figure 3.2, Panel 3.3). The two ATP are used to add a phosphate group to each end of the glucose molecule. The first phosphorylation converts glucose to glucose 6-phosphate (G6P). After isomerization to fructose 6-phosphate (F6P), the second

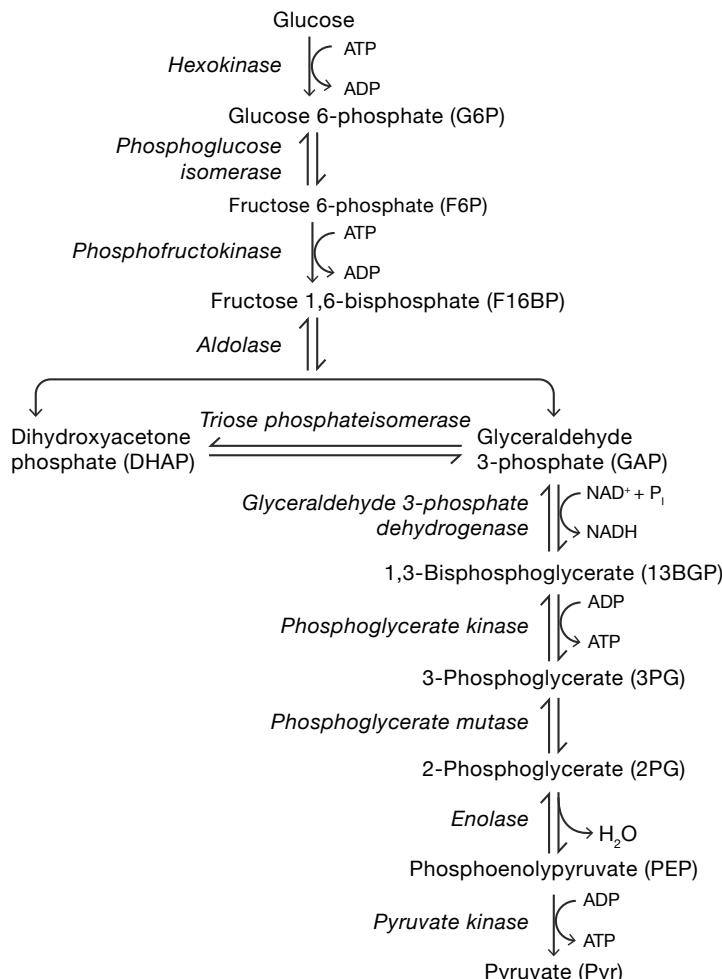


Figure 3.2. Glycolysis pathway. Only the chemical species are shown, not their stoichiometric relationships. The abbreviation of each compound is also shown. One mole of glucose yields two moles of pyruvate.

Panel 3.3. Glycolysis

- Each mole of glucose
 - Consumes 2 moles of ATP (to activate glucose to fructose 1,6-bisphosphate)
 - Produces 2 moles of NADH, 4 moles of ATP (by converting F16P₂ to 2 moles of pyruvate)
- Net:
 - Produces 2 pyruvate
 - Produces 2 ATP, 2 NADH
 - Requires 2 ADP, 2 NAD, plus glucose as reactants

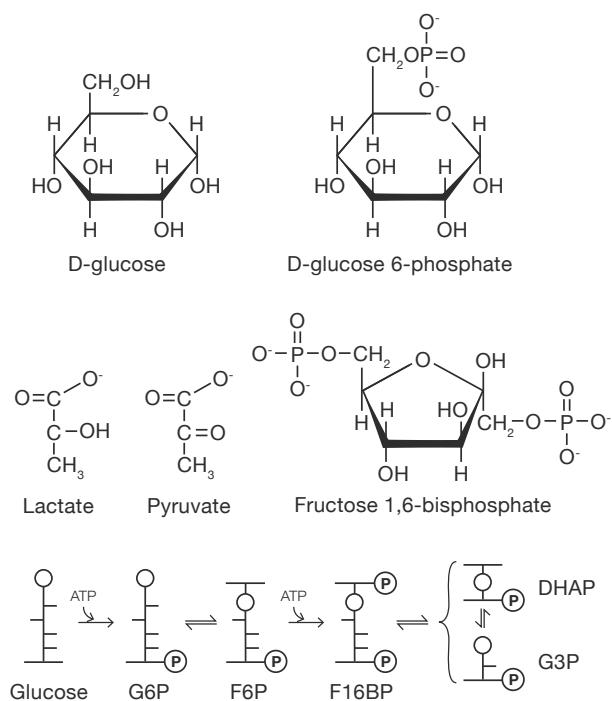


Figure 3.3. The structures of key compounds in glycolysis and a schematic representation of the hexose-splitting reactions in glycolysis. The carbonyl group in sugar is represented by a circle.

2 GAP to the end product of 2 pyruvates also converts 2 NAD⁺ and 4 ADP to 2 NADH and 4 ATP. The net energetic consequence of the conversion of glucose to 2 pyruvate in glycolysis is the generation of 2 ATP (recall 2 ATP are consumed to activate glucose) and 2 NADH. Note that NAD⁺ is often denoted as NAD in the text.

TCA cycle

The further oxidation of pyruvate takes place in the mitochondria. After passing through a transporter and entering the mitochondrial matrix, pyruvate is first decarboxylated to become acetyl-CoA while releasing 1 CO₂ (Figure 3.4a). The decarboxylation reaction breaks a carbon-carbon (C–C) bond. The energy of the C–C bond is preserved in the 1 NADH generated and in the high energy bond of acetyl-CoA. Acetyl-CoA is then fed into the TCA cycle where it is broken down into 2 CO₂ after going through one round of the TCA cycle. The pathway is cyclic, with four- to six-carbon skeletons cycling in a loop (Figure 3.4a). At the beginning of the cycle, acetyl-CoA uses its high energy bond to form a new C–C bond on the four-carbon oxaloacetate (OAA) to become six-carbon citrate. Citrate has three carboxylic acid groups, hence

phosphate is added to give fructose 1,6-bisphosphate (F16BP). The two phosphate groups, being nucleophilic centers, help pull their surrounding electron clouds toward the two ends of the molecule, thereby making the carbon-carbon bond in the middle of the glucose molecule susceptible to enzymatic cleavage (Figure 3.3). The six-carbon F16BP becomes two three-carbon compounds: glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP). These two compounds are interconvertible through a reversible reaction. The continued utilization of GAP toward downstream reactions effectively draws DHAP toward GAP. The original 1 mole of glucose now becomes 2 moles of GAP and moves further downstream in glycolysis. The conversion of

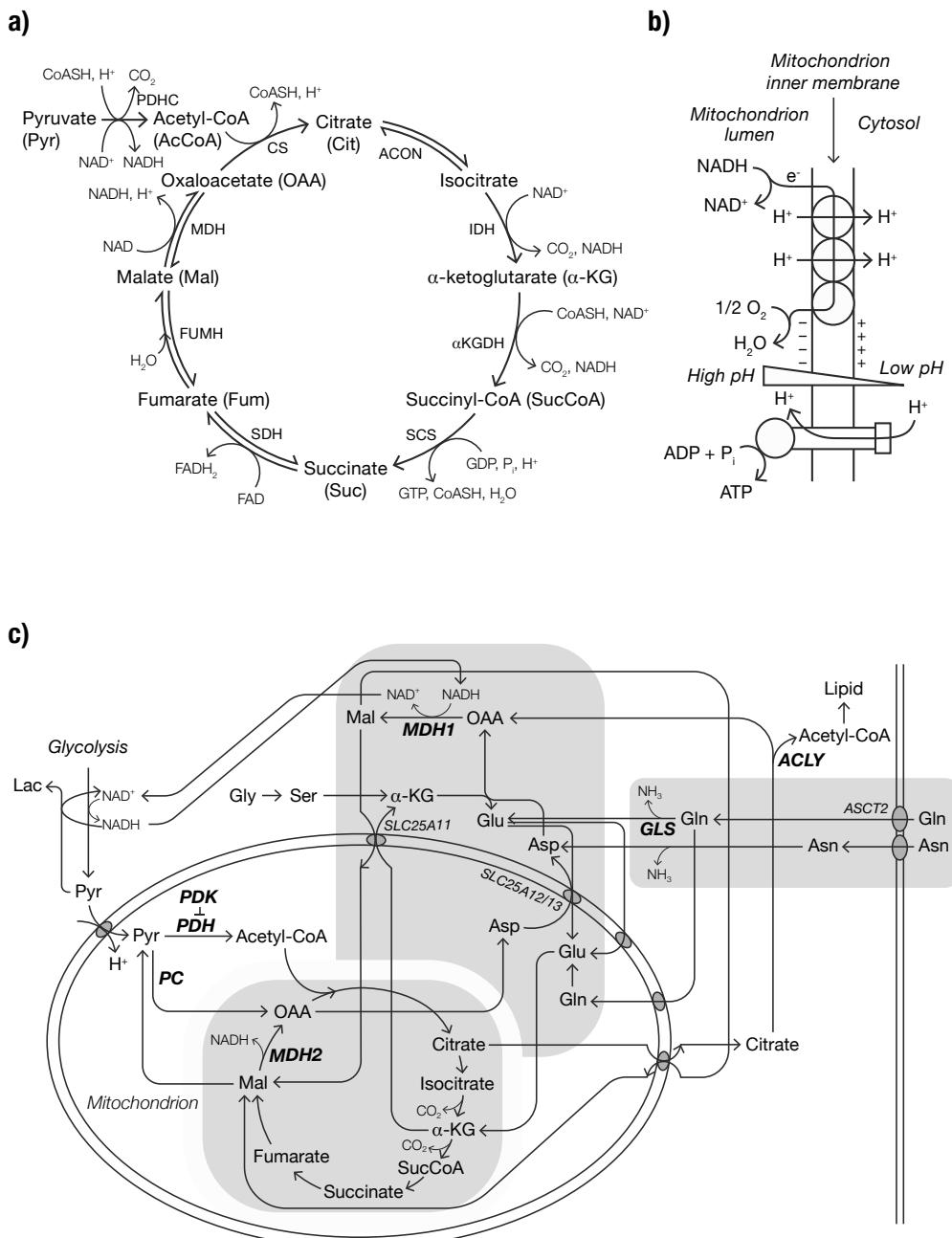


Figure 3.4. (a) The tricarboxylic acid cycle. (b) Electron transfer chain and oxidative phosphorylation. (c) The metabolite reaction network centering around a mitochondrion. Many metabolites, including α -KG, Glu, Mal, Asp, OAA, and citrate, are shared in multiple reactions/co-transports.

the name “tricarboxylic acid cycle” (Panel 3.4). The TCA cycle is also known as the citric acid cycle and the Krebs cycle. The structure of citrate and some other TCA cycle intermediates and their derived amino acids are shown in Figure 3.5.

The 6 carbon atoms in glucose which enter the mitochondria as 2 pyruvate are converted into 6 CO₂ after one round of the TCA cycle. This is as seen in the oxidation reaction of glucose, where 1 mole of glucose

Panel 3.4. The TCA Cycle

- Pyruvate enters a mitochondrion
- 1 CO₂ is released in pyruvate dehydrogenase reaction, becoming acetyl-CoA (2 C compound)
- Acetyl-CoA enters the TCA cycle by combining with OAA (4 C compound) to become citric acid (6 C compound, has 3 carboxylic acid groups)
- In each cycle, 2 CO₂ are released to regenerate OAA and produce NADH and FADH₂

generates 6 moles of CO₂ (Panel 3.1). However, no molecular oxygen participates in any reaction in glycolysis or the TCA cycle. The 6 CO₂ are released through decarboxylation reactions; they are derived from the carbon skeleton of pyruvate, citrate, and α-ketoglutarate without the participation of molecular oxygen. In two of these reactions, the carboxylic acid group next to a 2-keto group is released (catalyzed by pyruvate dehydrogenase and α-ketoglutarate dehydrogenase) and the energy from the breakup of the CO–COO⁻ bond is preserved in the high-energy compounds acyl-CoA (acetyl-CoA and succinyl-CoA, respectively) and

NADH. In the other case, the isocitrate dehydrogenase reaction, one of the three carboxylic acid groups in isocitrate is released and 1 NADH is generated. In the second half of the TCA cycle, succinyl-CoA is further oxidized to regenerate OAA via fumarate and malate and to preserve the chemical potential energy in NADH, FADH₂, and GTP.

Electron transfer and oxidative phosphorylation

In the oxidation of glucose, if the C–C bond is broken by directly reacting with oxygen, as in the case of the combustion of coal or wood, a very high temperature is necessary to provide the activation energy needed to trigger the reaction. Furthermore, the chemical potential energy in the C–C bond would be released as heat. Cells utilize decarboxylation reactions to break C–C bonds and release CO₂ while preserving the chemical potential energy in NADH.

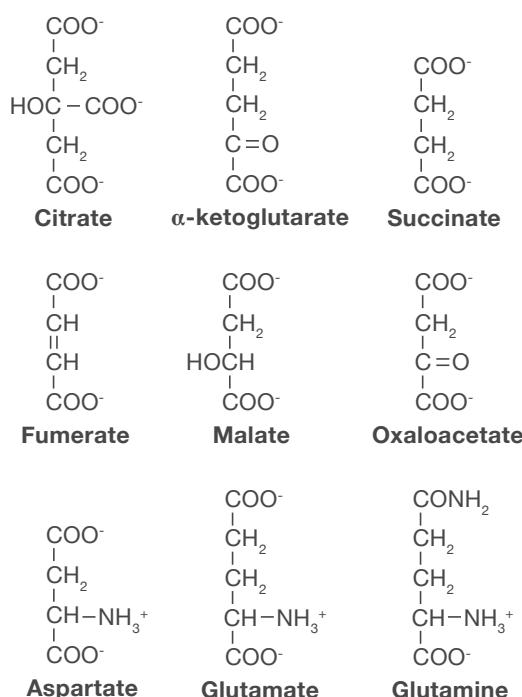


Figure 3.5. Compounds in the TCA cycle and the amino acids that can be derived from their carbon skeletons.

Oxygen is thereby used to extract the chemical potential energy from NADH and FADH₂ in order to generate ATP that can be used in cellular work. A total of 6 oxygens participate in the oxidation of the NADH/FADH₂ generated in glucose oxidation, thus accounting for the 6 O₂ required to oxidize 1 glucose as shown in the stoichiometric equation of glucose oxidation (Panel 3.1). This process of transferring chemical potential energy from NADH to ATP and reacting with oxygen is carried out using electron transport and oxidative phosphorylation (oxphos).

Extraction of the chemical potential energy of NADH and FADH₂ takes place through an electron transfer chain residing in the mitochondrial inner membrane (Panel 3.5). The high-energy electrons of NADH and FADH₂ enter the electron transport chain to move down the energy ladder, mediated by electron carriers including flavin, the iron-sulfur complex, heme, and copper ions which are embedded in a number of large enzyme complexes such as NADH dehydrogenase. At the end of the electron transfer chain, the electron is received by oxygen which then reacts with a proton to form H₂O (Figure 3.4b).

The energy released is then used to trigger a proton pump to drive H⁺ out of the mitochondrial inner membrane. The export of H⁺ from the mitochondrion creates a ~1.0 unit pH difference across the membrane, as well as about -120 mV of electric potential. Because of the higher pH (lower proton concentration) and excessive negative charge inside the mitochondrial membrane, there is a propensity for the proton ions outside the mitochondrion to cross the mitochondrial membrane and enter the mitochondrial matrix. The proton ion enters the mitochondrion through an ATP synthase embedded in the mitochondrion's inner membrane. While protons pass through ATP synthase, a series of protein conformation changes bring an ADP and a phosphate together to synthesize ATP. The electron transfer and the generation of ATP are often referred to as “oxidative phosphorylation” (Panel 3.6).

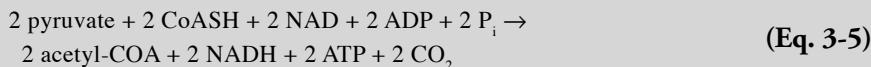
A general estimation of the energy yield in oxidative phosphorylation is 3 ATP per NADH and 2 ATP per FADH₂. However, the amount of ATP generated per mole of NADH or FADH₂ (hence also per mole of glucose) varies somewhat among different species due to their variable expressions of ATP synthase. For mammals, about 30–32 moles of ATP

Panel 3.5. Electron Transport

- NADH and FADH₂ enter the electron transport chain, pass their high energy electron to electron carriers
- As an electron passes the energetic ladder, it pumps protons out of the mitochondrion, increasing the pH inside the mitochondrion by 1.0 and generating a negative charge of ~120 mV across the mitochondrion's inner membrane
- At the bottom of the energetic ladder, the electron and proton react with oxygen to form water

Panel 3.6. Oxidative Phosphorylation Pathway

- The higher concentration of protons in the cytosol and the negative charge inside the mitochondrion drives the proton to move into the mitochondrion
- The proton passes through ATP synthase and converts ADP and P_i to ATP

Panel 3.7. Energetic Yield of Glucose Oxidation**Cytosol****Mitochondria**

- In glycolysis and the TCA cycle, glucose carbons never react with O₂
- The energy is preserved in 12 NADH/FADH₂ that is converted to ATP in electron transport and oxidative phosphorylation
- The overall energetic yield is ~30 ATP

are generated per mole of completely oxidized glucose (Panel 3.7). Older literature tends to list the number as 36 moles of ATP per mole of glucose. Under some physiological conditions, the electron transfer chain and oxidative phosphorylation are uncoupled. Instead of generating ATP, the energy from NADH is released as heat to maintain body temperature. This is seen in hibernating animals, for example.

The amount of energy, 2 ATP and 2 NADH (or the equivalent of 6 ATP, since 1 NADH in the cytosol can be roughly considered to be 2 ATP), produced from splitting glucose into two moles of pyruvate is only about 1/6 of what can be generated from the complete oxidation of glucose to CO₂ and H₂O. The vast majority of energy conversion in glucose oxidation therefore occurs in the mitochondria.

Pentose Phosphate Pathway (PPP)

The PPP is an important shunt from glycolysis that supplies five-carbon sugars (in the form of sugar phosphate) and NADPH. Five-carbon sugar is converted into ribose and deoxyribose to make nucleotides and deoxynucleotides for RNA and DNA synthesis. NADPH supplies the reduction potential used in many biosynthetic reactions (Figure 3.6, Panel 3.8).

The PPP consists of an oxidative segment and a monosaccharide transformation segment. In the first segment, glucose 6-phosphate from glycolysis is oxidized to generate 2 NADPH and then decarboxylated to form the five-carbon ribulose 5-phosphate. The molecular transformation segment converts the five-carbon sugar phosphates to 1 three-carbon (GAP) and 2 six-carbon sugar phosphates (F6P) to rejoin glycolysis (Panel 3.8).

Cells use two different nicotinamide-adenine dinucleotides as reductive chemical potential energy carriers: NADH and NADPH. NADH is used to store chemical potential energy in glycolysis, the TCA

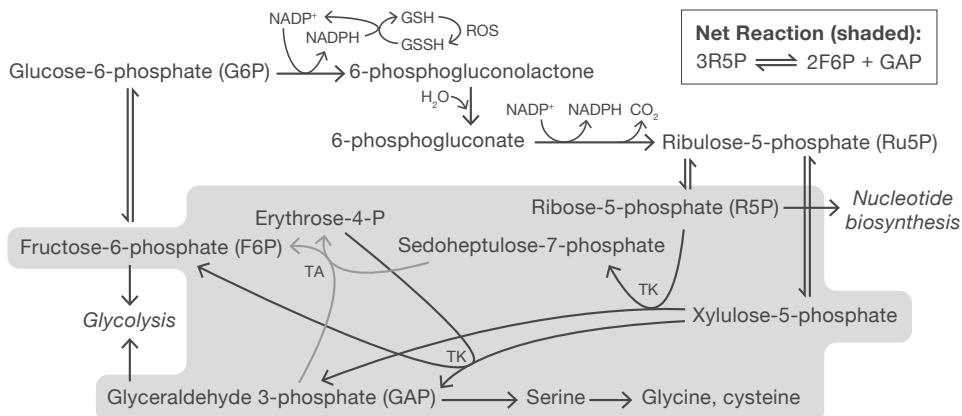


Figure 3.6. The pentose phosphate pathway with its oxidative branch and molecular transformation branch. Isomerization, transaldolase, and transketolase reactions are all reversible. Only one direction of the transformation (or non-oxidative) reactions (in the shaded area) is shown to illustrate the pairing of the reactants and the product of each reaction.

Panel 3.8. Pentose Phosphate Pathway (PPP)

Oxidative Segment

- Releases 1 CO₂, generates 5-carbon sugar phosphate for nucleotide synthesis
- Produces 2 NADPH

Overall Reaction:



Molecular Transformation

- Interconverts 5-carbon sugar phosphate to 3-carbon and 6-carbon
- Enables NADPH and 5-carbon sugar to be produced at different ratios

Overall Reaction:



cycle, and lipid catabolism. Eventually, NADH is used to derive ATP in the mitochondria.

NADPH, on the other hand, carries a chemical potential that is used in biosynthetic reactions (e.g., the synthesis of lipids or nucleotides). NADPH is also used to reduce oxidized glutathione and to regenerate it. The reduced form of glutathione is important in maintaining the cell's reductive environment and in the suppression of reactive oxygen species (ROS).

The second segment of the PPP is a molecular conversion pool that allows a 2-C aldehyde ($\text{CHO}-\text{CH}_2-$) unit or 3-C keto units ($\text{CH}_2\text{OH}-\text{CO}-\text{CHOH}-$) to be translocated among a number of 3-C to 5-C aldoses and ketoses. These reversible reactions together allow the interconversion of carbohydrate molecules that are three to seven carbons in length. This “mixing pool” enables 5-C sugars from the first segment of the PPP to be connected to glycolysis through 6-C fructose 6-phosphate or 3-C glyceraldehyde 3-phosphate.

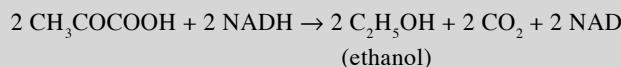
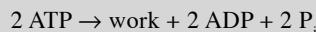
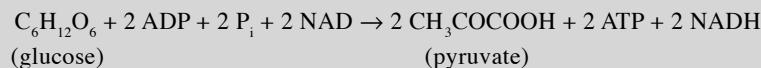
The first segment of the PPP generates 5-C ribulose and NADPH at a molecular ratio of 1:2. However, cells do not always need those two compounds at a 1:2 proportion. The molecular conversion in the second segment allows the pentose-phosphate:NADPH ratio to be increased or decreased according to cellular needs. If ribose is needed in a higher proportion, GAP and F6P can be used to generate more pentose phosphate. Conversely, if the pentose phosphate is needed in a smaller proportion, the extra pentose can be converted to F6P and GAP to re-enter glycolysis.

Lactate Formation

In the complete oxidation of glucose to 6 CO_2 , 10 NADH and 2 FADH_2 are generated. Among them, 2 NADH are produced from glycolysis and the rest from the TCA cycle. They are oxidized in the electron transport chains in the mitochondria, consuming 6 O_2 . This regenerates the NAD and FAD that are needed in order to continue the oxidation of glucose. Without the regeneration of NAD from electron transfer, the continued oxidation of glucose would run out of NAD as a reactant and stop. In the absence of oxygen, the reactions in the TCA cycle that are dependent on a continuous supply of NAD also cease to operate. Microorganisms that are capable of anaerobic metabolism can divert the pyruvate to a more reduced compound, such as ethanol or lactate. The fermentative metabolism, in the course of reducing pyruvate, also oxidizes NADH and regenerates NAD to keep glycolysis running to supply cellular energy (Panel 3.9).

Under some physiological conditions, some tissue cells can metabolize glucose just like fermentation in microorganisms. For example,

Panel 3.9. Yeast Fermentation Converting Glucose to Ethanol



- Regenerate NAD to allow glucose utilization to continue

during exercise, glucose consumption in muscle cells increases to boost energy output, often exceeding the capacity of the TCA cycle in the mitochondria. To keep the higher level of energy generation going, pyruvate is converted to lactate. The reaction converts NADH back to NAD that can be recycled to keep glycolysis running. The lactate produced is then excreted out of muscle cells (Panel 3.10).

Mammalian cells in culture and cancer cells have a high glucose consumption rate and generate pyruvate at 1:2 molar ratio. But only a small portion of the pyruvate generated is transported into mitochondria to further oxidize to CO_2 . Cells appear to have a limited capacity to translocate pyruvate into the mitochondria. The rest of the pyruvate generated in glycolysis is converted to lactate. This occurs in spite of the presence of sufficient oxygen. The phenomenon is therefore different from anaerobic fermentation in bacteria or yeast, and is referred to as “aerobic glycolysis.”

Lactate synthesis is catalyzed by lactate dehydrogenase. This reversible reaction converts 1 pyruvate and 1 NADH into 1 lactate and 1 NAD. In the conversion of glucose to 2 pyruvate in glycolysis, 2 ATP and 2 NADH are generated while also consuming 2 ADP and 2 NAD. Continued glucose metabolism through glycolysis requires continued supplies of both ADP and NAD as reactants. ATP formed in glycolysis is used by cells to perform many tasks, such as synthesis, maintaining osmotic balance, etc. It is continually being consumed in various cellular reactions and is converted back to ADP to resupply the reactant for glycolysis.

NADH is normally converted back to NAD through the electron transport chain in the mitochondria. To be regenerated in the electron transfer chain, the reducing equivalent of the cytosolic NADH generated in glycolysis must first enter the mitochondria and the regenerated NAD must be exported out of the mitochondria, as will be discussed later. Like the transport of pyruvate into the mitochondria, cells’ capacity for transferring the reducing equivalent of NADH into the mitochondria is limited. The lactate dehydrogenase reaction in cytosol allows for

Panel 3.10. Aerobic Glycolysis

Lactate Dehydrogenase Reaction



- Cultured cells and cancer cells undergo glycolysis and produce lactate even at high oxygen concentration
- This propensity toward lactate production is not for lack of oxygen (anaerobic glycolysis)
- At a high glycolysis flux, not all NADH can be oxidized by electron transfer in the mitochondria
- Lactate production serves to regenerate NAD to keep glycolysis going

Panel 3.11. Energetic Yield of Aerobic Glycolysis**Oxidation of Glucose to Pyruvate****Reduction of Pyruvate (Regeneration of NAD)****Net:**

NAD regeneration from NADH to take place, thereby enabling glycolysis to continue at a high flux (Panel 3.11). But, as a consequence, lactate must be excreted and it eventually accumulates in the medium.

Under typical culture conditions, proliferating cells in culture convert 85–90% of their glucose intake to lactate. A significant portion of the other 10–15% of glucose is converted to CO₂ while the rest is incorporated into cell mass. At the completion of glycolysis, 2 ATP and 2 NADH are generated. This is in contrast to about ~30 ATP generated upon the complete oxidation of glucose. The 90% of glucose converted to lactate generates 1.8 ATP (2 ATP x 0.9), while the other 10% of glucose generates about 3 ATP (30 ATP x 0.1). Aerobic glycolysis generates a significant portion of the total energy of glucose catabolism in proliferating cells.

Not all cells in our body convert a large portion of the glucose they take up into lactate. The vast majority of cells in our body are in a quiescent (non-proliferating) state. They consume less glucose than proliferating cells. Excessive glucose consumption and lactate production, known as the Warburg effect, was first observed between normal tissues and cancer cells. While normal cells have a lower glucose flux, cancer and other fast-proliferating cells consume a larger amount of glucose and convert much of that glucose to lactate.

Carbon Flow and the Supply of Biosynthetic Precursors

Among all of the pathways in the cellular metabolic network, glycolysis has the highest flux in terms of moles of substrate and reaction intermediates passing through. For proliferating cells in culture, the molar flux (based on the number of moles of each compound) or carbon flux (based on the number of moles of carbon atoms, i.e., the number of carbons in the compound multiplied by the number of moles of the compound) of glycolysis is normally several times higher than that of the TCA cycle. PPP flux usually constitutes only about 5% of glucose intake.

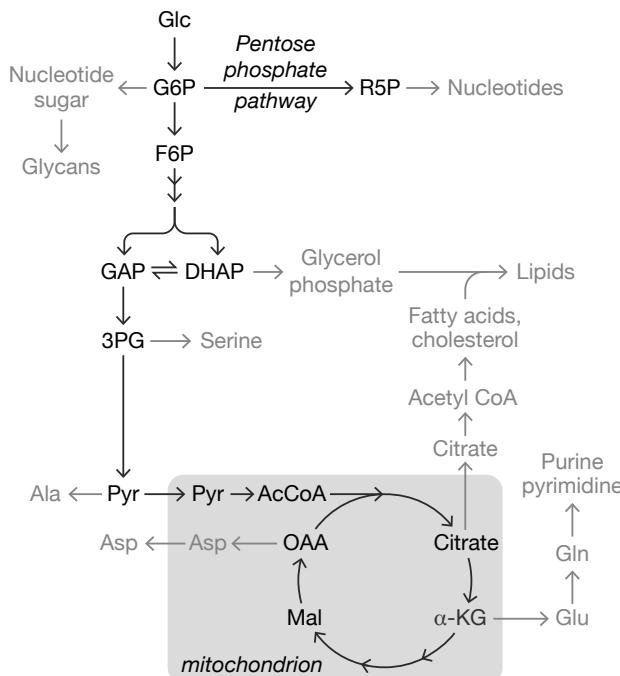


Figure 3.7. Energy metabolism pathways supply precursors for biosynthesis. The degradation of nutrients and cellular materials also feeds into these pathways.

Glycolysis and the TCA cycle also supply precursors to build cellular components. The culture medium does not necessarily supply cells with the right balance of all of the components that they need to synthesize cell mass and maintain cellular functions. The three main pathways for energy metabolism (glycolysis, the TCA cycle, PPP) also provide the precursors for the synthesis of some cellular materials. For example, DHAP in glycolysis supplies glycerol phosphate, which is used in the synthesis of phospholipids (Figure 3.7). Glucose 6-phosphate and fructose 6-phosphate are both sources of nucleotide sugars for glycan synthesis, such as UDP-galactose, UDP-glucose, and GDP-mannose. Except for liver cells (hepatocytes), cells in culture do not express enzymes involved in gluconeogenesis; that is, they cannot make hexose from lactate or amino acids. So, even if cells can derive energy from lactate and amino acids, they will still need hexose to synthesize ribose and glycans. α -ketoglutarate provides a carbon skeleton for glutamate and glutamine while oxaloacetate is used to make aspartate and asparagine.

Cells in culture take up a large quantity of amino acids from medium usually in excess of what is needed to make cell mass and product. Furthermore, the amino acids taken up are not necessarily of the stoichiometric amount that is needed for synthesis. The surplus of nitrogen is either excreted as ammonia or transferred as an amino group to

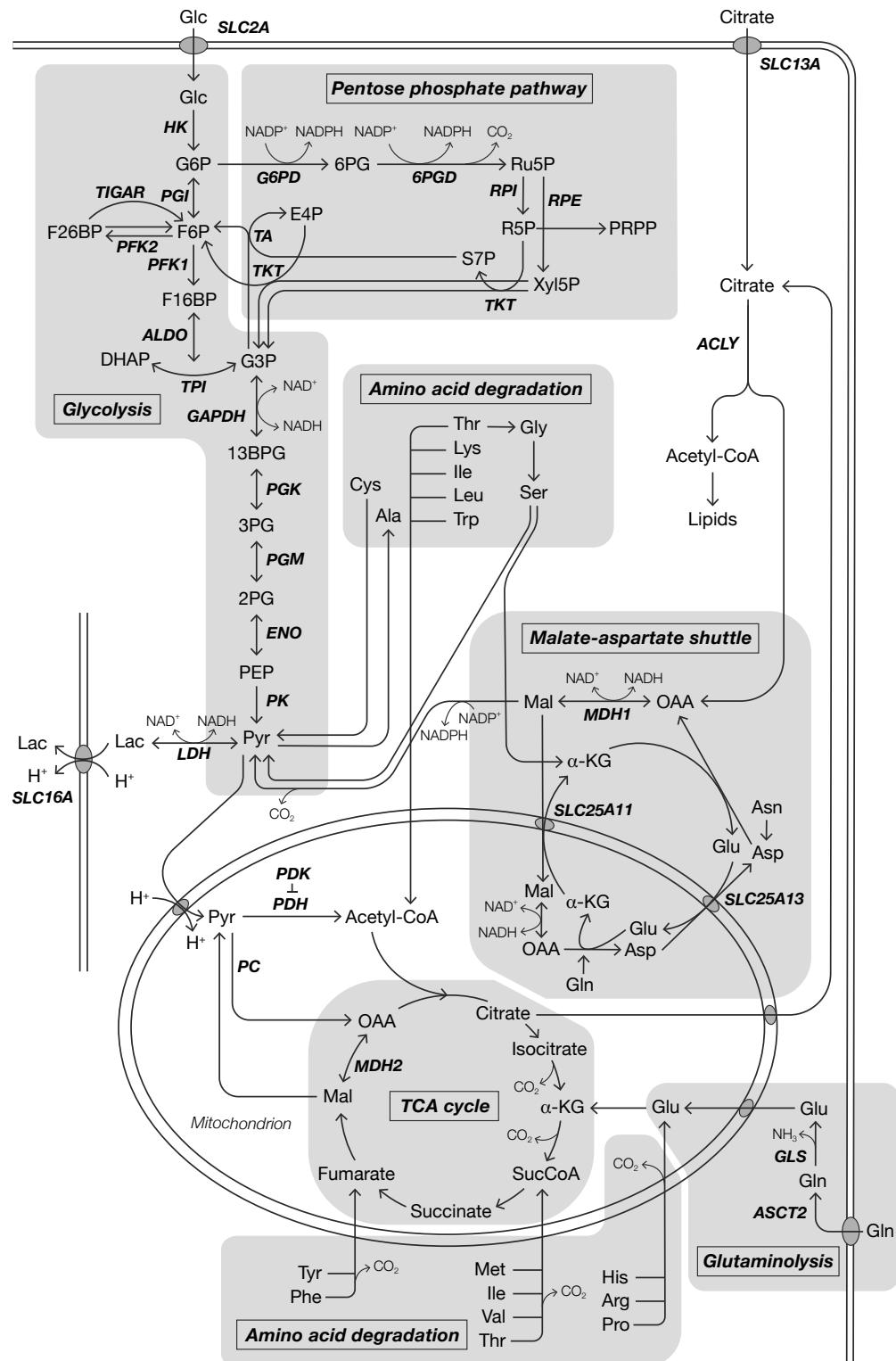


Figure 3.8. Key pathways in energy metabolism and their interconnections.

pyruvate to form alanine, and then excreted. Since alanine is much less growth-inhibitory than ammonium, pyruvate has some moderating effect on ammonium toxicity. The carbon skeletons of the amino acids enter the carbon metabolism pathway through pyruvate, acetyl-CoA, and some TCA cycle intermediates (α -ketoglutarate, succinyl-CoA, oxaloacetate) where they are degraded in the TCA cycle or converted to other amino acids.

At the start of the TCA cycle, one acetyl-CoA combines with one OAA to become one citrate (Figure 3.4a). At the end of the cycle, one OAA must be regenerated to take on another acetyl-CoA for a second round of the cycle. If a portion of the carbon flow initiated from citrate is diverted from the cycle, or if extra carbon enters into the cycle, then the amount of OAA returning at the end will not be what is needed for starting the second round of the TCA cycle.

Examples of carbon infusion include the entry of excess aspartic acid into OAA or glutamic acid through α -KG. A prominent diversion of carbons from the TCA cycle occurs in the export of citrate to the cytosol, where it is converted to acetyl-CoA for fatty acid synthesis. α -KG may also be converted to glutamate and glutamine. Glutamine is then taken to nucleotide synthesis.

To balance the withdrawal and the infusion of TCA cycle intermediates, a number of reactions are in place, including the conversion of pyruvate to oxaloacetate and the conversion of glutamine to glutamic acid and then to α -ketoglutarate (Figure 3.8). Under typical culture conditions, a significant flux of citrate exports from the mitochondria and glutamate to nucleotide synthesis are needed to support cell growth. The deficit in the OAA supply is made up for by a high consumption rate of glutamine. The replenishment of the carbon deficit in the TCA cycle, called anaplerosis, will be discussed later.

Regulation of Glucose Metabolism

DIFFERENT TISSUES IN MULTICELLULAR ORGANISMS play different physiological roles that often require them to have their particular metabolic characteristic. For example, muscle cells can ramp up glucose consumption quickly to generate energy during exercise; liver can take up the breakdown product of fat and convert it to glucose (gluconeogenesis); and adipose tissue can convert excess glucose to lipids for storage. All of these tissues have the same glycolysis pathway to metabolize glucose, but the pathway of each tissue also bears special characteristics that allow it to fulfill each tissue's specific function. The diversity of the metabolic capability in different tissues is endowed by the expression of different isozymes in different tissues.

Isozymes and Allosteric Regulation

Different isoforms of the same enzyme or transport protein, although performing the same reaction, may have different catalytic rates (k_{cat}) or affinities for a substrate (K_M) (Panel 3.12). Note that K_M is the substrate concentration at which the reaction rate is at half of its maximum. An isozyme that has a low K_M for its substrate can catalyze the reaction at a low substrate concentration, while one that has a high K_M will become active only at higher substrate concentrations. For example, all lactate dehydrogenase isoforms catalyze the reversible reaction of the conversion of pyruvate and NADH to lactate and NAD, but they have different K_M 's to pyruvate and lactate. LDHC favors the conversion of lactate to pyruvate, while LDHA favors the reverse reaction.

In the case that an enzyme can accept a number of different substrates, different isozymes may have different substrate preferences. For example, mammals have many different isoforms of the glucose transporter. They

have different affinities to glucose. Many of them can also transport some other monosaccharides such as galactose or glucosamine.

An enzyme may also be subject to allosteric regulation, meaning that its activity is modulated by the binding of an effector molecule to the enzyme. For example, the binding of the product of the reaction can decrease the enzyme activity (i.e., feedback inhibition). In many biosynthetic pathways in microbial systems, the first enzyme in the pathway is feedback inhibited by the product of the pathway. Upon the accumulation of the product, its activity is decreased to reduce the flux through

the pathway and to prevent wasteful accumulation of the product. Different isozymes may be subject to different allosteric regulations. Isoforms may also respond differently to hormonal or signaling stimuli.

Isozymes are thus important for giving different cells different metabolic characteristics. While in some cases isozymes of the same enzyme are products of alternative splicing in transcription, in other cases isozymes are coded by entirely distinct sequences in the genome.

Combination of Isozymes Gives Pathway Specific Characteristics

A pathway serving a relatively simple function, such as the aspartate amino acid biosynthesis pathway in *E. coli*, may be controlled by feedback regulation in one or a small number of “rate-limiting” enzymes. However, glycolysis in mammals is a complex pathway serving many functions under a wide range of physiological conditions, from during

Panel 3.12. Isozymes in Glycolysis

- Different tissue cells express different glycolytic isozymes with different kinetics and allosteric and gene expression regulations
- F26BP and F16BP play key regulatory roles
- Key regulatory enzymes of glycolysis with multiple isoforms: HK, PFK, PFKPB, PK
- Cancerous, fast-growing, and cultured cells express isozymes that favor aerobic glycolysis

a feast to under severe starvation. In a growing cell, glycolysis not only supplies pyruvate for energy generation in the TCA cycle, but also supplies PPP, sustains the biosynthesis of glycerol-phosphate (for lipids) and serine (through 3-phosphoglycerate), and maintains the supply of pyruvate for the biosynthesis of many cellular constituents. The needs of each branched pathway differ under different physiological conditions and in different tissues, so the supply rate to each branched pathway also differs. That supply rate is adjusted by controlling the concentration of the glycolysis intermediate at various branching points. Mammals have evolved to cope with such a wide range of needs in glycolysis by expressing different combinations of isoforms. The interactions of feed-forward and feedback inhibition and activation exerted by those isozymes can give rise to very diverse and complex metabolic behaviors that depend heavily on the environment.

Cells in culture are derived from various tissues. They often bear the metabolic characteristics of their tissues of origin. However, cells *in vitro* are also different from their counterpart in the tissue. For one thing, the cultured cells are proliferating, unlike their quiescent counterparts *in vivo*. Many cells in culture are continuous cell lines or are even transformed. Their growth control differs from tissue cells. All these factors contribute to enzyme and isoform composition and the metabolic properties of cells in culture.

Isozymes are often named after the tissue in which they are the dominant isoform. However, the expression of isozymes is not tissue-exclusive. The liver isoform of PFK is widely expressed in many tissues. The expression of isozymes in a tissue cell is not limited to one form. Different isoforms of the same enzyme are often co-expressed in the same tissue or the same cell. Different combinations of isozymes give rise to different kinetics and regulatory behaviors that may meet different physiological needs.

With the available genomic tools, we can easily determine the relative expression of different isoforms of the key enzymes of glycolysis, and further evaluate how to influence cellular metabolism.

Key Isozymes in Regulating Glucose Metabolism

Four reactions in glycolysis play key roles in regulating its flux: hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), and 6-phosphofructo 2-kinase/fructose 2,6-bisphosphate (PFKFB) (Panel 3.12). These four enzymes along with pyruvate dehydrogenase kinase (PDK) regulate the flux of glucose carbon and its distribution at the pyruvate node. We will take a simplified view to largely divide glycolysis into two types of metabolism: one high flux in proliferating cells and the other low flux in quiescent cells (Figure 3.9). These two types of metabolism are influenced by the isoforms involved, the composition of the medium, and the growth rate, among other factors. With some isoform

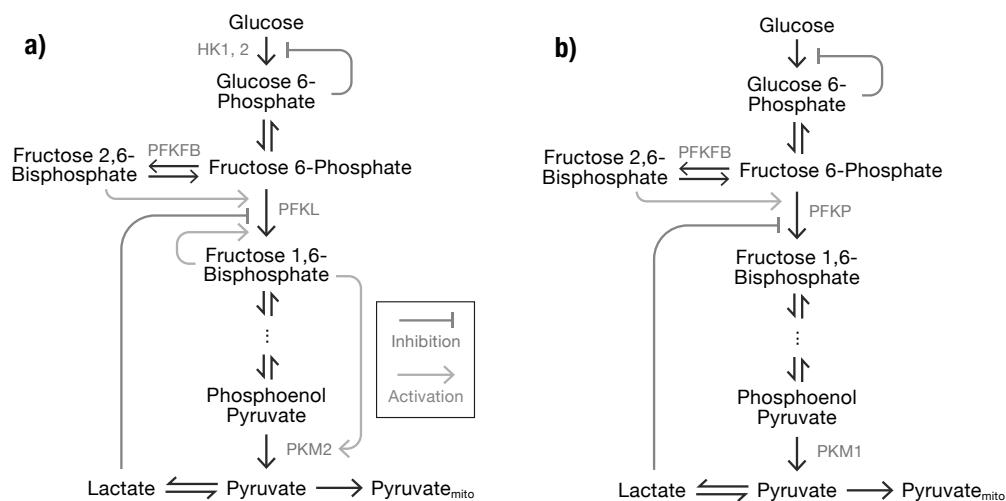


Figure 3.9. Allosteric regulation of glycolysis in (a) proliferating cells and (b) quiescent cells. The allosteric regulation present in the pathway is dependent on the isozymes expressed in the cell.

Table 3.1. Composition of Different Enzyme Isoforms Based on Transcript Expression (%)

	Heart Muscle	Liver	Cerebral Cortex	CHO K1	CHO S
PKM1	48	3	48	1	4
PKM2	52	37	52	99	96
PKL	0	60	0	0	0
PKR	0	0	0	0	0
PFKL	6	79	17	65	72
PFKM	64	16	36	23	20
PFKP	30	6	47	12	8
PFKFB1	0	52	0	12	27
PFKFB2	80	4	17	30	22
PFKFB3	18	43	79	55	46
PFKFB4	2	1	3	4	6
HK1	94	54	94	75	91
HK2	5	19	4	25	9
HK3	1	27	2	0	0

combinations, a number of reaction steps are activated by the accumulation of F2,6P and F1,6P (Figure 3.9a). Upon full activation, one may see a 5-fold or higher increase in glucose consumption, whereas with the isoform combination depicted in Figure 3.9b, the degree of activation is much lower. Below, we will describe the allosteric regulation of a few major enzymes that play key roles in determining the flux. Table 3.1 lists the compositions of the isozymes of glycolysis in a few human tissues.

Hexokinase

Hexokinase-1 (HK1) is present in virtually all cells. It has a low K_m for glucose and phosphorylates glucose for glycolysis even at a very low intracellular concentration. Liver cells additionally express glucokinase (GCK or HK4), which has a high K_m and is active only when glucose

concentration is high. HK4 phosphorylates glucose for conversion to glycogen storage when glucose is in excess.

PFK

PFK converts F6P to become F16BP. It is pivotal in modulating the overall rate of glycolysis and is a key node in energy metabolism. Its activity is subjected to allosteric inhibition by ATP and citrate, and is activated by AMP. Citrate is a TCA cycle intermediate. The accumulation of citrate and ATP in the cytosol, along with a low concentration of AMP, is indicative of an abundance of cellular energy. Its activity is thus also suppressed by an abundance of cellular energy. PFK has three isozymes: liver (PFKL), muscle (PFKM), and platelet (PFKP). Among the three isoforms, PFKL and PFKM are activated allosterically by their own reaction product, fructose 1,6-bisphosphate (F16BP). Upon a flux increase and initial accumulation of F16BP, the feedback activation quickly escalates the activity of PFKL and PFKM to increase the glycolysis rate. PFKM is inhibited by lactate, a characteristic which may facilitate the reduction of glycolysis flux at high lactate levels. This enzyme likely plays an important role in triggering the lactate consumption seen in the late culture stage (when lactate accumulates to high levels) in some bioprocess fed-batch cultures.

6-Phosphofructo-2-kinase / fructo-2,6-phosphate phosphatase (PFKFB)

Fructose 2,6-bisphosphate (F26BP) is a shunted reaction product of glycolysis that plays a key regulatory role in rapidly adjusting the activity of PFK and the glycolysis flux (Figure 3.9). It is not metabolized further or used in any biosynthesis, but is converted back to fructose 6-phosphate (F6P). Its concentration is a sensitive indicator of the “state” of glycolysis and is used for regulating the glycolysis flux. Both the synthesis of F26BP and its reversion to F6P are catalyzed by the same enzyme, a bi-functional PFKFB (also known as PFK-2). The kinase activity of PFKFB catalyzes the synthesis of F26BP and the phosphatase activity catalyzes the hydrolysis of F26BP to F6P. All three PFK isozymes are activated by F26BP, which activates PFK-1 by allosterically increasing its affinity for F6P, even in the presence of inhibitors such as ATP or lactate.

PFKFB has four isozymes, each with different kinase and phosphatase activities, allowing each to respond differently to regulators. A high kinase to phosphatase ratio allows for a higher accumulation of F26BP to exert a more robust activation of PFK activity. The brain isoform, PFKFB3, has the highest kinase to phosphatase activity and is expressed in several tumor cells. This suggests that PFKFB3 may be accountable for the glycolytic phenotype of reported cancerous cell lines by allowing them to have high cellular F26BP levels.

Pyruvate kinase

The enzyme catalyzing the penultimate step of glycolysis, pyruvate kinase, has three isozymes in mammalian systems. The muscle isozyme is expressed as either of two splice variants, M1 or M2. Both muscle isoforms of PK are activated by phosphoenolpyruvate, but only PKM2 is also activated by F16BP. The M1 isoform is mostly expressed in adult tissues, whereas the M2 isoform is expressed in rapidly growing tissues, such as fetal and tumor tissues, and is also thought to be a critical player in the transformation leading to cancer.

Pyruvate dehydrogenase kinase (PDK)

Pyruvate dehydrogenase complex (PDHC), which catalyzes the conversion of pyruvate to acetyl-CoA (and generates a CO₂ and NADH), is a protein complex made of 30 copies of E1 (consisting of two subunits), 60 E2 and 12 E3 proteins. It has three serine residues that can be phosphorylated by PDK to render it inactive, and dephosphorylated by pyruvate dehydrogenase phosphatase to return it to active state. There are four different isoforms for PDK that have varying phosphorylation activity towards the three serine residues. They thus tune the activity of PDHC to regulate the carbon flux into the TCA cycle.

Growth Control and Metabolic Regulation

The regulation of cellular metabolism is tightly linked to the control of cell growth. The signaling pathways and transcription factors that regulate a cell's growth rate play regulatory roles in regulating glucose metabolism. The transformation that causes cells to switch from a quiescent state to a proliferating state also triggers metabolic changes to increase their glucose uptake and glycolysis flux (Panel 3.13).

p53 is a major tumor suppressor that plays key roles in cell cycle arrest, senescence, apoptosis, and the regulation of glucose metabolism and oxidative phosphorylation. It induces the overexpression of TIGAR (TP53-induced glycolysis and apoptosis regulator) under mild oxidative stress conditions (Figure 3.10). TIGAR contains a fructose 2,6-bisphosphatase catalytic activity domain, which mediates the degradation of fructose 2,6-phosphate and leads to a decrease in PFK-1 activity and the attenuation of the glycolytic flux.

p53 can also modulate the glycolytic rate by regulating the activity of PGM, GLUT1, and GLUT4 transporters. Furthermore, it up-regulates mitochondrial oxidative phosphorylation by upregulating the expression of SCO2 (synthesis of cytochrome c oxidase 2), which mediates the assembly and activity of the cytochrome c oxidase complex.

AKT is a serine/threonine kinase that plays a key role in the regulation of cell proliferation and glucose metabolism. AKT exists in an active/phosphorylated form and an inactive/unphosphorylated form. The

Panel 3.13. Signaling Pathway / Growth Rate Control of Glycolysis

- Insulin signaling
 - Positively regulates growth rate
 - Regulates glucose and amino acid metabolism through AKT
- p53 (tumor suppressor) suppresses glucose uptake and glycolysis
- MYC (proto-oncogene) stimulates glycolysis
- Fast-growing (tumor) cells have fast glycolysis (and lactate production)

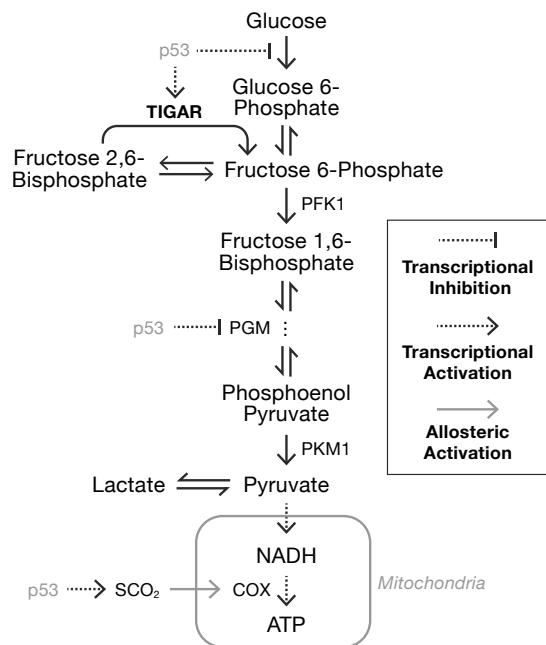


Figure 3.10. Tumor suppressor p53 negatively regulates glycolysis flux. TIGAR has phosphatase activity that converts F26BP to F16BP.

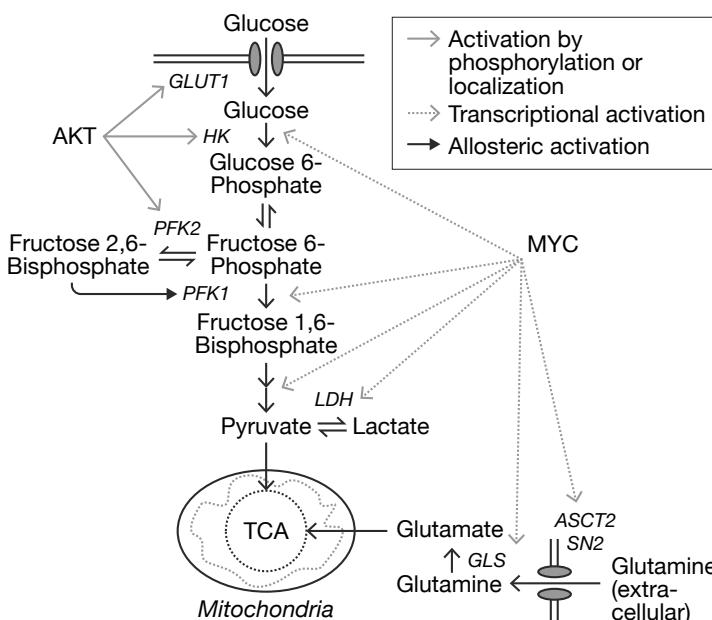


Figure 3.11. Signaling kinase AKT and transcription factor MYC positively regulate energy metabolism.

AKT signaling cascade has been shown to activate the transcription of GLUT1 and mediate the association of HK with the outer mitochondrial membrane (Figure 3.11). The phosphorylated form of AKT (pAKT) can increase the phosphorylation of PFKFB to shift its kinase/phosphatase ratio to increase the formation of fructose 2,6-bisphosphate levels, which in turn increases PFK activity and glycolysis flux. A decrease in the growth rate of the cells observed during the course of the culture is accompanied by a decrease in the pAKT activity.

MYC is a proto-oncogene whose pleiotropic regulatory roles include energy metabolism. It directly modulates the transcript level of glycolysis enzymes. Glycolytic enzymes have MYC canonical E-boxes in their promoter and are deregulated when MYC is overexpressed.

Regulation through the AKT signaling pathway provides a link between energy metabolism and cell proliferation in the tissue. Oncogenic transformation involves not only alterations in growth control but also invokes the expression of different isoforms or elevation of expression levels of key glycolysis enzymes that are hallmarks of fast growth. Virtually all proliferating cells in culture consume glucose at a high rate and convert the bulk of it into lactate.

Most cell lines of bioprocess importance have altered growth control that allows them to proliferate quickly in culture. During rapid growth, they exhibit a high glycolytic flux similar to other transformed cells. The decrease in glycolysis flux and the consumption of lactate as seen in some cultures occurs in the late culture stages when cell growth is already slow. Even for cells in culture, metabolism is linked to growth control.

Transport and Transporters

ENERGY METABOLISM TAKES PLACE in multiple cellular compartments that are separated by lipid bilayer membranes. First, glucose must cross the cytoplasmic membrane to enter glycolysis in the cytosol. Next, the products from glycolysis (pyruvate and NADH, via the malate-aspartate shuttle) are transported into the mitochondria. Finally, metabolites such as lactate are excreted through the cytoplasmic membrane. This crossing of molecules across the membrane is mediated by a number of transporters.

Glucose Transporters

Glucose transporters mediate the influx of glucose across the cytosolic membrane. There are two types of glucose transporters: GLUT and SGLT (Panel 3.14). The GLUT transporters are uniporters for facilitated transport, allowing glucose to move along its concentration gradient. The second type of glucose transporter, SGLT, is a co-transporter with Na^+ that is expressed in intestinal epithelial cells.

Panel 3.14. Two Main Types of Glucose Transporters

- GLUT transporters mediate facilitative diffusion across the plasma membrane
- SGLT, the sodium-dependent glucose co-transporters, are expressed primarily in small intestinal absorptive cells or renal proximal tubular cells. They use Na^+/K^+ ATPase pumps for active transport of glucose.

Panel 3.15. Glucose Transporter

- GLUT1 is highly expressed in all cells
- K_m is small for GLUT1. At the glucose concentration of the medium, it operates at its maximum rate.
 - K_m is the substrate concentration that gives a reaction rate of 1/2 the maximum. It is a measure of substrate affinity.

Table 3.2. Glucose Transporters

	<i>Class</i>	<i>Tissue expression</i>	<i>Affinity (sugar, K_m)</i>
GLUT1	I	Ubiquitous	Glucose, 1–2 mM
GLUT2	I	Liver, pancreas, intestine, kidney	Glucose, 16–20 mM; glucosamine, 0.8 mM
GLUT3	I	Brain, neurons	Glucose, 0.8 mM
GLUT4	I	Heart, muscle, adipose	Glucose, 5 mM
GLUT5	II	Intestine, testis	Fructose, 10–13 mM
GLUT7	II	Intestine, testis	Glucose, 0.3 mM; fructose, 0.1 mM
GLUT9	II	Kidney, liver	Fructose, 0.6 mM
GLUT6	III	Brain, spleen, leukocytes	Glucose, 5 mM
GLUT8	III	Testis, brain, liver	Glucose, 6 mM
GLUT10	III	Liver, pancreas	Glucose, 0.3 mM
GLUT12	III	Heart, muscle, prostate	Not well known

The GLUT transporters have twelve transmembrane regions and intracellular carboxyl and amino termini. According to common sequence motifs, they are divided into three subclasses (Table 3.2). GLUT1 is ubiquitous, appearing in almost all cells (Panel 3.15). It can transport glucose and galactose in a concentration-dependent manner that is described by Michaelis-Menten kinetics (Figure 3.12). Its K_m for glucose is low (~1 mM). At the glucose concentration used in culture medium, the flux of GLUT1 is at its maximum. In some cells, GLUT1 is under the regulation of the transcription factor HIF-1 (hypoxia inducible factor). Under hypoxic conditions, the expression of GLUT1 is upregulated to increase the uptake rate of glucose. The K_m of GLUT1 for galactose is rather high. When galactose is used as the only sugar, even at a high galactose concentration, the uptake rate and lactate production rate is low.

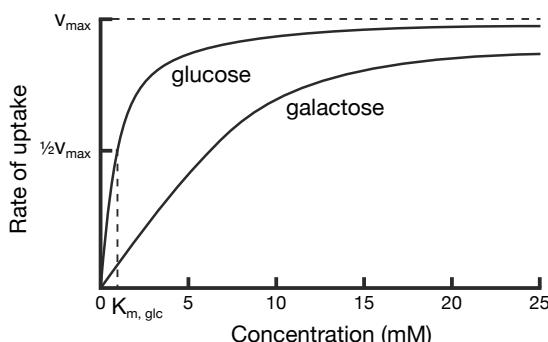


Figure 3.12. Michaelis-Menten kinetics plot for a GLUT1 transporter.

the Na⁺ concentration is low intracellularly but is high in the medium and in body fluid. The large sodium concentration difference and negative electric potential across the cytoplasmic membrane gives rise to a high propensity of Na⁺ to enter the cell. Thus, the chemical potential energy of the sodium concentration difference and the electric potential is used to drive the uptake of glucose against a concentration gradient.

SGLT transport is abundant in intestinal epithelial cells and is responsible for moving glucose from the gut into the intestinal epithelial cells. The glucose is then exported into the bloodstream on the other side of the cellular barrier.

Lactate Transporters

Lactate produced in glycolysis is excreted across the cytoplasmic membrane through monocarboxylate transporters (MCT). Lactate can also be consumed in some tissues and by cultured cells under some conditions. The uptake of lactate by cells is also mediated by MCT. Monocarboxylate transporters are largely divided into two categories: facilitated diffusion type monocarboxylate transporters and sodium-coupled monocarboxylate transporters (sMCT). The latter is expressed only in some tissue cells (kidney, colon epithelial cells) that can utilize the concentration gradient of sodium ions across its apical surface membrane to take up the solute. The solute transported by MCT and sMCT is not limited to lactate, and can also transport pyruvate, propionate, and other short-chain fatty acids.

Lactate is a negatively charged molecule. Its movement across the cellular membrane causes a charge imbalance and creates an electric potential across the membrane as a barrier for further transfer across the membrane, unless measures are taken to counteract the charge imbalance. The monocarboxylate transporters are a family of co-transporters that couple the transport of lactate to the transport of a hydrogen ion in the same direction to maintain the charge balance (Figure 3.13). MCT is thus a symporter; its mechanism of transport is facilitated diffusion.

A few other notable GLUT transporters are insulin-responsive GLUT4 and fructose-transporting GLUT5. In addition to GLUT1, cells in culture and in different tissues may express other GLUT transporters at different proportions. The expression of different transporters will give them different responses to the concentrations of glucose or other sugars.

SGLT, another class of glucose transporter, transports two sodium ions and one glucose molecule into

A number of MCTs are expressed in different tissue cells. Four of them, with 12 transmembrane helices, have been shown to mediate reversible transport of lactate, pyruvate, and ketone bodies. By far, lactate is the most important species transferred. MCT1 favors the uptake of lactate, while MCT4 favors the excretion of lactate produced in glycolysis.

Lactate transport is enhanced by a large difference in lactate concentration between intracellular and extracellular environments. However, pH also affects the flux of lactate through MCT, with the enhancing or retarding effect being dependent on the direction of the proton gradient. MCT allows for lactate transport in both directions, for excretion as well as uptake. Keeping medium pH at a lower level reduces lactate production during the rapid growth period, but enhances lactate consumption in the stationary phase.

Mitochondrial Pyruvate Carrier (MPC)

Proliferating cells in culture typically channel about 1/10–1/20 of the carbons from their glucose intake to the mitochondria as pyruvate, where it is broken down to CO_2 . The molar flux of pyruvate into the mitochondria is thus about 1/5–1/10 of that of the glucose consumption rate or 1/10–1/20 of the lactate excretion flux. Assuming an average cell diameter of 15 μm , a mitochondrial diameter of 1 μm , and 1000 mitochondria per cell, the total surface area of mitochondria in a cell is in the same order of magnitude as the cytoplasmic membrane. Thus, the pyruvate flux across the mitochondrial membrane ($\mu\text{mole}/\mu\text{m}^2\text{-h}$) is perhaps not as high as that of lactate passing through the cytoplasmic membrane.

The precise nature of the mitochondrial transport of pyruvate has been elusive until recently. Two proteins, mitochondrial pyruvate carriers MPC1 and MPC2, are involved in pyruvate transport. MPC1 and MPC2 have only 3 transmembrane helices. They are likely to form a protein complex (currently thought to be a heterodimer) to provide the transport activity. The transporter is also a symporter, co-transporting a proton to maintain charge neutrality. The 1.0 unit pH difference and -140 mV electric potential between the cytosol and the mitochondrial matrix provides a strong driving force for pyruvate transport into the mitochondria.

Not all pyruvate is generated from glycolysis. Pyruvate may also arise from catabolism of alanine and a few other amino acids. It may also be generated from acetyl-CoA shuttling, as will be discussed later in this chapter. Under some conditions, pyruvate may also be diverted to

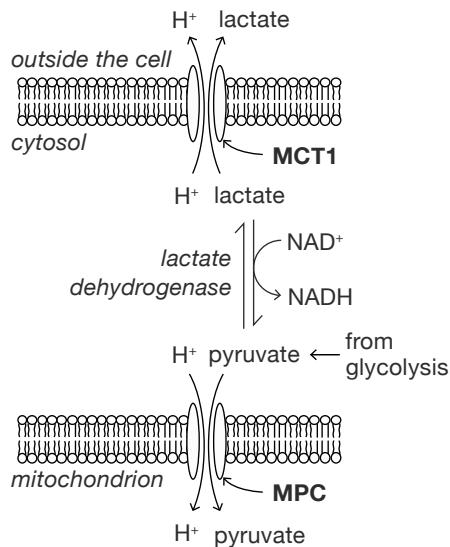


Figure 3.13. A monocarboxylate transporter for lactate and a mitochondria pyruvate carrier for pyruvate.

synthesize alanine. During rapid cell growth, these fluxes are relatively small compared to pyruvate synthesis from glycolysis. By and large, the flux of pyruvate into the mitochondria is estimated to be the difference between glucose flux in glycolysis and lactate production flux (Figure 3.9).

Metabolic Homeostasis and Lactate Consumption

CELLS IN CULTURE HAVE BEEN SELECTED for their capability to proliferate. The makeup of their glycolytic enzymes directs their metabolism to consume much glucose and convert most of it to lactate. Lactate accumulation in culture inhibits cell growth and hastens the decline of cell viability in the stationary phase. The phenomenon of high glucose consumption and high lactate production is seen in virtually all proliferating cells in culture. The problem is particularly acute in fed-batch cultures, in which continued glucose feeding raises both glucose and lactate to many times higher than their physiological levels. To achieve a high cell concentration and high productivity, it is beneficial to direct cell metabolism to a metabolic state that minimizes the accumulation of lactate.

States of Glucose Metabolism

High flux state

The “default” glucose metabolism is at a high flux state, in which cells consume glucose at a high level and convert most of it to lactate. Figure 3.14a illustrates a typical metabolic flux of a cultured mammalian cell line. If 100 moles of glucose are consumed in glycolysis, 200 moles each of pyruvate and NADH are generated. Here, for simplicity we neglect the diversion of carbons to the PPP, lipid biosynthesis, etc. Consider a scenario of a high flux state in which 90% (180 moles) of the pyruvate is channeled to lactate and excreted, and the rest (20 moles) of the pyruvate enters the mitochondria for the TCA cycle. Those 180 moles of pyruvate converted to lactate will also regenerate 180 moles of NADH to NAD. The remaining 20 moles of NADH will enter the mitochondria, where it is converted to NAD, consuming oxygen and generating H_2O and ATP. In total, 200 moles of NAD are regenerated to sustain glycolysis at a steady state flux of 100 moles of glucose. NADH generated in glycolysis in the cytosol cannot enter the mitochondria, as there is no appropriate transporter. Instead, it is transported via the malate-aspartate shuttle, as will be discussed next.

Neglecting the other cytosolic reactions that involve pyruvate, the molar fluxes of pyruvate and NADH into the mitochondria are equal (Panel 3.16). Overall, 10% of glucose is oxidized to CO_2 while 90%

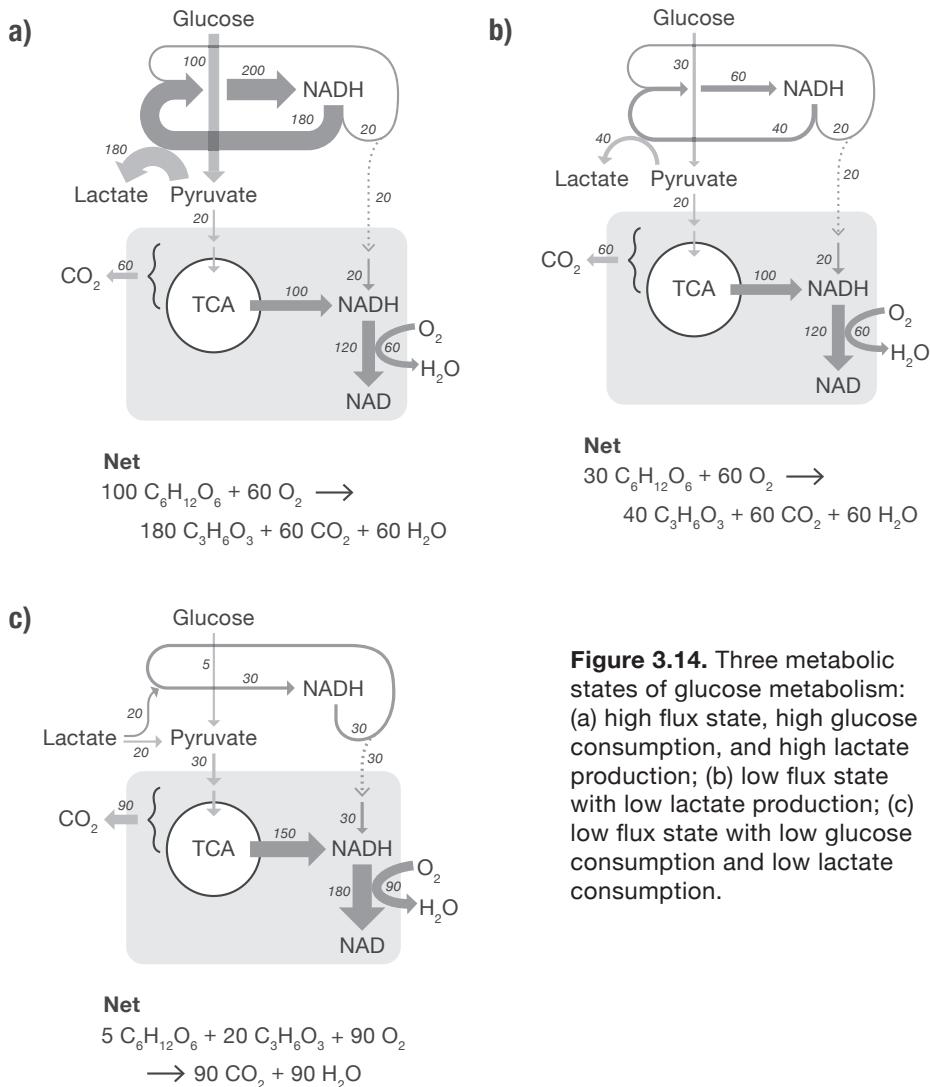


Figure 3.14. Three metabolic states of glucose metabolism: (a) high flux state, high glucose consumption, and high lactate production; (b) low flux state with low lactate production; (c) low flux state with low glucose consumption and low lactate consumption.

Panel 3.16. Pyruvate and NADH Balance

- Pyruvate and NADH are generated in glycolysis at a 1:1 ratio
- Both are transferred into the mitochondria and converted by lactate dehydrogenase (LDH)
- Altogether, 12 reducing equivalents (NADH / FADH₂) are generated to react with 6 O₂, generating 6 H₂O
- 2 of the 12 reducing equivalents are generated in the cytosol (in glycolysis), 10 in the mitochondria
- The 2 NADH generated in the cytosol need to be transferred into the mitochondria to feed into the electron transport chain, or oxidized to NAD in the LDH reaction

is converted to lactate. The 10% of glucose that is completely oxidized consumes 60 moles of O₂, as seen in the stoichiometric equation (Panel 3.1). This is carried out by the carbon and reducing equivalent entering the mitochondria to react with 60 moles of O₂ and produce 60 moles of CO₂ and H₂O (Eq. 3-1, Panel 3.1). The fluxes are thus all balanced.

Above all, there appears to be a limit in the capacity of MPC or in the malate-aspartate shuttle to allow a much greater flux of carbon from glucose to enter the mitochondria. Virtually all cells in culture resort to high flux metabolism to generate energy. There have been many hypotheses to explain the benefit of the Warburg effect to fast-growing cells, and why no organism has evolved a way to support fast proliferation without producing a large amount of lactate.

Low flux, lactate-producing state

Cells may enter a low glycolytic flux state under some conditions. Under most laboratory culture conditions, such as in a Petri dish, where cell density, lactate, and other metabolites are at low levels, the metabolism is mostly at a high flux state. As the metabolites accumulate and the growth rate decreases, as in a fed-batch culture, the combined effects of reduced AKT activation and possible lactate inhibition of PFKL lead the metabolism to a low flux state.

Even fast-growing cells can be guided to a low flux state by controlling glucose at low levels. Such a glucose flux control in yeast has been known for decades as the Crabtree effect. Even with plenty of oxygen, *Saccharomyces cerevisiae* ferments glucose to ethanol if glucose is kept at high levels. By controlling glucose at low levels to reduce its consumption, cells return to an oxidative metabolism with a reduced glucose flux and without ethanol production. This approach of restricting glucose supply and controlling glucose at low levels to reduce glycolysis flux and thereby reduce lactate flux has been demonstrated in hybridoma and CHO cell culture. By controlling glucose at very low levels or by replacing glucose with another sugar such as galactose or fructose that is only taken up slowly, even fast-growing cells can be manipulated at a low flux state.¹⁻⁴

As the cell growth rate decreases in culture, the AKT activation of the glycolytic enzymes also reduces,⁵ and it becomes more common to observe a switch to a low flux state. This may be facilitated by employing a slowly consumed sugar in the culture medium.

A typical flux distribution of cells in a low flux state, regardless of whether it is at a fast-growing or slow-growing state, is shown in Figure 3.14b. The prerequisite of entering a low lactate-producing state is low glucose uptake. In Figure 3.14, we assume that the flux into the mitochondria is fixed by the capacity of MPC. In practice, the value may increase somewhat by a fraction but not by a large margin. Glycolysis

generates 60 moles of NADH, of which 20 are regenerated to NAD through oxidation in the mitochondria (via the malate-aspartate shuttle) and 40 through lactate production. This allows a steady state flux of 30 moles of glucose through glycolysis.

Low flux, lactate-consumption state

Lactate transportation across the cytoplasmic membrane by MCT and lactate/pyruvate conversion by LDH are both reversible; one requires the co-transport of H⁺ and the other the co-substrate NAD/NADH. They operate in unison in the same direction depending on the intracellular levels of NAD, NADH, pyruvate, and lactate, and the intracellular and extracellular concentration difference of lactate and H⁺. Their concentrations, in turn, are affected by the glycolysis generation rate and the mitochondrial intake rate of pyruvate. At a high glucose flux state, the pyruvate production rate is so high, exceeding its mitochondrial intake, that lactate is always excreted as described above. Conversely, at a low glycolysis flux state, the pyruvate production rate is low and it is possible to observe lactate consumption.

In vivo, lactate consumption occurs under some condition in some tissues, such as in the muscle, liver, and brain. *In vitro*, lactate consumption by cultured cells is seen when the glycolysis flux is low, typically after the rapid growth stage is over and when lactate has accumulated to a significant level. The propensity and rate of lactate consumption is thus affected by pH, where a low pH (high H⁺) will facilitate lactate import and consumption.

A scenario of flux at a lactate consumption rate is shown in Figure 3.14c. The reverse reaction of LDH generates pyruvate and NADH. Both pyruvate and NADH are further oxidized in the mitochondria. Since the overall capacity of MPC and the intake rate of pyruvate into the mitochondria is limited, the lactate consumption flux is also limited. It never reaches the magnitude of the flux of glucose at its high flux state. On a per cell basis, the specific lactate consumption rate is relatively small.

In a fed-batch culture, one may observe the three metabolic states happening in order (Figure 3.15). In the fast-growing state, the metabolism is at a high flux state. As the growth rate slows down, it enters a low flux state with little lactate production, and then it may enter the lactate consumption state.

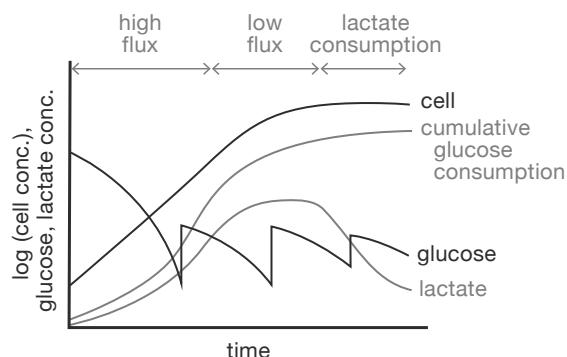


Figure 3.15. The occurrence of three metabolic states in fed-batch culture.

Glucose consumption in the lactate-consumption state

The lactate consumed by cells cannot be converted to glucose in most cultured cells. A number of reactions in glycolysis are irreversible. The conversion of pyruvate to glucose in the reverse direction of glycolysis, called gluconeogenesis, requires the expression of a few additional enzymes to counter these irreversible reactions. In mammals, gluconeogenesis primarily occurs in the liver. During the period that cells are consuming lactate, many intermediates derived from glycolysis are still needed for

Panel 3.17.
Lactate Consumption in Cultured Cells

- Lactate consumption is associated with a low glucose flux and occurs in a slow growth stage
- It is transported into the cell by MCT, converted to pyruvate by LDH, and generates 1 NADH
- Both pyruvate and NADH enter the mitochondria for energy generation
- While consuming lactate, glucose is also consumed at a low rate to supply NADPH and other intermediates

maintaining cellular functions. For example, dihydroxyacetone phosphate (DHAP) is needed for supplying glycerol 3-phosphate for lipid synthesis and NADPH, derived in the PPP, is needed for reductive biosynthesis and for maintaining the cell's redox balance. Furthermore, glucose 6-phosphate is required to synthesize the glucosamine and galactose that are used in glycan synthesis for the production of recombinant proteins. The glycolysis pathway thus remains active during the lactate-consumption stage. The glucose consumption rate is small, but not zero (Panel 3.17).

NADH Balance Malate-Aspartate Shuttle

A total of 12 moles of reducing equivalent (10 NADH and 2 FADH₂) are produced when 1 mole of glucose is completely oxidized to CO₂ through glycolysis and the TCA cycle. The 12 mole reducing equivalents consume 6 moles of O₂ in oxidative phosphorylation, consistent with the stoichiometry of glucose oxidation (1 glucose/6 O₂). Among the 12 NADH/FADH₂, 10 are produced in the TCA cycle in the mitochondria and the other 2 NADH are produced in cytosolic glycolysis (Panel 3.16). The two reducing equivalents produced in the cytosol must then be transported into the mitochondria where their oxidation consumes the sixth molecule of O₂.

NADH does not pass through the inner membrane of the mitochondria. Rather, it passes its reducing potential through a carrier system called the malate-aspartate shuttle. This system takes the reducing equivalent into the mitochondria through an exchange of molecules between the mitochondria and the cytosol. On the cytosolic side, NADH is oxidized to NAD and transfers its reducing equivalent to malate by reducing oxaloacetate. Malate is then transported across the mitochondrial membrane via an antiporter with α-KG going in the opposite direction. Once inside the mitochondria, the reducing equivalent is transferred back to NADH by oxidizing malate to oxaloacetate (Figure 3.16).

The shuttle employs two antiporter systems involving malate/ α -KG and aspartate/glutamate (hence the name “malate-aspartate shuttle”) to ensure the carbon flow and reducing equivalent flow are balanced by one other. The net result of a cycle of the shuttle is the transfer of a reducing equivalent from the cytosol to the mitochondria, or the conversion of a NADH to NAD in the cytosol and a simultaneous conversion of a NAD to NADH in the mitochondria. All other components involved in the shuttle then return to their original state. This is shown in Figure 3.16, with the fluxes in opposite directions shown in gray with different arrowheads. On each side of the mitochondrial membrane, the same aminotransferase reaction (converting a glutamate/OAA pair to α -KG/aspartate pair, or vice versa) occurs, but operates in the opposite direction.

The transfer of the reducing equivalent of NADH from the cytosol into the mitochondria is therefore dependent not only on the NADH concentration but also on the concentrations of α -KG, malate, aspartate, and glutamate in the two compartments. Furthermore, diversions of fluxes of those shuttle components may also perturb the transport of the reducing equivalent.

Other Carbon Fluxes across the Mitochondrial Membrane

Acetyl-CoA shuttle

Acetyl-CoA is the building block of fatty acids and cholesterol. It is generated primarily through the oxidative decarboxylation of pyruvate in the mitochondria. However, fatty acid biosynthesis takes place in the cytosol. Acetyl-CoA does not pass through the bilayer membrane. Instead, it is exported to the cytosol via an indirect process called the acetyl-CoA shuttle (Figure 3.7). Citrate, formed by condensation of OAA and acetyl-CoA in the TCA cycle, is diverted from the TCA cycle and transported into the cytosol by an antiporter with one malate being transported in the opposite direction (Panel 3.18). Once in the cytosol, citrate is

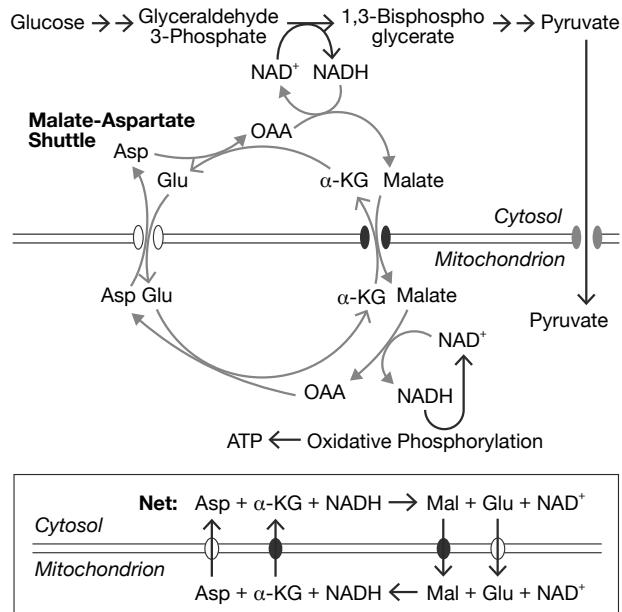


Figure 3.16. The malate-aspartate shuttle, for the transfer of the reducing equivalent of NADH from the cytoplasm to the mitochondria.

Panel 3.18. Acetyl-CoA Shuttle**In the Mitochondrial Matrix**

- Pyruvate carboxylase
 $\text{Pyruvate} + \text{CO}_2 + \text{ATP} \rightarrow \text{OAA} + \text{ADP}$
- Malate dehydrogenase
 $\text{Malate} + \text{NAD} \rightarrow \text{OAA} + \text{NADH}$
- Citrate synthase
 $\text{OAA} + \text{acetyl-CoA} \rightarrow \text{citrate} + \text{CoA}$

On the Mitochondrial Inner Membrane

- Citrate-malate transporter
 - An antiporter for citrate export from and malate import to the mitochondria
- Malate, α -ketoglutarate transporter
 - Transports malate into the mitochondria
- Mitochondrial pyruvate carrier (MPC)
 - Transports pyruvate into the mitochondria

In the Cytosol

- Citrate lyase
 $\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{OAA} + \text{acetyl-CoA}$
- Malate dehydrogenase
 $\text{OAA} + \text{NADH} \rightarrow \text{malate} + \text{NAD}$
- Malic enzyme
 $\text{Malate} + \text{NADP} \rightarrow \text{pyruvate} + \text{NADPH} + \text{CO}_2$

Panel 3.19. Metabolic Roles of Glutamine

- Essential for most cultured cells, consumed at a high rate
- Synthesized through glutamine synthetase *in vivo*
- Not an essential nutrient for mammals
- Used in nucleotide and protein synthesis
- Maintains carbon balance in the TCA cycle (anaplerotic metabolism)
- Releases NH_3 when entering the TCA cycle

split into OAA and acetyl-CoA, consuming one ATP. Acetyl-CoA is then used for lipid biosynthesis. OAA gets reduced to malate at the expense of one NADH. Malate is then transported back to the mitochondria, or is converted to pyruvate after releasing a CO_2 and consuming one NADPH. Pyruvate then recycles into the mitochondria. The process of making fatty acids using acetyl-CoA is thus energetically expensive.

Therefore, citrate and malate cross the mitochondrial membrane at a significant rate to sustain lipid biosynthesis. Citrate is not only present in the mitochondria, but also in the cytosol. Its accumulation can lead to allosteric inhibition of the conversion of F6P to F16BP in glycolysis.

Anaplerotic metabolism

As discussed earlier, the TCA cycle does not run perfect cycles all the time. When citrate, OAA, and other compounds are withdrawn from the cycle to serve other biosynthetic roles, they must be replenished in order to sustain the cycling at a steady state. This replenishment is mostly carried out by glutamine through anaplerotic reactions (Panel 3.19). Cells in culture consume glutamine at a high rate. The amount of glutamine consumed is in excess of that needed for biomass synthesis. Nearly half of the glutamine is converted to glutamate, which then enters the TCA cycle via α -KG to replenish OAA. α -KG can also be converted to isocitrate through

an isocitrate dehydrogenase I catalyzed reaction at the expense of 1 NADPH. Isocitrate then becomes citrate for export out of the mitochondria. Another enzyme, pyruvate carboxylase, converts pyruvate to OAA and can also serve to replenish OAA to allow the TCA cycle to continue.

Other fluxes across the mitochondria

Each pyruvate catabolized through the TCA cycle generates about 15 ATP and 3 CO₂. These products are exported to the cytosol. The exportation of ATP necessitates the importation of an equal amount of ADP and PO₄³⁻ for its synthesis. Besides these major species, many other molecules (including amino acids and nucleotides) are transported into the mitochondria for DNA, RNA, and protein synthesis.

Transportation across the mitochondrial inner membrane is dynamic and complex. Many compounds crossing the membrane are charged, yet their transport should not disrupt the proton and electric potential gradient that is fundamental to the energy generation function of the mitochondria. The transport across the mitochondrial membrane must be tightly regulated. Our understanding of that regulation is still rather limited.

Amino Acid Metabolism

Glutamine and Its Role in Energy Metabolism

MOST CULTURED CELLS CONSUME glutamine at a very high level, second only to glucose. Its molar consumption rate is about 1/5 to 1/10 that of glucose for many cell lines. Glutamine is not an essential amino acid for mammals; it becomes essential when cells are isolated from tissue and begin to grow in culture. Many tissues express glutamine synthetase that converts glutamic acid to glutamine by incorporating an ammonium at the expense of an ATP. The transcript level of this enzyme varies in cultured cells. Some cell lines can be readily adapted to grow in the absence of glutamine, apparently by increased glutamine synthetase activity, while most cells are cultured in glutamine-supplemented medium.

Glutamine is a major amino acid constituent of cellular proteins. Additionally, its amide group supplies the nitrogen in the synthesis of purine and pyrimidine bases, which are the backbone of nucleic acids. However, the amount of glutamine consumed by cells far exceeds what is needed for synthesizing cellular components.

A large portion of glutamine is converted to glutamate by glutaminase in the cytosol or mitochondria. Glutamate in the mitochondria is converted to α-KG via glutamate dehydrogenase (GDH), which releases an ammonium and NADH. Glutamate is also converted to α-KG via an aminotransferase reaction that transfers its amino group to the receiving OAA or pyruvate, forming aspartate or alanine, respectively. α-KG then enters the TCA cycle. The aminotransferase reaction retains the amino group in an amino acid, while the dehydrogenase reaction loses the amino group to ammonium. The former is likely to be favored in proliferating cells. Through α-ketoglutarate, glutamine is a major contributor to

central metabolic flux by fulfilling its anaplerotic role as discussed above. The conversion of glutamine to α -KG releases one or two ammonium, depending on the path taken (aminotransferase or glutamate dehydrogenase). The ammonium generated is excreted to the extracellular environment. The ammonium that is released from glutamine contributes to the waste metabolite accumulation.

Amino Acid Metabolism

In addition to providing the building blocks for protein synthesis, amino acids are also used in the biosynthesis of many other cellular components. They contribute the nitrogen atom and amino group in the synthesis of nucleoside bases and nitrogen-containing sugars. They also provide part of their carbon skeleton to form groups like the methyl group (Panel 3.20). Mammals can synthesize only some of the twenty amino acids used in the translational synthesis of proteins. The nine (depending on the species) essential amino acids that mammals cannot synthesize must be acquired through diet. The non-essential amino acids alanine and aspartic acid (and asparagine) are synthesized from py-

ruvate and OAA, respectively by acquiring the amino group from donor amino acid glutamate through transaminase reactions. Glutamate is derived from glutamine supplied in the medium or formed by incorporating ammonium into α -KG at the expense of an NADH via a glutamate dehydrogenase reaction. The synthetic pathways of serine, cysteine, and arginine are expressed in many cells. Tyrosine is derived from phenylalanine. However, in cultured cells, the supply rates of these amino acids may be too low for proliferation.

It is important to remember that an industrial high-producing cell line diverts a large portion of its amino acid pool to making the recombinant product. Even though non-essential amino acids can be synthesized by cells, their

provision rate (i.e., the sum of uptake and synthesis) must be sufficiently high to prevent the misincorporation of amino acids into proteins. It has been shown that the depletion of the non-essential amino acid asparagine in culture medium can lead to amino acid misincorporation into the product protein.

Amino Acid Transport

Amino acids are taken up by cells through a large number of amino acid transporters (Figure 3.17). Most amino acid transporters transfer a

Panel 3.20. Amino Acids in Cellular Biosynthesis

- Glutamine (amide group) is used as an amino group donor in adenosine (AMP), guanosine (GMP), and cytosine (CTP) biosynthesis
- Aspartic acid and glycine are also used in nucleic acid synthesis
- Methionine is a methyl group donor. Tryptophan is used in NAD synthesis.
- Glutamate participates in a large number of reactions. The flux of its synthesis or supply is expected to be high.

group of amino acids with similar chemical characteristics, such large neutral (uncharged side chain) amino acids, cationic, or anionic amino acids. The uptake rate of a particular amino acid is thus not only dependent on its own concentration, but also on the concentrations of other amino acids that compete for the same transporter. One amino acid may be taken up through more than one transporter, albeit with different affinities. Transport of amino acids across the cytoplasmic membrane is thus rather complex, and changing the concentration of one or several amino acids may affect the uptake rates of many.

Amino Acid Degradation and Growth Inhibition

In many industrial processes, especially in fed-batch cultures, concentrated amino acid solution is fed to the culture intermittently (see Chapter 9), causing some amino acids to accumulate to high levels

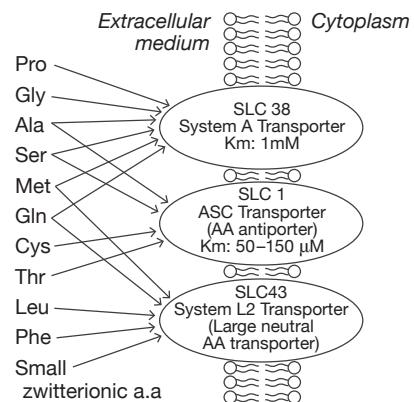


Figure 3.17. Major amino acid transporters.

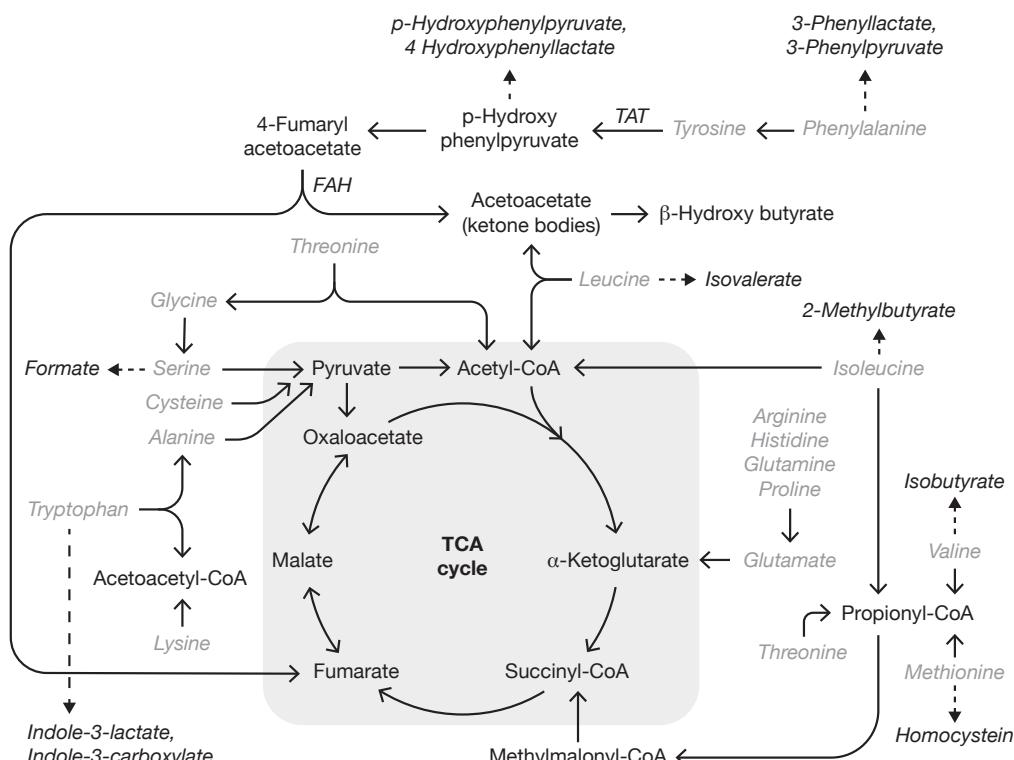


Figure 3.18. Entry of excess amino acids into catabolic pathways. The dashed line shows the amino acid degradation product that may be excreted into the culture medium.

Panel 3.21. Amino Acid Degradation

- Excess amino acids taken up by cells must be excreted
- The nitrogen (amino group) is removed from the carbon skeleton by transamination or oxidative/non-oxidative deamination. The excess nitrogen is excreted as ammonium ions or amino acids (e.g., alanine, proline, asparagine)
- The fate of the carbon skeleton is to enter the TCA cycle through pyruvate, acetoacetyl-CoA, oxaloacetate, or α -ketoglutarate
- The degradation of aromatic and aliphatic amino acids generates growth-inhibitory metabolites

transiently. This high level of amino acids may lead to excessive consumption. The amino acids taken up by cells in excess of cellular need are stripped of their nitrogen atoms and metabolized to pyruvate, acetyl-CoA, propionyl-CoA, or TCA cycle intermediates to enter energy metabolism (Figure 3.18, Panel 3.21). Some catabolic products are secreted into the medium. The catabolism of amino acids also releases ammonium. Ammonium and some catabolic products are growth inhibitory when accumulated to high levels. The catabolism of methionine, valine, and isoleucine gives rise to propionyl-CoA that is then carboxylated to methylmalonyl-CoA and further to succinyl-CoA, which enters the TCA cycle. In animals and humans, insufficient reactivity in the carboxylation reaction can lead to propionic acidemia. In culture, the accumulation of aliphatic amino acid degradation products may also lead to growth inhibition.⁶ Similarly,

the accumulation of the catabolic intermediates of aromatic amino acids (phenylalanine and tyrosine), especially fumarylacetoacetate, can be fatal in animals deficient in its degradation enzyme if left untreated. A number of inborn diseases of amino acid catabolism are caused by the accumulation of catabolic metabolites. The catabolism of amino acids typically takes place in the liver. Although cultured cells are not derived from individuals with inborn errors, the expression level of those catabolic enzymes in those cells may not be programmed to catabolize amino acids efficiently. At a very high cell concentration in the reactor, and with excessive feeding of amino acids, the catabolic products, both ammonium and reaction intermediates, may become growth inhibitory.

Lipid Metabolism

Lipid Transport

LIPLIDS SERVE MANY FUNCTIONS in the body. Phospholipids are critical components of the cell membrane, glycerides store energy, and sterols are not only constituents of the cell membrane but also hormones. Although their role in cultured cells is primarily as the constituent of cell membranes, they are also important in signaling, protein trafficking, and protein-membrane interactions (Panel 3.22). However, as cells grow and the membrane expands, lipids must be supplied at a sufficient rate

through biosynthesis and media provision. A balanced lipid composition in the cellular membrane is important in maintaining cellular vitality. However, our understanding of the balanced state of cellular lipids is rather limited.

The content of lipids in cell culture media varies widely, from high-serum-containing media that is rich in various lipids and lipoproteins to very lean media with only minimal amounts of lipid precursors, such as ethanolamine and a small number of fatty acids. Fatty acids and lipid precursors like ethanolamine may be directly dissolved in media. Most lipids are supplied as serum lipoproteins, conjugated to serum albumin, as liposomes, or as solubilized conjugates, such as sorbitol-fatty acid esters. Although some cells can be cultured in lipid-free media, most cell culture media contains some fatty acids and lipids.

Cellular uptake of lipids is mediated by receptors of the lipoprotein lipid carriers, by endocytosis of membrane vesicles, or by diffusion. Cells readily take up fatty acids, phospholipids, and cholesterol from the medium and incorporate them into cellular lipids. The cellular uptake of fatty acids is a passive, non-energy-dependent process. After being taken up by cells, fatty acids quickly become esters; the intracellular levels of free fatty acids are quite low. Cholesterol is complexed to low density lipoprotein (LDL) in the body and is taken up by cells through the LDL receptor. For cells in culture, cholesterol is often supplied as a conjugate with serum albumin, or as complexes with cyclodextrin.

The lipid composition in the bilayer membrane affects membrane fluidity and permeability. Not all lipid bilayer membranes are the same. The lipid composition of the outer leaflet and the inner leaflet of the cytoplasmic membrane, for example, differ from each other. The plasma membrane is enriched in cholesterol, while the amount of cholesterol in the ER and other organelles is much less. There is very little cholesterol in the inner mitochondrial membrane. After being taken up by cells, lipids have to be distributed to different organelles to give them distinctive membrane lipid compositions. Their transport is mediated by a number of lipid transport proteins or by membrane vesicles for those organelles.

After processing in the endoplasmic reticulum (ER) and the Golgi apparatus, recombinant proteins are transported via membrane vesicles to the cytoplasmic membrane for secretion. Membrane homeostasis and organelle biogenesis, therefore, also affect the productivity of recombinant proteins.

Panel 3.22. Functions of Lipids

- Contribute to membrane fluidity
- Involved in protein trafficking and membrane fusion events (e.g., polyphosphoinositide)
- Involved in the attachment of cytoskeletal proteins to membranes (anionic lipids, e.g., phosphatidylserine)
- Form microdomains or ‘rafts’ enriched in specific subsets of membrane proteins (cholesterol and sphingolipids)

Panel 3.23. Subcellular Localization of Lipid Metabolism

Cytosol

- NADPH synthesis (pentose phosphate pathway)
- Isoprenoid and early cholesterol synthesis
- Fatty acid synthesis

Mitochondria

- Fatty acid oxidation
- Acetyl-CoA synthesis
- Ketone body synthesis
- Fatty acid elongation

Endoplasmic Reticulum

- Phospholipid synthesis
- Cholesterol synthesis (late stage)
- Fatty acid elongation
- Fatty acid desaturation

Peroxisome

- Cholesterol precursors synthesis
- The final steps of cholesterol synthesis

Fatty Acid Metabolism

Most cells have the capability of synthesizing various fatty acids. Under starvation conditions, cells also perform β -oxidation to degrade fatty acids into acetyl-CoA in the mitochondria or peroxisomes (Panel 3.23). Acetyl-CoA then enters the TCA cycle and generates energy.

Fatty acids are synthesized from acetyl-CoA in the cytosol. The first step of fatty acid synthesis involves adding a CO_2 to acetyl-CoA to form malonyl-CoA, which then reacts with acetyl-CoA to become a four-carbon fatty acyl-CoA. This is a case where CO_2 is an essential substrate for biosynthesis, not merely a catabolic product. Fatty acid synthesis, therefore, involves the step-wise elongation processes of using three-carbon malonyl-CoA to add a two-carbon unit to fatty acyl-CoA in each cycle. NADPH is also used to provide energy in this reductive synthesis reaction. There are a number of fatty acid synthetases that can synthesize fatty acids to different lengths.

The fatty acid products from elongation reactions are all saturated fatty acids. Double bonds are then synthesized by unsaturation reactions after saturated fatty acids have been made.

Cholesterol and Its Biosynthesis

Cholesterol is a 27-carbon molecule that has a hydrocarbon chain linked to aliphatic rings (Figure 3.19). A hydroxyl group attached to the aliphatic ring allows it to interact with the head group of phospholipids while the hydrocarbon chain is well embedded in the membrane. Mammals require cholesterol as a constituent of cellular membranes and as a precursor for the synthesis of steroid hormones, bile acids, and lipoproteins (Panel 3.24). Cholesterol is relatively insoluble and resides exclusively in various cell membranes. Its regulation is particularly important since excess cholesterol forms solid crystals, leading to cell death.

Cholesterol constitutes ~10% of the dry weight of plasma membranes, and plasma membrane cholesterol accounts for 65% to 80% of total cellular cholesterol. Cells in culture obtain cholesterol either by *de novo* synthesis or through receptor-mediated uptake of exogenous low-density lipoproteins.

Cholesterol is synthesized from acetyl-CoA, which is condensed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGCS) to form HMG-CoA. HMG-CoA is converted to mevalonate by HMG-CoA

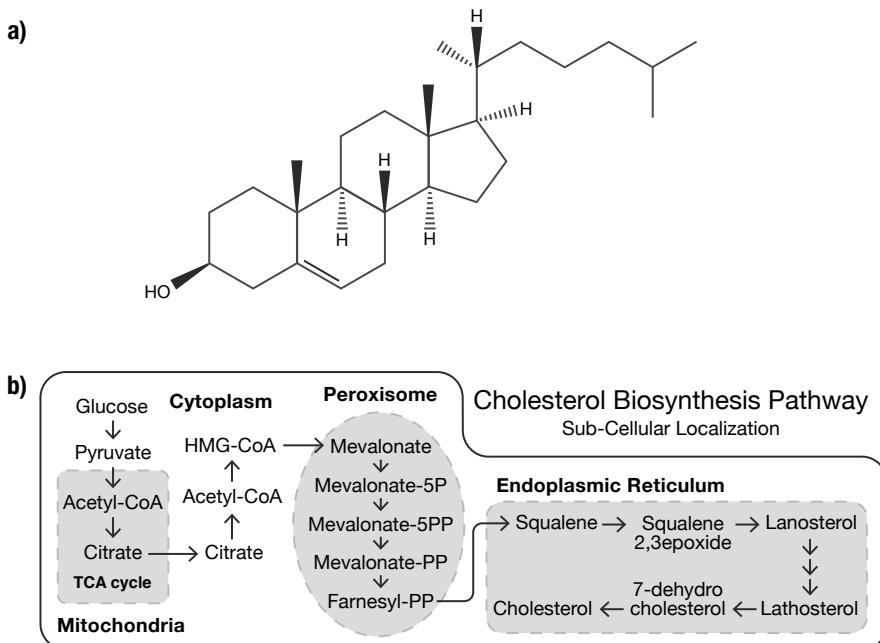


Figure 3.19. (a) The structure of cholesterol. (b) The segregation of the cholesterol biosynthesis pathway in the mitochondria, peroxisome, and endoplasmic reticulum.

reductase (HMGCR) (Figure 3.19b). This enzyme is the target of statins, the class of drugs that suppresses cholesterol synthesis in patients.

Further synthesis of mevalonate to farnesyldiphosphate takes place in peroxisomes. Subsequent condensation of two molecules of farnesyl diphosphate to form squalene, lanosterol, lathosterol, and finally cholesterol occurs in the ER.

Out of the 18 key enzymes taking part in cholesterol biosynthesis, 5 enzymes reside in the peroxisome and 13 reside in the ER. HMGCS is upstream of HMGCR and is found in the cytosol. Thus, there are at least three different sub-cellular compartments involved in cholesterol biosynthesis.

Although cholesterol in mammals is synthesized primarily in the liver, most cells have the capability of synthesizing cholesterol for their own growth requirements. NS0 cells lack an enzyme, 17-HSD, which converts lanosterol to lathosterol. In NS0 cells, 17-HSD is silenced through methylation of a CpG island upstream of its promoter, leading to the cell line's dependency on cholesterol for growth.

Panel 3.24. Cholesterol

- A component of membranes and a precursor of steroid hormones, bile acids, and lipoproteins
- Resides exclusively in cell membranes
- Excess cholesterol forms solid crystals, leading to cell death
- Constitutes ~10% of dry weight of plasma membranes
- Cells obtain cholesterol by *de novo* synthesis and by receptor-mediated uptake of plasma lipoproteins

Glycan Biosynthesis and Protein Glycosylation

Glycan Heterogeneity

AVAST MAJORITY OF RECOMBINANT therapeutic proteins are glycoproteins. These proteins have carbohydrates, in the form of branched oligosaccharides, attached to them. The glycosylation of proteins, along with disulfide bond formation, is the most common post-translational modification in recombinant protein products. Glycans are classified as O-linked or N-linked glycans (Panel 3.25). O-glycans attach to the polypeptide through the -OH group of serine or threonine. N-glycans link to protein through the amide group of asparagine. For N-linked glycans, the asparagine is in an Asn-X-Thr/Ser recognition sequence, where X indicates no specificity. For O-glycans, no specific recognition sequence is known.

The glycans attached to proteins are oligosaccharides of varying sizes. They are structurally heterogeneous (Panel 3.26). Glycans that attach to the same attachment site of different glycoprotein molecules often have different monosaccharide compositions and sequences. Even glycans of the same sequence may have different linkage bonds (glycosidic bonds) between monosaccharides. Such heterogeneity is called microheterogeneity.

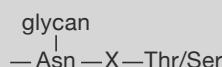
Multiple glycosylation sites are often present on a protein molecule. Not all glycan attachment sites on a protein molecule may be occupied. Different protein molecules may have different combinations of occupied

Panel 3.25. Protein Glycosylation

- Many secreted proteins and cell surface proteins are glycosylated
- All IgG antibodies produced by mammalian cells are glycoproteins, with an N-linked oligosaccharide attached to each heavy chain in the hinge region at Asn₂₉₇

N-linked Glycosylation

- Attachment of an oligosaccharide to the protein through the amine group of an asparagine



O-linked glycosylation

- Attachment of an oligosaccharide to the protein through the hydroxyl group of a serine or threonine

Panel 3.26. Heterogeneity In Glycoforms

Macroheterogeneity

- When multiple sites of glycosylation are present in a protein, the occupancy on different sites differs on different molecules
- Possible occupancy:

Glycosylation	Glycosylation
Site 1	Site 2
+	+
-	-
+	-
-	+

Microheterogeneity

- The structure of the glycan occupying the same site differs among different molecules

and free sites; such differences in the occupancy of different attachment sites among protein molecules is called macroheterogeneity.

The microheterogeneity and macroheterogeneity of glycoproteins give them complex glycosylation patterns. The diversity of glycans is seen on secreted glycoproteins in the human body's circulation, as well as on cell surface proteins. The glycan pattern (i.e., the distribution of abundance levels of different glycan structures) of a protein may vary at different developmental or disease states. In biomanufacturing, glycoproteins produced by the same cell line are also heterogeneous in their structure. However, for the manufacturing of therapeutic biologics the glycan profile is specified in the filing of the product for regulatory approval. Upon drug approval, the glycosylation pattern of the manufactured product must be within the range of specification. Those who wish to produce a biosimilar biologic after the patent of an innovative product expires must reproduce the protein with comparable or better (biobetter) quality with the acceptable glycan profile. The heterogeneity of glycans thus poses a special challenge for biosimilar production.

Major Types of N-Glycans

A few commonly seen N-glycans are shown in Figure 3.20. A glycan is first formed in the ER, with 9 mannose extended from 2 N-acetylglucosamine. The 9 mannose is normally trimmed down to 3 and extended further. Glycans that are not trimmed and still have 5 or more mannose residues are called high mannose glycans. High mannose glycans are not abundant in humans, but appear prominently or even dominantly in

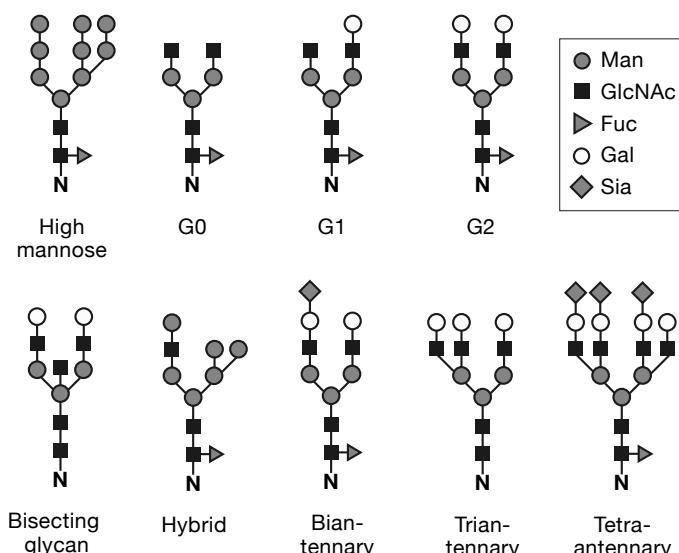


Figure 3.20. Different types of N-glycans in glycoproteins.

yeast, plants, and insects. They may cause an immunogenic response in humans. The trimmed mannose can then be extended to become a biantennary, triantennary, or tetraantennary glycan. These glycans, which are sometimes referred to as complex glycans, may be extended fully to have sialic acid at the terminal, or can be terminated sooner. Glycans with both untrimmed mannose and an antennary extending are called hybrid glycans. A glycan may be fucosylated or unfucosylated. The mannose at the base is extended to two antennaries through its C3 and C6 to two more mannoses. Sometimes the C4 of the mannose at the base is linked to a bisecting N-acetylglucosamine. Bisecting sugar will affect fucosylation and further glycan extension.

IgG molecules typically have simpler glycan structure with only two antennaries. After extending by an N-acetylglucosamine, it may be further galactosylated in 0 to 2 branches. These glycans with 0,1, or 2 galactose are referred to as G0, G1, and G2 structures.

Role of Glycans

The glycan structure on a glycoprotein affects its half-life in blood circulation and its immunogenicity. For IgG molecules, N-glycosylation in the Fc region affects their biological activities. The presence of glycans on interferon produced in mammalian cells prolongs its clearance from blood, as compared to its non-glycosylated counterpart produced in *E. coli*. Higher sialic acid content on erythropoietin (EPO) increases its circulation half-life. Under-sialylated glycoproteins are cleared by liver uptake via the hepatic asialoglycoprotein binding protein faster. It has been postulated that glycosylated recombinant proteins are better retained by the extracellular matrix, thus giving them a longer bioavailability *in vivo* than their unglycosylated variants.

Glycans on glycoproteins may also affect their biological activities. The effect of glycan structure on biological activities is relatively well understood in IgG. Many therapeutic antibody IgG molecules facilitate the killing of target cells through antibody-dependent cellular cytotoxicity (ADCC). ADCC activities of those antibodies are affected by the glycan structure at position Asn₂₉₇ on the CH₂ domain in the Fc region of the IgG heavy chain. Unlike many other glycans on glycoproteins, which are often thought of as being “coated” on the exterior of proteins, the glycan at Asn₂₉₇ is in a “pocket” inside the folded IgG molecule. However, the glycan structure at Asn₂₉₇ affects the binding of IgG to the Fc receptor on the effector cells and its interactions with the complement component C1q. IgG molecules that do not have a fucose on their mannose core have a greater than 10-fold higher ADCC activity compared with those with a fucose. Bisection of the glycan on Asn₂₉₇ also appears to affect ADCC activity. However, bisection and afucosylation occur together, making their effects difficult to distinguish. The effects of galactosylation

on IgG activity is less clear. However, a decrease in galactosylation has been related to some autoimmune diseases.

Protein Folding and Glycosylation in the ER

N-glycosylation starts while the protein molecule is still being translated and folded in the ER (Figure 3.21). The translation and translocation into the endoplasmic reticulum of a protein molecule takes a finite amount of time. The translation rate is in the order of 10 amino acids per second. It takes about half a minute to synthesize an average protein and many minutes for a large protein like the heavy chain of IgG. During that period, protein molecules must be protected from misfolding or aggregation. Many chaperone proteins play important roles in the folding process. The addition of glycans to the protein facilitates the folding process (Panel 3.27). Glycans also serve as a quality control signal to ensure the molecules passed on to the Golgi apparatus for further processing are all properly folded.

N-glycosylation is initiated by the transfer of a preassembled oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, an oligosaccharide of three glucose, nine mannose, and two N-acetylglucosamine) to the asparagine in a recognition sequence of a nascent protein in the ER lumen (Figure 3.21).⁷

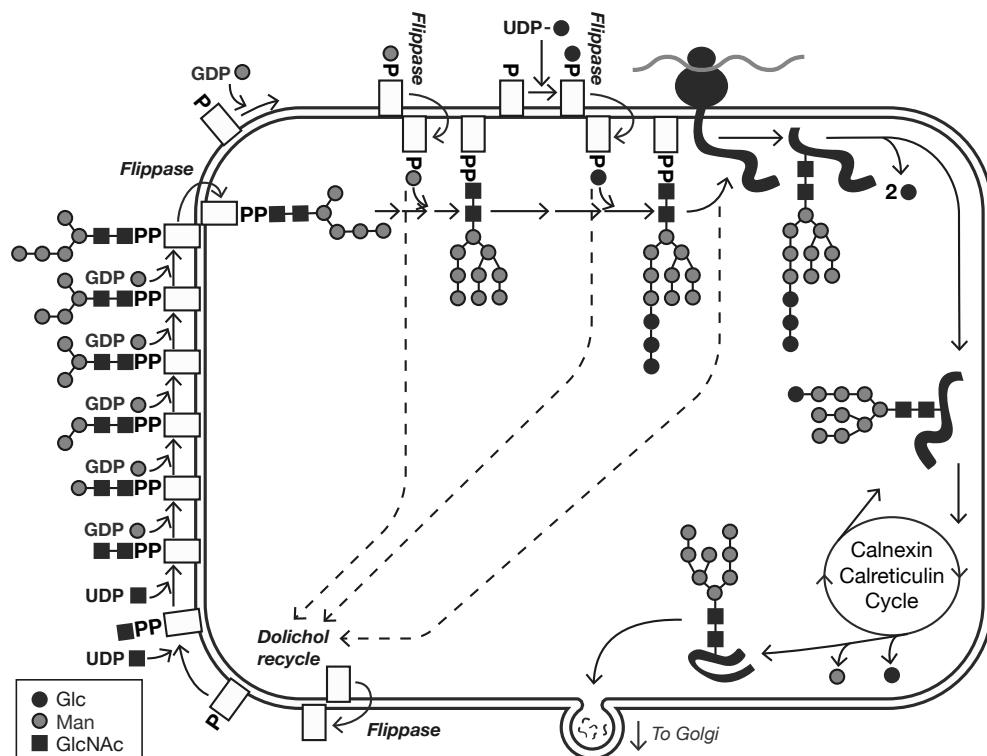


Figure 3.21. N-glycan processing of glycoproteins in the endoplasmic reticulum.

Panel 3.27. Effects of Glycans

- Facilitates protein folding in the ER
- Increases protein solubility
- Affects biological activities
 - Fucose for ADCC activity
- Affects half-life in circulation and pharmacokinetics

The assembly of the high mannose backbone starts on the outside surface of the ER. The glycan is linked to a membrane-anchored dolichol carrier through a pyrophosphate group. After the seven-sugar backbone is formed (with five mannose and two N-acetyl glucosamine), it flips over to the interior of the ER. No transporters are needed for the transport of the backbone glycan; rather, a flippase catalyzes their translocation into the ER lumen. Once inside the ER, the backbone acquires an additional four mannose and three glucose to become a mature core.

The mature core is then transferred to a binding site (Asn-X-Thr/Ser) on a nascent protein molecule. However, not all glycan binding sites may receive the core glycan. This might be due to competition between local protein folding, which could make the glycosylation site inaccessible and the transfer of the glycan core to the site impossible. Hence, on the same glycosylation site of a protein, some molecules may be occupied by a glycan while others may not.

After translation, protein molecules are assisted by many chaperone molecules to undergo the folding process. The three glucose on the glycan core serve as a quality control signal for the proper folding of these glycoprotein molecules. The three glucose are quickly removed from the glycan to generate a monoglucosylated intermediate. But a glucosyltransferase quickly adds a glucose to any protein molecule that is not yet correctly folded. The presence of the monoglucosylated glycan signals the protein molecule liable to bind to the ER lectins calnexin and calreticulin, and the oxidoreductase ERp57. ERp57 facilitates the formation of disulfide, a critical step in protein folding. The folding process continues until the absence of the glucose residue allows the folded molecule to dissociate from the lectin. The “correctly” folded protein molecule is then transported to the Golgi apparatus by a membrane vesicle.

Misfolded proteins, with the de-glucosylated mannose exposed, are subjected to the removal of their terminal α 1,2-bonded mannose. Trimming of the mannose then serves as the signal to divert the unfolded molecules to the ER disposal system, through which they are exported to a proteasome for degradation.

Well-folded glycoprotein molecules are enclosed in membrane vesicles of the ER, where they bud and then translocate to the Golgi apparatus. Once there, they fuse with the Golgi body membrane and the glycoprotein cargos are released into the lumen of the Golgi apparatus.

Glycan Extension in the Golgi Apparatus

Inside the Golgi, mannose is trimmed further from the N-glycan core, reducing the number of mannoses from nine to three to form the

tri-mannosyl ($\text{Man}_3\text{GlcNAc}_2$) core structure (Figure 3.22). However, incomplete trimming does occur, leading to the synthesis of high-mannose-type glycans ($\text{Man}_{5-9}\text{GlcNAc}_2$) (Figure 3.20). After mannose trimming, more monosaccharide units are added to the glycan one at a time. The extension of the glycan from the 3-mannose core creates complex-type glycans, while the extension from those with 4-5 mannose creates hybrid-type glycans.

Three monosaccharide units constitute most of the extended N-glycans: N-acetyl glucosamine, galactose, and sialic acid. Different glycosyl-transferases are involved in these reactions. Each enzyme adds a different monosaccharide and forms a specific glycosidic linkage to the growing core glycan. Each glycosidic bond is defined by the two sugars being linked and the position of the carbon on each sugar. The incoming monosaccharide provides the activated carbonyl group (by linking to a nucleotide), and the receiving carbohydrate moiety on the growing glycan on the protein provides a hydroxyl group for forming the glycosidic bond. For example, the two-terminal mannose residues each use its C1 (carbonyl carbon) to link to C3 and C6 of the base mannose, respectively (Figure 3.22).

Each terminal mannose can be further extended by the addition of a N-acetyl glucosamine (GlcNAc). The terminal receiving mannose has three hydroxyl groups, a pair of which (carbon 2 and carbon 4, or carbon 2 and carbon 6) can be extended by the addition of a GlcNAc. The extension reaction does not take place on all of the available reaction (receiving) sites. If one of each of the two-terminal mannoses of the $\text{Man}_3\text{GlcNAc}_2$ core are extended by the addition of a GlcNAc, it becomes a biantennary glycan (Figure 3.20). If more than one hydroxyl group is

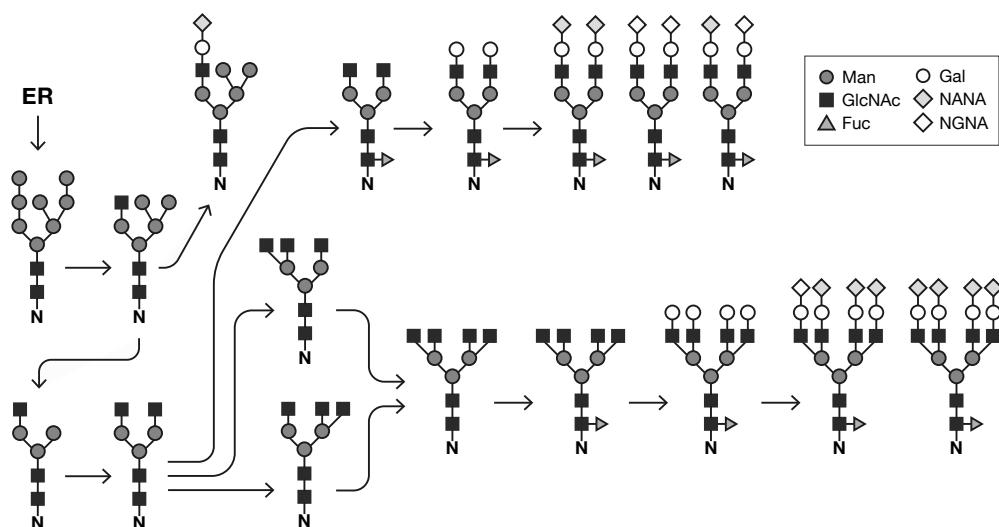


Figure 3.22. N-glycan extension in the Golgi apparatus.

extended on either one or both terminal mannoses, then the glycan becomes tri- or tetra-antennary.

There are a number of different glycosyltransferases, each of which recognizes different pairs of incoming nucleotide sugars and substrate glycans, and which catalyzes the formation of different glycosidic bonds. A number of glycosyltransferases do allow for some flexibility in glycosidic bond formation. In mammals, some glycosyltransferases have a large number of isozymes. Their expression may be tissue-specific. Some isozymes are co-expressed in some cells. Roughly a couple dozen glycosyltransferases are prominent in contributing to the glycan heterogeneity in recombinant proteins.

Each growing glycan, thus, has multiple available reaction paths for extension. Many of those different reaction paths will lead to different products (Figure 3.22). However, in other cases, the reactions of adding sugars to different branches of the glycan may occur in different orders but lead to the same product. In the extension of the first layer GlcNAc to the 3-mannose core, the addition of GlcNAc to the base mannose (catalyzed by N-acetylglucosaminyltransferase III (GnT-III)) suppresses further extension of the glycan. This bisecting GlcNAc thus has a strong effect on glycan structure. The enzyme fucosyltransferase has the opposite effect of increasing structural diversity; it adds a fucose to the base sugar (GlcNAc), thus making two versions of most glycans, fucosylated or unfucosylated. Both the bisecting GlcNAc and the core fucose affect the biological activities of many protein molecules (e.g., the ADCC activity of IgG, and the binding of EGF to EGFR).

The web of glycan extension reactions forms a complex network which, when drawn out graphically, indeed resembles a network of diverging and converging paths leading to a number of different fully-extended N-glycan structures (Figure 3.23). Adding to the complexity is the compartmentalization within the Golgi apparatus. The Golgi apparatus consists of stacks of membranous compartments commonly grouped into *cis*, medial, trans, and trans-Golgi network (TGN) cisternae. These cisternae are not biochemically homogeneous. The glycosyltransferase composition varies among different compartments, with the earlier enzymes more enriched in the early compartment and vice versa. As the secretory glycoproteins traverse through these Golgi compartments, the extension reactions will favor the addition of different glycans. An extending glycan that fails to acquire a particular sugar in an early compartment may thus not get extended further after moving on to a later compartment.

The reaction path for glycan extension in the Golgi apparatus is a rather long one. Protein molecules spend tens of minutes passing through the Golgi, but the residence time that each protein molecule spends in the Golgi is not uniform. Not all protein molecules will have fully extended glycans. In fact, often only a portion will be fully extended and

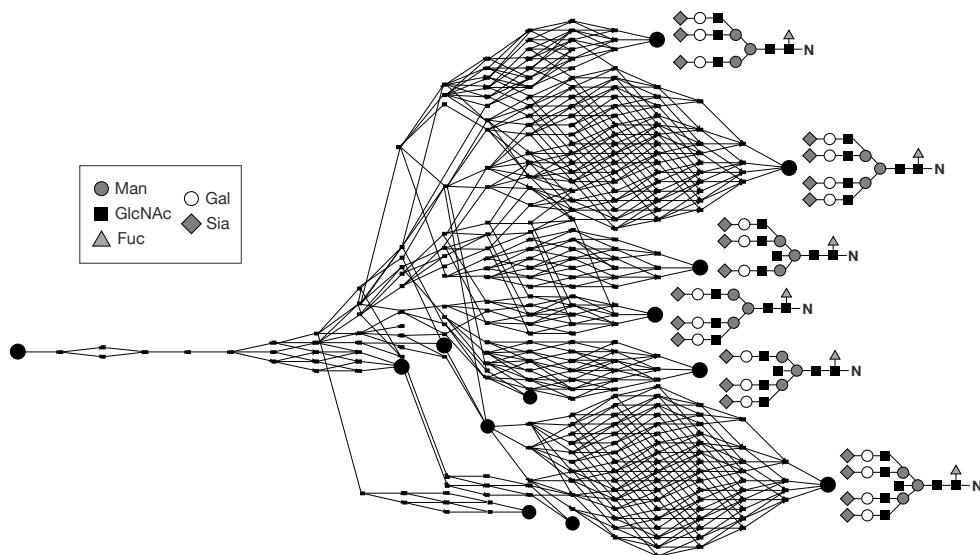


Figure 3.23. N-glycan extension reactions in the Golgi form a complex network. Only a small number of enzymatic reactions generate a very large number of different glycans. Each enzyme catalyzes the same reaction on many different extending glycans.

contain sialic acid. IgG molecules have glycans on Asn₂₉₇ of the heavy chain constant region and in some cases also in the hypervariable region. Sialylation is rarely seen in the glycan on Asn₂₉₇, but is more often seen in the one in the hypervariable region.

A very large number of glycan structures can be formed in the N-glycosylation pathway; typically only a fraction of all these possible configurations constitute the dominant types. The patterns of glycans on recombinant DNA proteins produced in different production cell lines but derived from the same host cell line are likely to be different. The pattern is also affected by different culture conditions.

O-Glycosylation

O-glycosylation is initiated in the endoplasmic reticulum or in the Golgi apparatus. Unlike N-glycosylation, which is initiated by the translocation of a preassembled core-oligoglycan (Man₉Glc₃) to the nascent protein, O-glycosylation starts by adding monosaccharides directly to the protein and extends mostly through one-by-one addition of more sugars. There is no known consensus sequence for the addition of O-glycans to serine and threonine, although clusters of O-linked glycosylation are often seen in regions with a high density of serine/proline/threonine. The OH- groups of serine and threonine can be linked to glucose, N-acetylglucosamine, galactose, N-acetyl galactosamine, fucose, xylose, or mannose. O-glycans can thus be more complex and heterogeneous than N-glycans, because they lack the consensus attachment amino

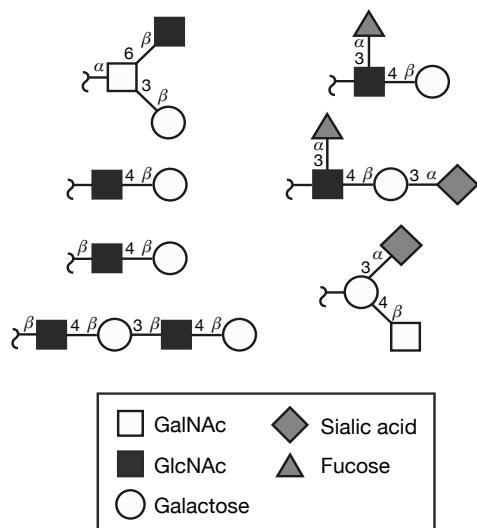


Figure 3.24. Some O-glycans synthesized in CHO cells. CHO cells have a relatively small number of enzymes involved in O-glycosylation.

acid sequence and a single common core glycan structure. O-glycans are abundantly represented in some glycoproteins. The O-glycans seen in recombinant proteins are mostly of the mucin-type structure with an N-acetylgalactosamine (GalNAc) as the first sugar. Etanercept (Embrel, a fusion protein of TNF α -Fc for rheumatoid arthritis treatment) has 3 N-glycosylation sites and 11 O-glycosylation sites. Most of those O-glycans are linked to serine/threonine through O-GalNAc, whereas in the O-glycan of factor VII, O-fucose and O-glucose are also seen (Figure 3.24).

The attachment of N-acetylgalactosamine to the $-OH$ group of serine/threonine is initiated by GalNAc-transferases. The presence of GalNAc recruits other enzymes to add more GalNAc to nearby serines/threonines. Subsequently, a number

of enzymes catalyze the addition of different sugars (glucose, galactose, GalNAc) to form different core O-glycans. This is followed by core extension (e.g., by adding more galactose) and O-glycan capping (e.g., by the addition of sialic acid, fucose, or sulfate). Like N-glycosylation, various glycosyltransferases are locally enriched in different compartments of the Golgi apparatus. Some glycosyltransferases are probably shared with N-glycan biosynthesis. Like N-glycan synthesis, the formation of the different cores of O-glycans is affected by the relative abundance of different enzymes in the tissue or the cell. The addition and extension of different glycans is also affected by the protein domain structure, although our knowledge with respect to the effect of protein domain on glycosylation is still rather limited.

Synthesis and Transport of Nucleotide Sugar Precursors

The substrate for glycosidic bond formation by glycosyltransferase is a nucleotide sugar. The carbonyl carbon of the sugar is the carbon that will form the glycosidic bond. Before it can react with the glycan substrate, the carbonyl carbon of the sugar substrate (glucose 1-phosphate, galactose 1-phosphate, N-acetyl-glucosamine-phosphate, or mannose 1-phosphate) needs to be “activated” by reacting with a nucleotide (NTPs: UTP, CTP, GTP) to form NDP-sugar or CMP-sialic acid at the expense of an equivalent of 1 ATP. Different sugars are linked to

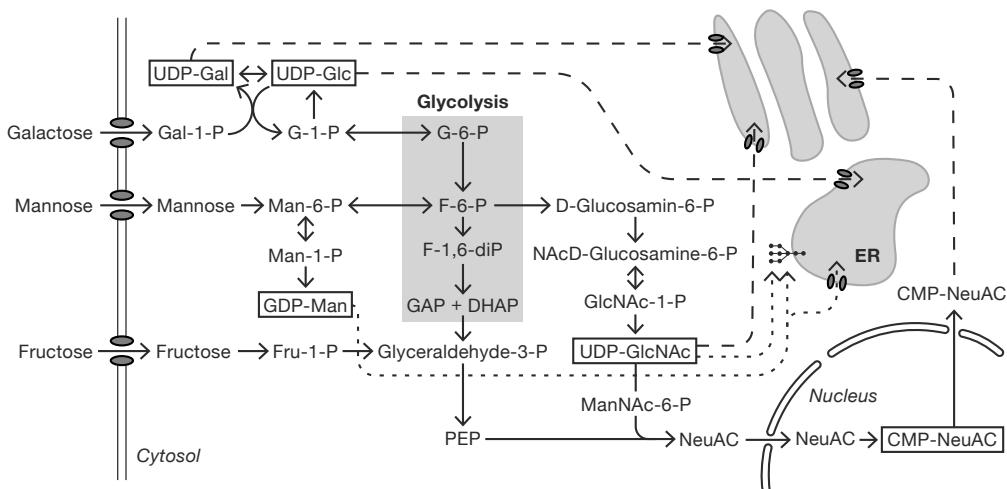


Figure 3.25. Biosynthesis of the precursors of glycans.

different NDPs. Uracil is used for glucose- and galactose-based sugars (e.g., UDP-glucose and UDP-galactose), guanyl for mannose and fucose, and cytidyl for sialic acid.

Mannose, galactose, N-acetylglucosamine, and fucose are synthesized in branches of the glycolysis pathway (Figure 3.25). All four sugars are activated at their first (carbonyl) carbon. Therefore, they link to glycans through the formation of (1→n) glycosidic bonds. For example, UDP-GlcNAc is added to a growing core by the formation of an N-acetylglucosamine β (1→n) mannose bond. The linkage can be through a number of possible carbons on mannose (e.g., 2, 3, 4, or 6) depending on the position of the mannose in the glycan. Upon giving away its sugar, an NMP is formed as the reaction product, and is transported back to the cytosol (Figure 3.25).

Sialic acid is a generic name for the derivative of 9-carbon neuraminic acid and is also the name of the most commonly seen N-acetylneuraminic acids. The second carbon of neuraminic acid is activated; thus CMP-2-sialic acid will form a sialyl (2→n) bond with galactose. The synthesis of all the precursor sugars occurs in the cytosol, including a nine-carbon neuraminic acid and N-acetyl neuraminic acid. Similarly, all nucleotide sugars are formed in the cytosol, except for CMP-sialic acid. The activation of sialic acid to CMP-sialic acid occurs in the nucleus.

The backbone of N-linked glycan is synthesized on the cytosolic side of the ER membrane through the membrane-anchored dolichol. The nucleotide sugars used in the formation of the backbone, GDP-mannose and UDP-N-acetyl glucosamine, are synthesized in the cytosol and directly react with dolichol or with the growing glycan backbone. The

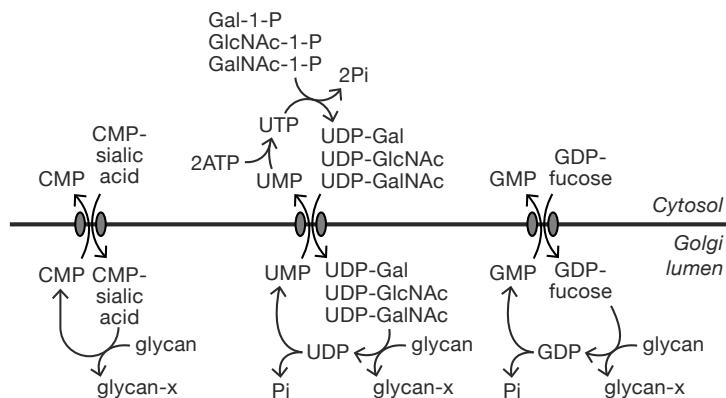


Figure 3.26. Transport of nucleotide-sugar precursors into organelles.

assembled backbone is then “flipped” into the ER and the subsequent reactions occur inside the ER. Transporters are used to supply the nucleotide sugars involved in the subsequent reactions, including GDP-mannose and GDP-glucose in the ER.

The nucleotide sugars for the extension reactions in the Golgi apparatus are also transported through transporters (Figure 3.26). These include CMP-sialic acid, GDP-fucose, UDP-N-acetylglucosamine, UDP-galactose, and the activated sulfate donor (3'-phosphoadenosine, 5'-phosphosulfate). All of these transporters are antiporters, requiring an equal molar exchange of the import of nucleotide sugar and the export of the corresponding NMP (as a product of glycosyltransferase reactions) during the process.

Glycan Diversity among Species

A concern about recombinant protein therapy is the possible elicitation of immunogenicity. An antibody elicited by and against the protein therapeutic can result in neutralization of the therapeutic protein and may result in an unintended drop in efficacy, thus causing serious adverse clinical effects.

The potential immunogenicity of recombinant therapeutics may arise from an aglycosylated protein core or from the glycans associated with it. There are at least two mechanisms by which glycans on a protein may affect the immunogenicity of a human therapeutic: 1) by being a foreign glycan structure, or 2) by shielding a segment of the protein that is otherwise antibody inductive.

Recombinant human therapeutic proteins produced in different organisms or cells are often differently glycosylated (such as those from CHO versus yeast) or aglycosylated (such as from CHO versus *E. coli*) (Panel 3.28).⁸ Comparison of those proteins indicates that the “shielding”

effect of minimizing immunogenicity is affected by the nature of the protein as well as by the source of the protein. The concerns about the immunogenicity of different glycoforms of the rDNA proteins produced in insect cells and in transgenic plants has hindered those technologies' application for rDNA therapeutic protein production. Glycosylated proteins produced in CHO and mouse myeloma cells are minimally immunogenic.

The glycosylation pathway is highly conserved in mammals. Nevertheless, divergence among different species is seen. Furthermore, the expression pattern of glycosylation genes in different tissues or in cells derived from different tissues is often different. Host cells derived from different species or from different tissues may produce recombinant proteins with different glycosylation patterns. For example, the sialic acid in glycans produced in animal cells can be different from that produced in human cells. Human glycans have terminal N-acetylneuraminic acid (NANA). NANA can be further hydrolyzed to N-glycolylneuraminic acid (NGNA) in most other mammals. However, humans have a truncated CMP-sialic acid hydroxylase that renders it unfunctional.⁹ Thus, glycoproteins produced in non-human cells have some NGNA that is not present in human glycoproteins. Similarly, glycoproteins expressed in CHO cells have only terminal α (2,3)-linked sialic acids, in contrast to α (2,6) and α (2,3) seen in humans, due to the near absence of expression of 2,6-sialyltransferase in CHO cells. Such differences in glycan composition have posed a concern; however, immunogenicity of recombinant proteins directly caused by variant glycans is still rare.

In the production of biosimilar biologics, a critical issue is the production of glycoproteins with "the same" glycoform distribution as the original innovative product. In almost all cases, the cell lines as well as the processes used for the production of the biosimilar are different from those employed in the production of the original innovative product. It is therefore a challenge to devise a robust process that can produce protein molecules with the desired or target glycosylation pattern. Many factors affect the glycosylation pattern of the product, including the expression pattern of glycosylation enzymes in the cell line, the nucleotide sugar supply, the cultivation conditions, and the metabolic state of the cells. The glycosylation pattern is also affected by signaling pathways and their modulators. Further complicating the issue is the fact that the glycosylation pathways in the endoplasmic reticulum and the Golgi apparatus are

Panel 3.28. Diversity of Glycosylation in Species

- The makeup of glycosylation enzymes is somewhat different among species, even among different tissues of the same species
- Possible immunogenicity of non-native glycans (e.g., high mannose glycan from most yeast)
- Glycans on CHO-produced proteins
 - Have NGNA, humans have only NANA
 - Have only α (2,3) sialic acid, whereas human proteins have both α (2,3) and α (2,6)

not only present to process product proteins, but also a very large fraction of all cellular proteins. Thus, the effect of modulating the pathway is not limited to the product protein but potentially includes other aspects of cell physiology. Due to the importance of glycoforms in the assessment of the quality of therapeutic proteins from a regulatory perspective, we will likely see growing efforts to better understand and control glycoforms in the near future.

Concluding Remarks

IN THIS CHAPTER, we presented a brief overview of the broad areas of cellular metabolic processes. We covered the core of energy metabolism, the process of glucose utilization through glycolysis, the PPP, and the TCA cycle, and examined how all of these affect cell growth behavior and productivity. Through interconnected pathways, the central corridor of energy metabolism also influences the synthesis and glycosylation of the product proteins. The excessive consumption of glucose and glutamine and the corresponding accumulation of lactate and ammonium in culture contribute to growth inhibition and low productivity. Lactate consumption in the late stage of culture has been positively associated with a high productivity. There are, therefore, ample incentives to better understand cell metabolism and search for new ways of manipulating it that can better redirect the process. In recent years, we have developed a better understanding of the link between glycolytic regulation and growth control. We have also established better tools to probe the relationship between metabolic flux distribution and other aspects of physiology that influence both productivity and product quality. With the benefit of global physiological perspectives, we continue to gain a deeper understanding of metabolism. Global views at a systemic level will significantly enhance our capacity to manipulate cell metabolism and thus increase productivity and product quality.