METABOLISM AND REGULATION | BIOTECHNOLOGY
PROBLEM-BASED LEARNING #1 | PBL1

PBL1.1 Read and discuss (from a critical metabolic point of view) the following text:

> Humans transform energy from food into motion, heat, and thought. Energy is conserved. The energy we expend during a day comes from the food we eat. A typical energy intake from food is 2,500 food calories per day. One food calorie is equal to 4,184 joules of energy. A human consuming 2,500 food calories takes in approximately 10.5 million joules in energy from food a day. This sounds like a lot. However, a day has 86,400 seconds, and therefore the *rate* at which our bodies transform this energy is 10.5 million joules divided by 86,400 seconds, or about 120 watts. Far from illuminating a whole city, a human being has about the same power rating as *one* light bulb.
>
> Peter M. Hoffmann (2012) *Life's ratchet. How molecular machines extract order from chaos*. Basic Books, New York.

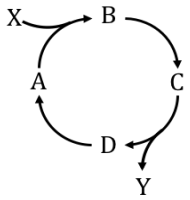
PBL1.2 *Be critical when reading textbooks* Do you agree with the following sentence? *Parallel pathways of catabolism and anabolism must differ in at least one metabolic step in order that they can be regulated independently*. Source: Garrett RH and Grisham CM (2007) *Biochemistry*, Fig. 17.8.

PBL1.3 René Descartes proposed that living beings are, quite literally, machines. The comparison between organisms and machines has often been used along the history of biology. This mechanicism is still present in the era of the computers and comparisons between cells and computers are commonplace. See, for instance, Fig. 1 of Andrianantoandro E et al. (2006) Synthetic biology: new engineering rules for an emerging discipline. *Mol Syst Biol* **2**:2006.0028. Criticize Cartesian mechanicism using the concept of recursivity (or metabolic circularity).

METABOLISM AND REGULATION | BIOTECHNOLOGY
PROBLEM-BASED LEARNING #2 | PBL2

Part A

A.1 | The following diagram shows a simplified metabolic cycle:



(a) Give one example of catabolic cycle (Y = CO_2) and another of anabolic cycle (i.e., biosynthesis of Y from X) that could be represented by that simplified scheme. Consider that arrows might represent more than one chemical reaction. **(b)** In each case, identify the feeder substrate (A) that it is regenerated in each cycle turn. **(c)** Define anaplerotic reaction and represent it in the above scheme. Discuss the experimentally measurable changes that you would expect after the activation of an anaplerotic process. **(d)** Define cataplerotic reaction and apply the concept to intermediate D, modify the diagram accordingly, and write the overall equation of the global transformation.

A.2 | Show which parts (if any) of the citric acid cycle are utilized in each of the following reactions, what (if any) additional enzymes are needed, and write an overall chemical equation for each of the following pathways. **(a)** Catabolism of glutamate to CO_2. **(b)** Biosynthesis of glutamate from pyruvate.

A.3 | The tissues of the mammalian central nervous system contain a pyridoxal phosphate-dependent glutamate decarboxylase that catalyzes conversion of Glu to γ-aminobutyrate (GABA), an inhibitory synaptic transmitter. GABA is degraded by transimination with 2-oxoglutarate as the acceptor to yield succinic semialdehyde, which is then oxidized to succinate by an NAD-linked dehydrogenase. **(a)** Show how these reactions can operate as a shunt pathway that allows the citric acid cycle to function without the enzymes 2-oxoglutarate dehydrogenase and succinyl CoA synthase. **(b)** Is the shunt more or less efficient than the normal cycle from the standpoint of energy recovery? Explain.

A.4 | Assume that you have a reaction medium containing the pyruvate dehydrogenase complex, the respiratory machinery for the oxygen-dependent reoxidation of coenzymes, and all the enzymes of the TCA cycle but none of the metabolic intermediates. **(a)** If you add 3 mM each of pyruvate, coenzyme A, NAD^+, ADP, GDP, and P_i, what will happen? How much CO_2 will be evolved? **(b)** If in addition to the reagents specified in part (a), you add 1 mM of oxaloacetate, how much CO_2 will be evolved? **(c)** How much oxygen will be consumed in the two considered cases?

Part B

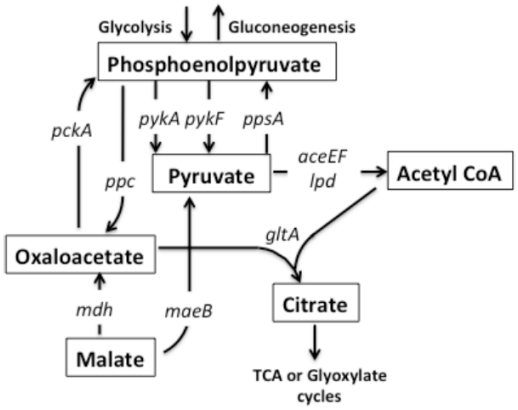
B.1 | In the early 1930s, Albert Szent-Györgyi reported the interesting observation that the addition of small amounts of oxaloacetate or malate to suspensions of minced pigeon breast muscle stimulated the oxygen consumption of the preparation. Surprisingly, the amount of oxygen consumed was about seven times more than the amount necessary for complete oxidation (to CO_2 and H_2O) of the added oxaloacetate or malate. Why did the addition of oxaloacetate or malate stimulate oxygen consumption? Why was the amount of oxygen consumed so much greater than the amount necessary to completely oxidize the added oxaloacetate or malate?

B.2 | The accumulation of trehalose, a disaccharide formed by two glucose molecules, has a demonstrated role in cellular resistance to dehydration. In cultures of the yeast *Saccharomyces cerevisiae* grown in the presence of acetate, it has been found that a defect in the normal operation of

the glyoxylate cycle greatly decreases the desiccation tolerance of yeast. Provide a metabolic explanation to this observation. Hint: glucose can be derived from oxaloacetate via gluconeogenesis.

B.3 | People with beriberi, a disease caused by thiamine deficiency, have elevated levels of blood pyruvate and 2-oxoglutarate, especially after consuming a meal rich in glucose. How are these effects related to thiamine deficiency?

B.4 | The figure shows the reactions in the vicinity of pyruvate in *Escherichia coli* occurring under aerobic conditions. The initials in italics indicate the genes coding for the enzyme activities present.



(a) Give the name of an ATP-producing reaction, an ATP-consuming reaction, one producing NADPH, one producing NADH, one carboxylation, one decarboxylation, and one oxidation. **(b)** What are the names of two enzymes catalyzing the same reaction but encoded by different genes (such as of *pykA* and *pykF*)? **(c)** Why is the production of acetyl-CoA from pyruvate associated with three genes (*aceE*, *aceF* and *lpd*)? **(d)** It has been experimentally verified that the *pckA* gene is not essential for *E. coli* grown both in the presence of glucose or acetate, while the double mutant for *pckA* and *ppsA* grows with glucose, but not in acetate. Explain and complement the diagram with the necessary reactions.

Part C

C.1 | In 1941, radiolabeled compounds were used to corroborate the reactions of the citric acid cycle. When oxaloacetate labeled with ^{14}C in position 4 was used, *all* the label was recovered as CO_2. However, scientists were expecting to recover only half of the initial label. Explain why. Predict the expected results when using [U-^{14}C]-acetate.

C.2 | Write out the equation by which acetyl-CoA and pyruvate can be converted into L-glutamate, which can be observed in living animals by ^{13}C NMR. If [2-^{13}C] sodium acetate was injected into the animal, what labeling pattern could be anticipated in the L-glutamate?

C.3 | Suppose that [2-^{13}C] acetate is added as sole C source to a culture of *Escherichia coli*. In which position(s) will the produced oxaloacetate be labeled?

C.4 | Anticipate the positions that could be labeled in L-glutamate when an actively growing autotrophic bacteria using the Arnon cycle is fed with $^{13}CO_2$.

Part D

D.1 | The detailed biochemistry of the citric acid cycle was determined by several researchers over a period of decades. In a 1937 article, Krebs and Johnson summarized their work and the work of others in the first published description of this pathway.

The methods used by these researchers were very different from those of modern biochemistry. Radioactive tracers were not commonly available until the 1940s, so Krebs and other researchers had to use nontracer techniques to work out the pathway. Using freshly prepared samples of pigeon breast muscle, they determined oxygen consumption by suspending minced muscle in buffer in a sealed flask and measuring the volume (in μL) of oxygen consumed under different conditions. They measured levels of substrates (intermediates) by treating samples with acid to remove contaminating proteins, then assaying the quantities of various small organic molecules. The two key observations that led Krebs and colleagues to propose a citric acid cycle as opposed to a linear pathway (like that of glycolysis) were made in the following experiments.

Experiment I. They incubated 460 mg of minced muscle in 3 mL of buffer at 40 °C for 150 minutes. The addition of citrate increased O_2 consumption by 893 μL compared with samples without added citrate. They calculated, based on the O_2 consumed during respiration of other carbon-containing compounds, that the expected O_2 consumption for complete respiration of this quantity of citrate was only 302 μL.

Experiment II. They measured O_2 consumption by 460 mg of minced muscle in 3 mL of buffer when incubated with citrate and/or with 1-phosphoglycerol (glycerol 1-phosphate; this was known to be readily oxidized by cellular respiration) at 40 °C for 140 minutes. The results are shown in the table.

Sample	Substrate(s) added	μL O_2 absorbed
1	No extra	342
2	0.3 mL 0.2 M 1-phosphoglycerol	757
3	0.15 mL 0.02 M citrate	431
4	0.3 mL 0.2 M 1-phosphoglycerol and 0.15 mL 0.02 M citrate	1 385

(a) Why is O_2 consumption a good measure of cellular respiration?

(b) Why does sample 1 (unsupplemented muscle tissue) consume some oxygen?

(c) Based on the results for samples 2 and 3, can you conclude that 1-phosphoglycerol and citrate serve as substrates for cellular respiration in this system? Explain your reasoning.

(d) Krebs and colleagues used the results from these experiments to argue that citrate was "catalytic" – that it helped the muscle tissue samples metabolize 1-phosphoglycerol more completely. How would you use their data to make this argument?

(e) Krebs and colleagues further argued that citrate was not simply consumed by these reactions, but had to be regenerated. Therefore, the reactions had to be a cycle, rather than a linear pathway. How would you make this argument?

Other researchers had found that arsenate (AsO_4^{3-}) inhibits 2-oxoglutarate dehydrogenase and that malonate inhibits succinate dehydrogenase.

(f) Krebs and coworkers found that muscle tissue samples treated with arsenate and citrate would consume citrate only in the presence of oxygen; and under these conditions, oxygen was consumed. Based on the known TCA cycle pathway, what was the citrate converted to in this experiment, and why did the samples consume oxygen?

In their article, Krebs and Johnson further reported the following. (1) In the presence of arsenate, 5.48 mmol of citrate was converted to 5.07 mmol of 2-oxoglutarate. (2) In the presence of malonate, citrate was quantitatively converted to large amounts of succinate and small amounts of 2-oxoglutarate. (3) The addition of oxaloacetate in the absence of oxygen led to production of a large

amount of citrate; the amount was increased if glucose was also added.

Other researchers had found the following pathway in similar muscle tissue preparations:

$$\text{succinate} \rightarrow \text{fumarate} \rightarrow \text{malate} \rightarrow \text{oxaloacetate} \rightarrow \text{pyruvate}$$

(g) Based only on the data presented in this problem, what is the order of the intermediates in the citric acid cycle? How does this compare with the known pathway? Explain your reasoning.

(h) Why was it important to show the quantitative conversion of citrate to 2-oxoglutarate?

The Krebs and Johnson article also contains other data that filled in most of the missing components of the cycle. The only component left unresolved was the molecule that reacted with oxaloacetate to form citrate.

Reference Krebs, H.A. & Johnson, W.A. (1937) The role of citric acid in intermediate metabolism in animal tissues. *Enzymologia* **4**, 148–156. [Reprinted (1980) in *FEBS Lett.* **117** (Suppl.), K2–K10.]

* * *

RAG.AH/N. 14th June 1937.

The Editor of NATURE presents his compliments to Mr. H. A. Krebs and regrets that as he has already sufficient letters to fill the correspondence columns of NATURE for seven or eight weeks, it is undesirable to accept further letters at the present time on account of the delay which must occur in their publication.

If Mr. Krebs does not mind such delay, the Editor is prepared to keep the letter until the congestion is relieved in the hope of making use of it. He returns it now, however, in case Mr. Krebs prefers to submit it for early publication to another periodical.

Nature rejects Krebs's paper, 1937

What would be the perfect revenge for a scientist whose paper is turned away from *Nature*? A Nobel Prize, of course. Such was the case for Hans Krebs, the biochemist who nabbed the award in 1953 for discovering the citric acid cycle, or "Krebs cycle" – the cellular pathway that converts carbohydrates, fats, and proteins into energy.

Krebs, a German-born Jew trained in medicine and chemistry, was forced out of his position at the University of Freiburg as the Nazis rose to power in the early 1930s. Fleeing the country for England, he joined the faculty of the University of Sheffield in 1935, where he achieved his groundbreaking discoveries.

In March 1937, Krebs and a colleague minced the breast of a freshly killed pigeon in their lab, suspended it in solution, and observed its metabolic rate decline over the next half hour. By adding a salt of citric acid, however, they were able to keep the tissue "alive" for three times as long. Additional experiments revealed the cyclical nature of the pathway, which regenerates citric acid with each cycle and releases ATP – the cell's primary energy currency.

"It is undesirable to accept further letters at the present time."

Krebs submitted his findings to *Nature*, only to receive a note that the journal had a backlog of "letters" and could not publish it without a significant delay. "This was the first time in my career, after having published more than fifty papers, that I experienced a rejection or semi-rejection," Krebs wrote in his memoir. He resubmitted his findings to the journal *Enzymologia* in Holland, where they were published within 2 months.

In 1988, 7 years after Krebs's death, an anonymous editor published a letter in *Nature* calling the rejection the journal's most "egregious error."

Borrell B. Nature rejects Krebs's paper, 1937. *The Scientist*, March 1, 2010.

Follow the link: http://www.the-scientist.com/?articles.view/articleNo/28819/title/Nature-rejects-Krebs-s-paper--1937/

METABOLISM AND REGULATION | BIOTECHNOLOGY
PROBLEM-BASED LEARNING #3 | PBL3

Part A

A.1 | In *Escherichia coli*, extracellular glucose is incorporated by the phosphotransferase system (PTS), which uses phosphoenolpyruvate (PEP) as a phosphorylating agent and produces glucose-6-phosphate directly inside the cell. How does the use of PTS in *E. coli* affect the net ATP performance of glycolysis when the Embden-Meyerhof-Parnas pathway works? What about in the case of the Entner-Doudoroff pathway?

A.2 | Avidine is a protein that binds to biotin with extraordinary affinity. In fact, it is a very specific inhibitor of biotin-dependent carboxylases. Why is it that when avidin is added to an animal cell extract, the conversion of glucose to lactate can be observed, unlike the conversion of lactate to glucose? Reason the answer with the help of a reaction diagram.

A.3 | Pathogens such as *Legionella pneumophila* have to cope with the oxidative stress produced by the defenses of the infected cells. Some authors have linked the pathogen's success with increased enzymatic activities associated to the pentose phosphate and Entner-Doudoroff pathways of the bacterium. What is the metabolic basis for this proposal?

A.4 | A microorganism carrying out alcoholic fermentation via the Embden-Meyerhof-Parnas (EMP) pathway undergoes a mutation that completely inactivates the enzyme triose phosphate isomerase. How will this affect the ATP yield of the pathway under anaerobic conditions? Consider now the effect of the same mutation on the ATP yield of alcoholic fermentation if the microorganism uses the Entner-Doudoroff (ED) pathway.

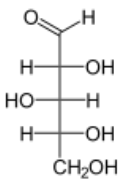
A.5 | The global reaction for alcohol fermentation (from glucose) may be summarized as follows:

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ H^+ \rightarrow 2\ C_2H_6O + 2\ CO_2 + 2\ ATP + 2\ H_2O$$

In an *in vitro* study with all necessary enzymes purified from the yeast *Saccharomyces cerevisiae*, 100 mmol glucose, 200 mmol ADP and 200 mmol of phosphate buffered at pH 7 were introduced into the test tube, but no detectable amount of CO_2 or ethanol was observed. Why? What and how much would you suggest adding to make the system work?

Part B

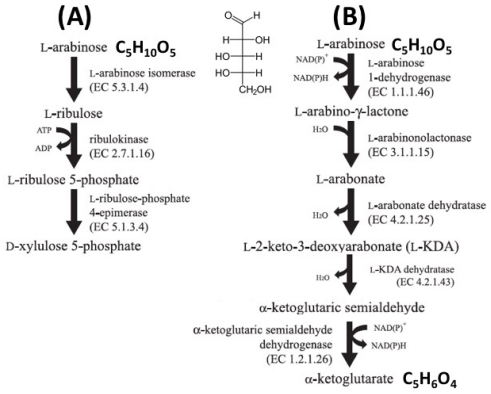
B.1 | The production of ethanol from lignocellulose requires an efficient fermentation of D-xylose.



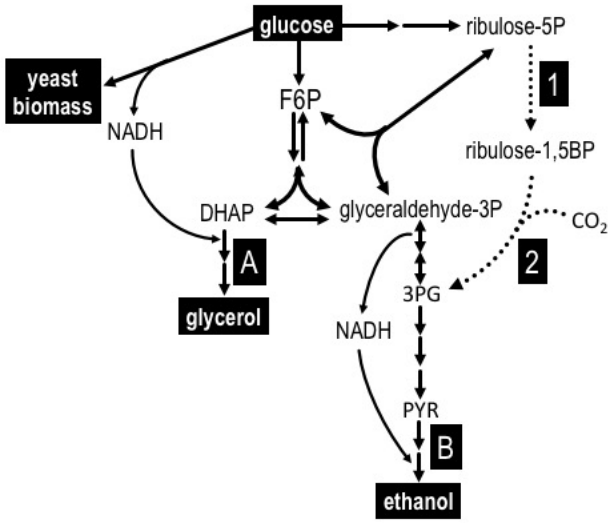
D-Xylose

The yeast *Saccharomyces cerevisiae* has been transformed with genes encoding one of the metabolic pathways of D-xylose ($C_5H_{10}O_5$). The genes coding for the XR-XDH pathway from *Pichia stipites*, encoding D-xylose reductase (XR, an NADPH-dependent enzyme that synthesizes D-xylitol from D-xylose) and D-xylitol dehydrogenase (XDH an NAD^+-dependent enzyme synthesizing D-xylulose from D-xylitol), were introduced in yeast. The endogenous D-xylulose kinase activity (XK) allows the synthesis of D-xylulose-5-phosphate, an intermediate of the pentose phosphate pathway. The resulting transformed yeast produced 0.38 g of ethanol per gram of xylose. Compare this result with the maximum theoretical stoichiometric yield from xylose to ethanol by the XR-XDH route.

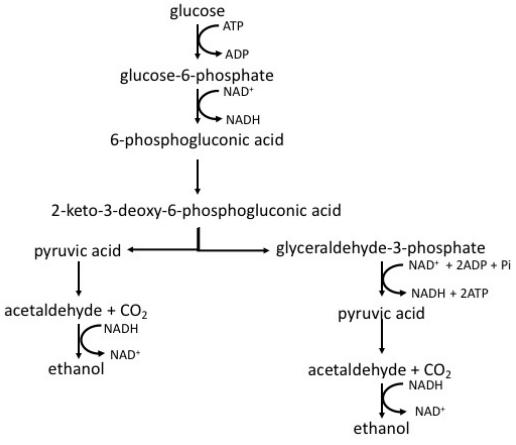
B.2 | The metabolic incorporation of L-arabinose into bacteria can occur in several ways. In many bacterial species, including *Escherichia coli*, the successive action of L-arabinose isomerase, ribulokinase and L-ribulosephosphate-4-epimerase allows the synthesis of D-xylulose-5-phosphate,



an intermediate of the pentose phosphate pathway (Figure A). However, in *Azospirillum brasiliense*, an alternative route has been described that is an analogue of the Entner-Doudoroff pathway with a different end product: instead of splitting the molecule with aldolase, the oxidation continues until alpha-ketoglutarate (Figure B). **(a)** If arabinose is totally catabolized to CO_2, calculate how many ATPs could be obtained and how much oxygen would be consumed in the case of both route A and route B. Hint: count reduced respiratory coenzymes! **(b)** In case B, if we use L-arabinose labeled with ^{13}C in position 1 under gluconeogenic conditions, we do not find any isotopic mark on glucose, why? **(c)** Which of the two systems is stoichiometrically more efficient for making glucose from arabinose?



B.3 | The performance of industrial ethanol production under anaerobic conditions is affected by the parallel production of glycerol. In anaerobic cultures of *Saccharomyces cerevisiae*, between 4 and 10% of the sugar consumed is lost as glycerol. This process (indicated in the scheme as A) is due to the need to adjust the NADH balance produced by certain biosynthetic reactions (biomass production). Several strategies have been tested to oxidize NADH with alternative processes to glycerol synthesis. Thus, Guadalupe-Medina and collaborators introduced enzymes of the Calvin cycle (indicated as 1 and 2 and discontinuous arrows) so that the reduction of CO_2 to ethanol would consume electrons generated in the production of biomass. The transformed yeasts synthesized much less glycerol and increased ethanol production. **(a)** Name the enzymes listed as 1 and 2. **(b)** What types of reactions occur in processes A and B? **(c)** Explain the metabolic logic behind the introduction of enzymes 1 and 2 to decrease glycerol synthesis. **(d)** How many moles of NADH are needed for the production of one mole of ethanol (C_2H_6O) from CO_2? **(e)** The transformed strain produced 0.44 g of ethanol per g of glucose consumed. What percentage of the theoretical maximum yield does this result represent? Data: molecular mass of glucose: 180 Da, molecular mass of ethanol: 46 Da. **Reference**: Guadalupe-Medina V et al. (2013) Carbon dioxide fixation by Calvin-cycle enzymes improves ethanol yield in yeast. *Biotechnology for biofuels* **6**:125.



B.4 | *Zymomonas mobilis* lives on the surface of plants such as Mexican agave (maguey). When the agave is ground, *Zymomonas* ferments plant sugars to alcohol via the Entner-Doudoroff route (see figure) and the "pulque" is obtained, from which tequila can be distilled. **(a)** Does the metabolic process fulfill the biochemical definition of "fermentation"? Why? **(b)** Name a coenzyme that acts catalytically in the process. **(c)** Write the balanced global equation of the glucose-to-ethanol transformation process by *Zymomonas*. **(d)** 100 mmol glucose, 50 mmol NAD^+, 50 mmol ATP, 50 mmol ADP and 100 mmol phosphate are mixed in a

test tube in the presence of all the purified *Zymomonas* pathway enzymes. How much ethanol and CO_2 will be obtained?
Note: *Z. mobilis* glucose-6-phosphate dehydrogenase can use NAD^+ as cofactor.

Part C

C.1 | A method to measure the proportion of metabolic flux for glucose consumption via glycolysis or the pentose phosphate pathway is feeding a cell culture with ^{13}C labeled glucose in position 1 and then measure the enrichment patterns in pyruvate. Reason the logic of this procedure and discuss whether it could also be used to differentiate between the Embden-Meyerhof-Parnas (EMP) and the Entner-Doudoroff (ED) pathways.

C.2 | A synthetic biology project from Victor de Lorenzo's laboratory at the National Centre for Biotechnology (CNB, CSIC) has succeeded in replacing the Entner-Doudoroff (ED) pathway of *Pseudomonas putida* by the Embden-Meyerhof-Parnas (EMP) pathway, using the so-called Glucobrick I (GBI), a plasmid that encodes the enzymes of the first part of the EMP pathway (transformation of hexose into triose phosphate). Wild type strains of *P. putida* use a combination of ED glycolysis with the pentose phosphate (PPP) pathway, in which both the oxidative and non-oxidative branches are operational. In an experiment to test the success of metabolic substitution in a *P. putida* strain with the inactivated Ed pathway and transformed with with GBI, the researchers used glucose marked with ^{13}C in position 1 and analyzed the isotopic label recovered in the produced pyruvate. Predict the result (both the isotope fraction recovered and its position within the pyruvate) in the wild-type bacteria (in a situation where the metabolic flow was 80% through the ED pathway and 20% through the PPP) and in the bacteria modified with the GBI construction, assuming that the EMP pathway is operational and captures 100% of the glucose flow. **Reference**: Sánchez-Pascuala A et al. (2019) Functional implementation of a linear glycolysis for sugar catabolism in *Pseudomonas putida*. *Metabolic Engineering* **54**:200–211.

C.3 | If [1-^{14}C]-glucose is added to a yeast culture active in alcoholic fermentation, where would you expect the isotopic mark to be? **(a)** On the C-1 of ethanol and CO_2. **(b)** Only on the C-1 of ethanol. **(c)** Only on C-2 ethanol. **(d)** On the C-2 of ethanol and CO_2. **(e)** In CO_2 only. Choose the correct sentence and justify the answer with a diagram of reactions.

C.4 | In the first bypass step of gluconeogenesis, the conversion of pyruvate to phosphoenol-pyruvate (PEP), pyruvate is carboxylated by pyruvate carboxylase to oxaloacetate, which is subsequently decarboxylated to PEP by PEP carboxykinase. Because the addition of CO_2 is directly followed by the loss of CO_2, you might expect that in tracer experiments, the ^{14}C of $^{14}CO_2$ would not be incorporated into PEP, glucose, or any gluconeogenesis intermediates . However, investigators find that when a rat liver preparation synthesizes glucose in the presence of $^{14}CO_2$, ^{14}C slowly appears in PEP and eventually at C-3 and C-4 of glucose. How does the ^{14}C label get into the PEP and glucose?

Part D

D.1 | Fermentation of plant matter to produce ethanol for fuel is one potential method for reducing the use of fossil fuels. Many microorganisms can break down cellulose and then ferment the glucose to ethanol. However, many potential cellulose sources, including agricultural residues and switchgrass, also contain substantial amounts of arabinose, which is not as easily fermented.

Escherichia coli is capable of fermenting arabinose to ethanol, but it is not naturally tolerant of high ethanol levels, thus limiting its utility for commercial ethanol production. Another bacterium, *Zymomonas mobilis*, is naturally tolerant of high levels of ethanol but cannot ferment arabinose. Deanda et al. (1996) described their efforts to combine the most useful features of these two organisms by introducing the *E. coli* genes for the arabinose-metabolizing enzymes into *Z. mobilis*.

(a) Why is this a simpler strategy than the reverse: engineering *E. coli* to be more ethanol-tolerant?

Deanda and colleagues inserted five *E. coli* genes into the *Z. mobilis* genome: *araA*, coding for L-arabinose isomerase, which interconverts L-arabinose and L-ribulose; *araB*, L-ribulokinase, which uses ATP to phosphorylate L-ribulose at C-5; *araD*, L-ribulose 5-phosphate 4-epimerase, which interconverts L-ribulose 5-phosphate and D-xylulose 5-phosphate; *talB*, transaldolase; and *tktA*, transketolase.

(b) For each of the three *ara* enzymes, briefly describe the chemical transformation it catalyzes.

The five *E. coli* genes inserted in *Z. mobilis* allowed the entry of arabinose into the nonoxidative phase of the pentose phosphate pathway, where it was converted to glucose 6-phosphate and fermented to ethanol.

(c) The three *ara* enzymes eventually converted arabinose into which sugar?

(d) The product from part (c) feeds into the pentose phosphate pathway. Combining the five *E. coli* enzymes listed above with the enzymes of this pathway, describe the overall pathway for the fermentation of arabinose to ethanol, assuming that *Z. mobilis* follows the ED pathway.

(e) What is the stoichiometry of the fermentation of arabinose to ethanol and CO_2? How many ATP molecules would you expect this reaction to generate? Calculate the theoretical ethanol yield (i.e., g of ethanol per g of arabinose) based on this stoichiometry.

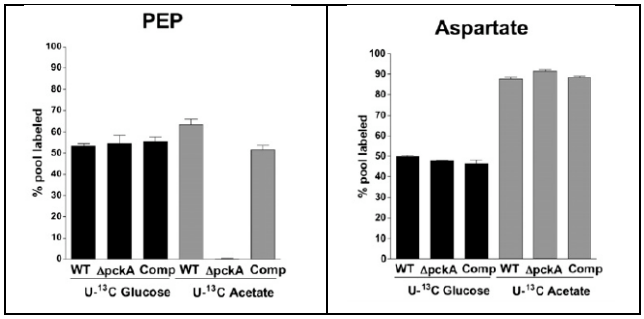
Another sugar commonly found in plant matter is D-xylose.

(f) What additional enzymes would you need to introduce into the modified *Z. mobilis* strain described above to enable it to use xylose as well as arabinose to produce ethanol? You don't need to name the enzymes (they may not even exist in the real world!); just provide the reactions they would need to catalyze.

Reference Deanda, K., Zhang, M., Eddy, C., & Picataggio, S. (1996) Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. *Appl. Environ. Microbiol.* **62**:4465–4470.

D.2 | *Mycobacterium tuberculosis*, the causal agent of tuberculosis, can survive for long periods of time in lung granulomas (lipid rich nodules formed by groups of macrophages). It has been shown that the infection success depends on the metabolic use of fatty acids and of an active gluconeogenic flux. In experiments with infected mice, it was shown that isocitrate lyase (ICL) [1] as well as phosphoenolpyruvate carboxykinase (PEPCK) [2] are essential activities for bacterial survival and virulence . For the study of the role of PEPCK in the gluconeogenesis from fatty acids, researchers used ^{13}C-labeled glucose or acetate and analyzed several metabolites by HPLC and MS. The following figures show the recovered label in PEP and Asp in wild-type bacteria (WT), PEPCK mutants (Δ*pck*A) and complemented mutants with a copy of the PEPCK gene (Comp). (a) Explain the need of ICL and

PEPCK for bacterial survival in the presence of fatty acids. (b) Explain the results of isotopic label with a diagram of the metabolic processes involved.



References [1] McKinney et al. (2000) *Nature* **406**:735. [2] Marrero et al. (2010) *PNAS USA* **107**:9819.

METABOLISM AND REGULATION | BIOTECHNOLOGY
PROBLEM-BASED LEARNING #4 | PBL4

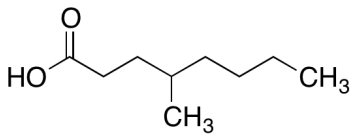
Part A

A.1 | The reaction for the activation of fatty acids before degradation (i.e., the generation of acyl CoA from free fatty acid) involves the pyrophosphorolytic consumption of ATP. Explain why, from a biochemical bookkeeping point of view, the equivalent of two molecules of ATP is used despite the fact that the left side of the adjusted reaction has only one molecule of ATP.

A.2 | Compare and contrast fatty acid oxidation and synthesis with respect to **(a)** site of the process; **(b)** acyl carrier; **(c)** reductants and oxidants; **(d)** stereochemistry of the intermediates; **(e)** direction of synthesis or degradation; **(f)** organization of the enzyme system.

A.3 | Avidin, a glycoprotein found in eggs, has a high affinity for biotin. Avidin can bind biotin and prevent its use by the body. How might a diet rich in raw eggs affect fatty acid synthesis? What will be the effect on fatty acid synthesis of a diet rich in cooked eggs?

A.4 | What are statins? What is their pharmacological function? Would the development of a "super statin" that inhibited all HMG-CoA reductase activity result in a useful drug? Explain.



A.5 | Hyrcinoic acid (4-methyl-octanoic) is a volatile acid present in goat and lamb fat and contributes to the characteristic flavors of sheep meat and goat cheese. Explain whether this acid can be completely degraded by β-oxidation and, if so, name the products that would be obtained in each oxidation cycle.

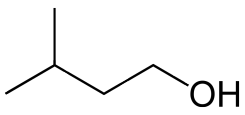
Part B

B.1 | Compare the ATP yield from the complete oxidation of glucose and hexanoic acid (also known as caproic acid, responsible for the "aroma" of goats). Why are fats better fuels than carbohydrates?

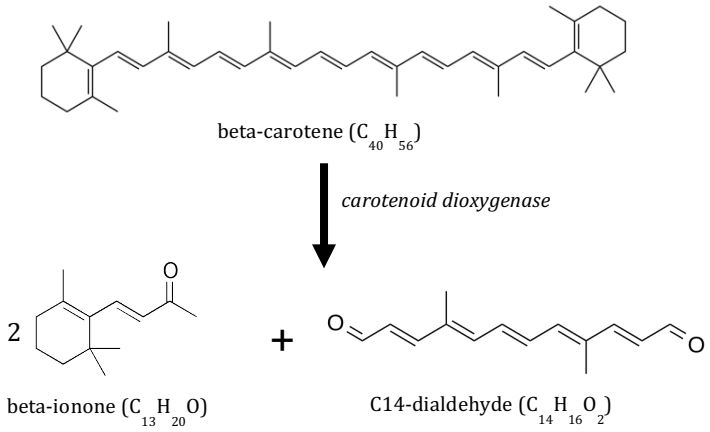
B.2 | How much ATP can be obtained from the complete oxidation of phytanic acid?

B.3 | Butyric acid ($C_4H_8O_2$) is one of the main products of intestinal fermentation in humans. The cells of the intestinal mucosa (colonocytes) are adapted to use this metabolite as the main energy source in aerobic metabolism. **(a)** Calculate the maximum production yield of butyrate by intestinal bacteria fermenting glucose (in terms of grams of butyrate produced per gram of glucose). **(b)** How many moles of ATP could the complete oxidation of a mole of butyrate by colonocytes yield? Use 2.5 and 1.5 ATPs per NADH and $FADH_2$ oxidized, respectively, as energy equivalent. **(c)** How much oxygen would be consumed during the complete catabolism of a mole of butyrate?

B.4 | Imagine that you work for a biotechnology company in the field of biofuels and that one of the products of interest as a gasoline additive is isopentanol ($C_5H_{12}O$, 3-methyl-1-butanol, see figure). You have two species of microorganisms: a fungus that synthesizes terpenes via the mevalonic acid (MVA) pathway and a bacterium that uses the deoxy-xylulose-5-phosphate (DXP) pathway. The company has access to cellulosic wastes as a carbon source for growth of microorganisms, and the CEO wants to know which microorganism is the best for isopentanol synthesis. What would you recommend? Clearly indicate the metabolic basis of your choice. Calculate the maximum theoretical stoichiometric yield of obtaining isopentanol from glucose ($C_6H_{12}O_6$) (expressed in grams of product per gram of consumed substrate) in the pathway that you have chosen as the best one.



B.5 | Ionones are a family of chemical compounds derived from carotenoids. They are aromatic molecules present in essential oils of plants, such as rose or violet. In a recent work, a strain of the oleaginous yeast *Yarrowia lipolytica* that produces beta-ionone ($C_{13}H_{20}O$) has been obtained from beta-carotene (figure). **(a)** If the only carbon source of this yeast is glycerol ($C_3H_8O_3$), what would be the theoretical maximum yield of beta-ionone production (i.e., g beta-ionone/g glycerol) if carotenoid synthesis operates via mevalonate? **(b)** The enzyme repertoire of this yeast includes a generic aldehyde dehydrogenase activity which can transform the C14-dialdehyde by-product into the corresponding dicarboxylic acid. Assuming that double carboxyl is not an impediment, could this acid be a substrate for beta-oxidation? If so, what products would be obtained?



Part C

C.1 | In vitro studies of fatty acid biosynthesis show that bicarbonate is an essential component of the reaction mixture. However, when [^{14}C]-HCO_3^- is used, there is no measurable label in the recently synthesized fatty acid. Why? On the other hand, adding [U-^{14}C]-acetyl-CoA to the soluble fraction of a rat liver extract, uniformly labeled palmitic acid is obtained. The addition of trace amounts of [U-^{14}C]-acetyl-CoA in the presence of an excess of unlabeled malonyl-CoA, produces palmitic acid labeled only in positions 15 and 16. Explain these experimental observations.

C.2 | Determine the ratio ^{3}H/^{14}C in the palmitate synthesized in an in vitro system in the presence of methyl-labeled acetyl-CoA with a ratio of 3, i.e., in this way $(^3H)_3{}^{14}C$–.

C.3 | What is the distribution of isotopic labeling in cholesterol synthesized from each of the following precursors? **(a)** Mevalonate labeled with ^{14}C in its carboxyl carbon atom; **(b)** malonyl CoA labeled with ^{14}C in its carboxyl carbon atom.

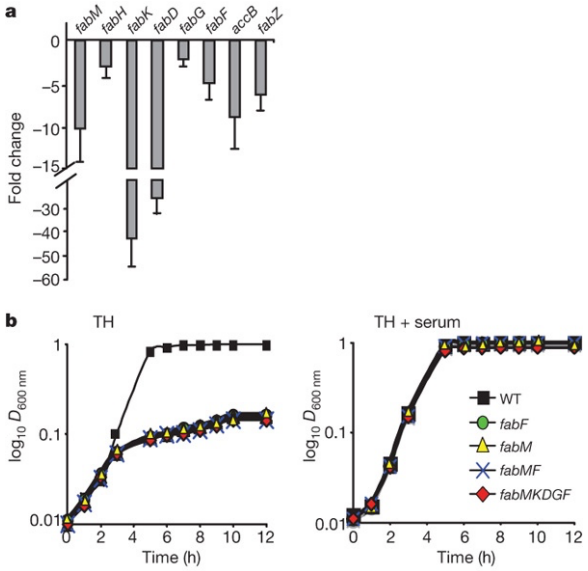
Part D

D.1 | Antimicrobial drugs, like cerulenin and triclosan, targeting the reportedly essential type II fatty acid FA synthesis (FASII), have been acclaimed for their efficacy against infections caused by multiresistant bacteria. However, Brinster et al. (2009) showed that the strategy for antibiotic development based on FASII pathway targets is fundamentally flawed because exogenous FA fully bypass inhibition of this pathway in both in vitro and in vivo conditions. **(a)** What is the molecular target inside FASII of cerulenin and triclosan?

Clinical isolates of Gram-positive pathogens were tested for sensitivity to cerulenin and triclosan. In standard medium lacking FA, all bacterial strains tested were sensitive to both drugs. However, inhibitor sensitivity was alleviated in all cases by supplying FA, either dispersed in Tween 80 or via human serum (data not shown). The predominant fatty acids in human serum, comprising ~80% of the total FA, are the unsaturated linoleic (C18:2 n-6) and oleic (C18:1 n-9) acids, and the saturated palmitic (C16:0) and stearic acids (C18:0). They were individually tested, dispersed in the detergent Tween 80, for their capacity to overcome cerulenin inhibition of *Streptococcus agalactiae* strain NEM316, which was used as a model (Table).

	OD_{600}[a]	
	NEM316	NEM316 + cerulenin [b]
Fatty acid addition[c]		
None	1.15 (± 0.03)	0.34 (± 0.03)
Tween 80	1.26 (± 0.02)	1.05 (± 0.03)
SFA		
C16:0	0.91 (± 0.08)	0.27 (± 0.01)
C18:0	1.14 (± 0.04)	0.31 (± 0.04)
UFA		
C18:1n-9	0.81 (± 0.02)	0.79 (± 0.02)
C18:2n-6	0.93 (± 0.05)	0.92 (± 0.05)

[a] OD_{600} were determined in 18 h TH broth cultures at 37°C. [b] Cerulenin was added at a final concentration of 4 μg ml^{-1}. [c] 0.1 % Tween 80, or 0.1 % fatty acids were used as indicated, except for C16:0, which was added at 0.01% v/v. Values are the mean of 3 independent experiments, each of which was performed in duplicate. SFA, saturated fatty acids; UFA, unsaturated fatty acids.



(b) What is the effect of added (saturated or unsaturated) FA on *S. agalactiae* growth in the presence of cerulenin?

To further study the effect of the serum, the authors performed quantitative polymerase chain reaction with reverse transcription (RT–PCR) to determine its effects on expression of the *fab* genes in *S. agalactiae* (Fig. a). **(c)** What is the effect of serum on the expression of the *fab* genes?

The challenge test for the FASII requirement is to remove FASII genes from the bacterial genome. They constructed *S. agalactiae* strains with deletions in *fabF*, *fabM*, and *fabMF*. Remarkably, a quintuple mutant defective in *fabMKDGF* could also be established. Growth of all these mutants was tested in a standard Todd Hewitt (TH) medium with or without human serum (Fig. b). The same effect on mutant growth was observed with added oleic or linoleic acids (not shown). **(d)** Why did the authors choose the genes *fabF* and *fabM* for deletion? What is the main conclusion of this experiment?

Reference Brinster S et al. (2009) Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. *Nature* **458**:83–86.

METABOLISM AND REGULATION | BIOTECHNOLOGY
PROBLEM BASED-LEARNING #5 | PBL5

Part A

A.1 | (a) Why has AMP-dependent protein kinase (AMPK) been called "cellular fuel gauge"? Predict the activation or inhibition effect of AMPK on the following enzymatic targets: acetyl-CoA carboxylase, phosphofructokinase-2, glycogen synthase. **(b)** Caffeine inhibits cAMP phosphodiesterase. Discuss the effect of caffeine on glycogen biosynthesis and triacylglycerol mobilization. **(c)** Find the logic behind the allosteric inhibition of carnitine-acyl transferase I (cytosolic) by malonyl-CoA.

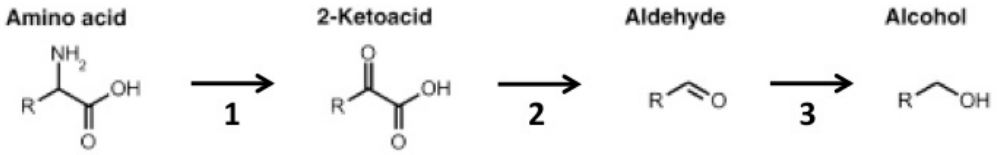
A.2 | Predict the major consequence of each of the following mutations: **(a)** loss of the AMP-binding site in muscle glycogen phosphorylase; **(b)** mutation of Ser 14 to Ala 14 in liver glycogen phosphorylase; **(c)** overexpression of phosphorylase kinase in hepatocytes.

A.3 | (a) Why should phenylketonurics avoid the ingestion of aspartame, an artificial sweetener? **(b)** Pyruvate carboxylase deficiency is a fatal disorder. Patients with this enzyme deficiency sometimes display some or all of the following symptoms: lactic acidosis, blood hyperammonemia, hypoglycemia, and demyelination of regions of the brain due to insufficient lipid synthesis. Provide a possible biochemical rationale for each of these observations.

A.4 | Which of the following compounds, if added to an active tissue preparation, might be expected to yield the greatest increase in urea production in terms of moles of urea produced per mole of added compound? **(a)** ammonia, **(b)** bicarbonate, **(c)** aspartate, **(d)** ornithine. Explain your decision.

A.5 | Some *Neurospora crassa* mutants defective in carbamoyl phosphate synthase I (CPS I) are Arg auxotrophs, whereas mutants of carbamoyl synthase II (CPS II) are uracil auxotrophs. At first sight, an active CPS II in the CPS I mutants could be expected to compensate their deficiency, and the reverse would be true in the CPS II mutants. Explain why this is not the case.

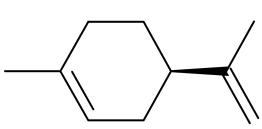
A.6 | (a) Compare the chemical mechanisms (and cofactors if needed) of enzyme catalyzed decarboxylation of the following: (i) a β-oxo-acid such as acetoacetate, (ii) an α-oxo-acid such as pyruvate, (iii) an amino acid such as L-glutamate. **(b)** The following figure represents the Ehrlich pathway for superior alcohols synthesis from amino acids in yeast:



For each of the reactions indicate: **(a)** class of reaction; **(b)** possible cofactor or coenzyme involved.

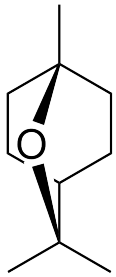
Part B

B.1 | An acellular system for the synthesis of monoterpenes from glucose ($C_6H_{12}O_6$) has been proposed. This synthetic biochemistry approach allows to increase production yields and avoids some problems such as the cellular toxicity of the products. One of the monoterpenes synthesized was limonene ($C_{10}H_{16}$), a substance that can have applications as biofuel and that until now had been achieved in cell fermentations with very low stoichiometric yields. In the acellular design, the researchers couple a conventional glycolytic route (EMP) with the mevalonate route (MV),



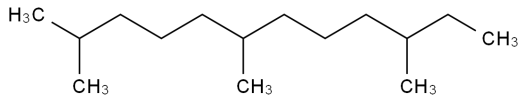
achieving, after 7 days of reaction, 0.22 g of limonene per g of glucose consumed. **(a)** What is the stoichiometric yield achieved expressed as a % of the maximum possible yield? **(b)** Now imagine that you are commissioned to redesign the system to increase theoretical stoichiometric yields (hint: reducing C losses in the form of CO_2). Propose modifications to the original metabolic design (EMP + MV) to achieve this goal and calculate the corresponding maximum yield.

B.2 | Eucalyptol (1,8-cineole, $C_{10}H_{18}O$, molecular mass 154 Da) is a candidate biofuel monoterpene.



The synthesis of eucalyptol has been studied in a yeast that efficiently metabolizes succinate (C_4H_6O, molecular mass 118 Da) and has a very active mevalonate pathway. **(a)** Point out on the formula the isoprene units that make up eucalyptol. **(b)** Calculate the theoretical maximum yield of eucalyptol biosynthesis from succinate, expressed as g of eucalyptol per g of succinate. **(c)** Calculate the yield if we were to install a reverse glyoxylate cycle for succinate catabolism in this yeast. **(d)** What is the metabolic explanation for the difference between the two yields?

B.3 | The Brazilian airline Gol has started tests with conventional fuel with 10% of farnesane, a sesquiterpene ($C_{15}H_{32}$, see figure). Amyris, a leading biotechnology company in biofuel research, is trying to synthesize farnesane from cane sugar (sucrose, a disaccharide $C_{12}H_{22}O_{11}$). Suppose you have a system based on a modified bacterium using the mevalonate pathway for isoprenoid synthesis. What is the maximum theoretical yield expected during farnesane production (expressed as grams of product per gram of sucrose)? Calculate the yield if the bacteria were also modified with the synthetic metabolic pathway called non-oxidative glycolysis (NOG). What is the metabolic explanation for the observed difference between the two yields?



Part C

C.1 | Cells were incubated with glucose labeled with ^{14}C in carbon 2. Later, uracil was isolated and found to contain ^{14}C in carbons 4 and 6. Account for this labeling pattern.

C.2 | Find the positions of GTP and ATP that will be labeled when using [^{15}N]-Asp during the de novo synthesis of purines. And what if we use [^{15}N]-labeled in the amide group of Gln?

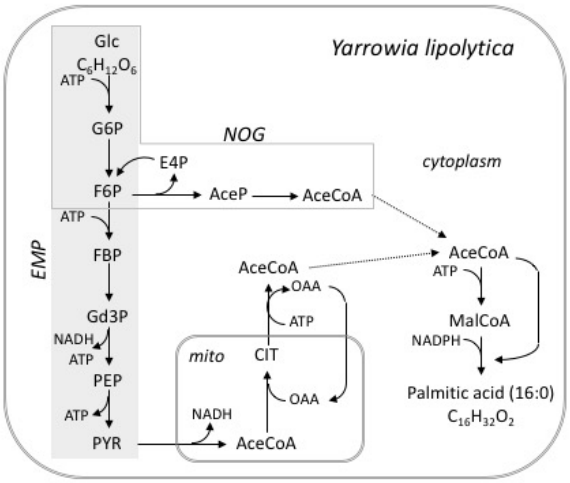
C.3 | We add [U-^{14}C]-succinate to cells active in pyrimidine biosynthesis. How can the succinate carbon atoms be incorporated into pyrimidines and in which positions?

C.4 | Formaldehyde reacts non-enzymatically with tetrahydrofolate yielding 5,10-methylentetrahydrofolate. How would you use this reaction with [^{13}C]-formaldehyde to label serine? Adding your [^{13}C]-serine to cells, you find label in proteins, glycogen, membrane lipids and nucleic acids. Explain.

Part D

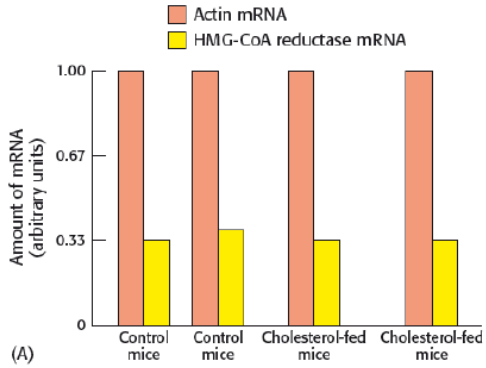
D.1 | In a study on lipid production in the fungus *Yarrowia lipolytica*, different metabolic engineering modifications have been made to increase the production of fatty acids using glucose as feedstock. The following diagram is a summary of the metabolic processes studied (the main transformations are presented, without specifying the stoichiometries). **(a)** One of the strategies to improve palmitate synthesis (16:0) was to increase the availability of reducing power in the cytoplasm. The *Y. lipolytica* genome contains a unique gene coding for an NAD^+-dependent malic enzyme located in the

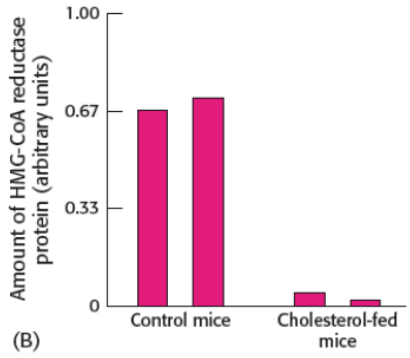
mitochondrial matrix, the activity of which has no significant effect on lipid synthesis. Explain why this enzyme does not affect the synthesis of fatty acids and propose a possible metabolic modification to increase the synthesis of NADPH in the cytoplasm. **(b)** If glucose is degraded by the EMP pathway (marked with gray background), calculate the final balance of ATP for the synthesis of one mole of palmitate. **(c)** The authors modified a strain of *Y. lipolytica* with the necessary enzymes so that the artificial NOG pathway (marked in box) was operative. Calculate how much palmitate (in g per g of glucose) would be obtained if all of the glucose is degraded by the NOG pathway and compare the result with the natural pathway performance (i.e., if all glucose was degraded through the EMP pathway).



Abbreviations Glc (glucose), G6P (glucose-6-phosphate), F6P (fructose-6-phosphate), FBP (fructose-1,6-bisphosphate), Gd3P (glyceraldehyde-3-phosphate), PEP (phosphoenolpyruvate), PYR (pyruvate), AceCoA (acetyl CoA), CIT (citrate), OAA (oxaloacetate), E4P (erithrose-4-phosphate), AceP (acetylphosphate), MalCoA (malonyl CoA), EMP (Embden-Meyerhof-Parnas pathway), NOG (non-oxidative glycolysis pathway).

D.2 | Mice were divided into four groups, two of which were fed a normal diet and two of which were fed a cholesterol-rich diet. HMG-CoA reductase mRNA and protein from their livers were then isolated and quantified. Graph A shows the results of the mRNA isolation. **(a)** Define the concept of *biological replicate* (or why control and treated mice are separated in two groups) versus *technical replicate* (no information available in this case). **(b)** What is the effect of cholesterol feeding on the amount of HMG-CoA reductase mRNA? **(c)** What is the purpose of also isolating the mRNA for the protein actin, which is not under the control of the sterol response element?





The HMG-CoA reductase protein was isolated by precipitation with a monoclonal antibody to HMG-CoA reductase. The amount of HMG-CoA protein in each group is shown in graph B. **(d)** What is the effect of the cholesterol diet on the amount of HMG-CoA reductase protein? **(e)** Why is this result surprising in light of the results in graph A? **(f)** Suggest possible explanations for the results shown in graph B and the molecular mechanisms involved.

D.3 | A metabolic engineering project seeks the possibility of producing isobutanol (Isobut) from glucose (Glc) in the bacterium *Escherichia coli*. A stoichiometric analysis was performed with the metabolic network with the additional reactions for Isobut synthesis under anaerobic conditions (see figure). The table shows only the elementary modes (EM) that produce Isobut. **(a)** Define the concept EM. **(b)** Calculate the theoretical maximum yield of Isobut production in *E. coli*. **(c)** Is it possible that the production of Isobut as a unique fermentation product under anaerobic growth conditions? Why? **(d)** Under anaerobic growth conditions, calculate the maximum yield of Isobut production.

Table **EM producing Isobut from Glc under anaerobic conditions**

EM	Reactions
1	1 Glc = 1 Isobut + 2 CO_2
2	1 Glc = 1 Isobut + 2 CO_2 + 2 ATP
3	50811 Glc + 25004 NH_3 = 2 Biomass + 242 Ethanol + 66240 CO_2 + 5508 Formate + 35475 Isobut
4	50811 Glc + 25004 NH_3 = 2 Biomass + 242 Ethanol + 71748 CO_2 + 5508 H_2 + 35475 Isobut

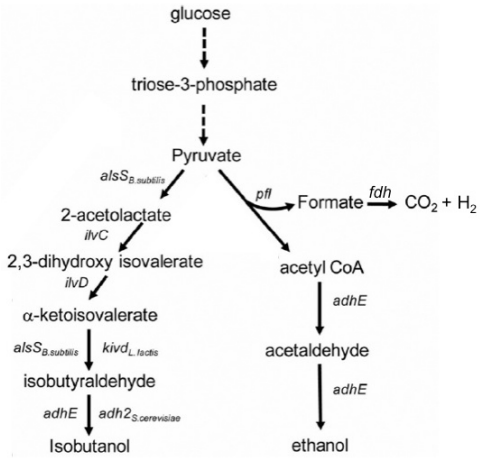


Figure **Detail of the anaerobic metabolism producing Isobut in modified cells of *E. coli*.** The enzymatic steps are named after its coding gene symbols: *pfl*, pyruvate-formate lyase; *fdh*, formate dehydrogenase; $alsS_{B.subtilis}$, acetolactate synthase from *Bacillus subtilis*; *ilvC*, 2,3-dihydroxyisovalerate oxidoreductase; *ilvD*, 2,3-hydroxy isovalerate dehydratase; $kivd_{L.lactis}$, α-ketoacid decarboxylase from *Lactobacillus lactis*; $adh2_{S.cerevisiae}$, alcohol/aldehide dehydrogenase from *Saccharomyces cerevisiae*; *adhE*, alcohol dehydrogenase from *E. coli*.