

## chapter

## 16

## The Citric Acid Cycle

- 1. Balance Sheet for the Citric Acid Cycle** The citric acid cycle has eight enzymes: citrate synthase, aconitase, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase.
- Write a balanced equation for the reaction catalyzed by each enzyme.
  - Name the cofactor(s) required by each enzyme reaction.
  - For each enzyme determine which of the following describes the type of reaction(s) catalyzed: condensation (carbon-carbon bond formation); dehydration (loss of water); hydration (addition of water); decarboxylation (loss of  $\text{CO}_2$ ); oxidation-reduction; substrate-level phosphorylation; isomerization.
  - Write a balanced net equation for the catabolism of acetyl-CoA to  $\text{CO}_2$ .

**Answer****Citrate synthase**

- $\text{Acetyl-CoA} + \text{oxaloacetate} + \text{H}_2\text{O} \longrightarrow \text{citrate} + \text{CoA}$
- CoA
- Condensation

**Aconitase**

- $\text{Citrate} \longrightarrow \text{isocitrate}$
- No cofactors
- Isomerization

**Isocitrate dehydrogenase**

- $\text{Isocitrate} + \text{NAD}^+ \longrightarrow \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADH}$
- $\text{NAD}^+$
- Oxidative decarboxylation

 **$\alpha$ -Ketoglutarate dehydrogenase**

- $\alpha\text{-Ketoglutarate} + \text{NAD}^+ + \text{CoA} \longrightarrow \text{succinyl-CoA} + \text{CO}_2 + \text{NADH}$
- $\text{NAD}^+$ , CoA, thiamine pyrophosphate
- Oxidative decarboxylation

**Succinyl-CoA synthetase**

- $\text{Succinyl-CoA} + \text{P}_i + \text{GDP} \longrightarrow \text{succinate} + \text{CoA} + \text{GTP}$
- CoA
- Substrate-level phosphorylation and acyl transfer

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**Succinate dehydrogenase**

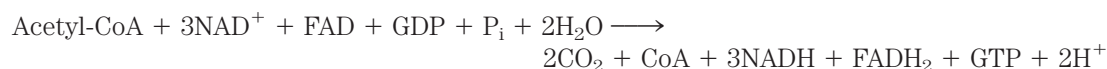
- (a) Succinate + FAD  $\longrightarrow$  fumarate + FADH<sub>2</sub>  
 (b) FAD  
 (c) Oxidation

**Fumarase**

- (a) Fumarate + H<sub>2</sub>O  $\longrightarrow$  malate  
 (b) No cofactors  
 (c) Hydration

**Malate dehydrogenase**

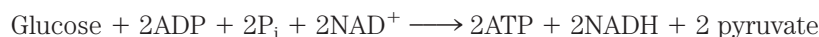
- (a) Malate + NAD<sup>+</sup>  $\longrightarrow$  oxaloacetate + NADH + H<sup>+</sup>  
 (b) NAD<sup>+</sup>  
 (c) Oxidation  
 (d) The net equation for the catabolism of acetyl-CoA is



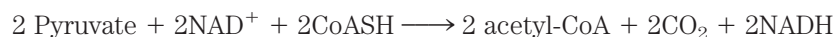
- 2. Net Equation for Glycolysis and the Citric Acid Cycle** Write the net biochemical equation for the metabolism of a molecule of glucose by glycolysis and the citric acid cycle, including all cofactors.

**Answer**

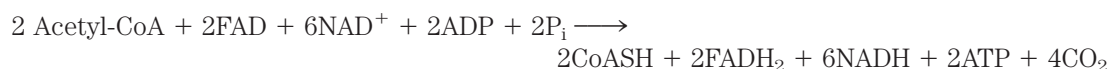
*Glycolysis:*



*Pyruvate dehydrogenase:*



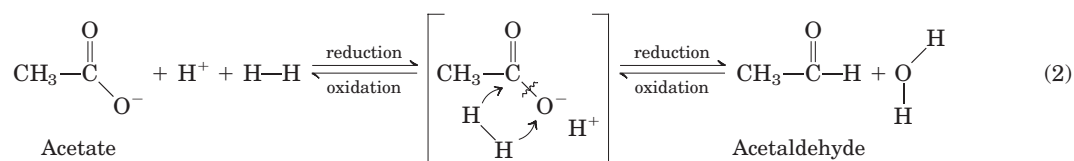
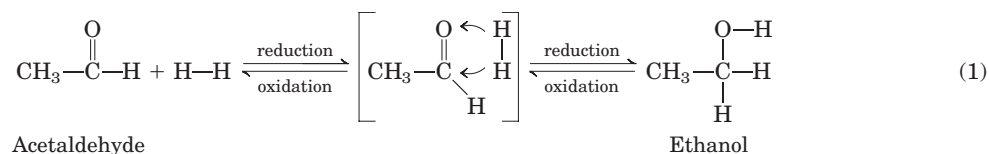
*Citric acid cycle:*



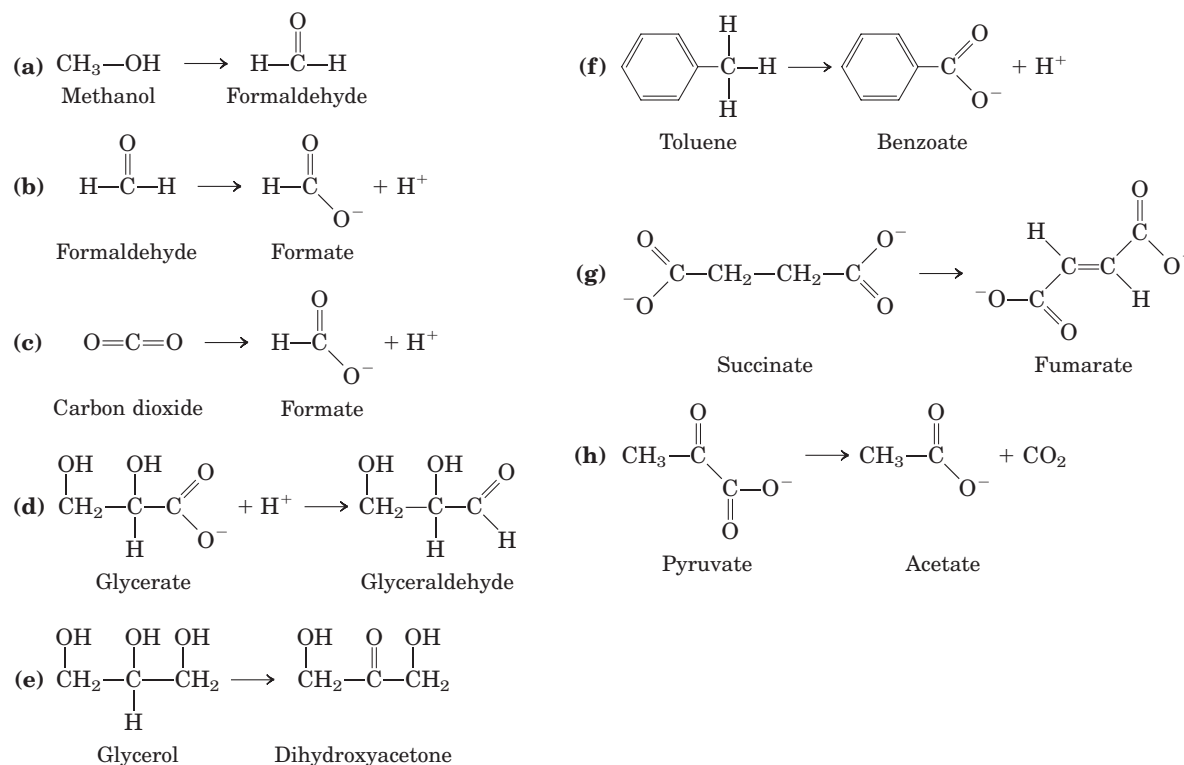
*Overall:*



- 3. Recognizing Oxidation and Reduction Reactions** One biochemical strategy of many living organisms is the stepwise oxidation of organic compounds to CO<sub>2</sub> and H<sub>2</sub>O and the conservation of a major part of the energy thus produced in the form of ATP. It is important to be able to recognize oxidation-reduction processes in metabolism. Reduction of an organic molecule results from the hydrogenation of a double bond (Eqn 1, below) or of a single bond with accompanying cleavage (Eqn 2). Conversely, oxidation results from dehydrogenation. In biochemical redox reactions, the coenzymes NAD and FAD dehydrogenate/hydrogenate organic molecules in the presence of the proper enzymes.



For each of the metabolic transformations in (a) through (h), determine whether oxidation or reduction has occurred. Balance each transformation by inserting H—H and, where necessary, H<sub>2</sub>O.



**Answer** Keep in mind that oxidation is the loss of electrons and accompanying H<sup>+</sup>, whereas reduction is the gain of electrons (or H—H).

- (a) Oxidation: Methanol  $\longrightarrow$  formaldehyde + H—H  
 (b) Oxidation: Formaldehyde  $\longrightarrow$  formate + H—H  
 (c) Reduction: CO<sub>2</sub> + H—H  $\longrightarrow$  formate + H<sup>+</sup>  
 (d) Reduction: Glycerate + H—H + H<sup>+</sup>  $\longrightarrow$  glyceraldehyde + H<sub>2</sub>O  
 (e) Oxidation: Glycerol  $\longrightarrow$  dihydroxyacetone + H—H  
 (f) Oxidation: Toluene + 2H<sub>2</sub>O  $\longrightarrow$  benzoate + H<sup>+</sup> + 3H—H  
 (g) Oxidation: Succinate  $\longrightarrow$  fumarate + H—H  
 (h) Oxidation: Pyruvate + H<sub>2</sub>O  $\longrightarrow$  acetate + CO<sub>2</sub> + H—H

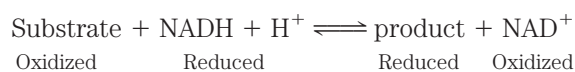
- 4. Relationship between Energy Release and the Oxidation State of Carbon** A eukaryotic cell can use glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) and hexanoic acid (C<sub>6</sub>H<sub>14</sub>O<sub>2</sub>) as fuels for cellular respiration. On the basis of their structural formulas, which substance releases more energy per gram on complete combustion to CO<sub>2</sub> and H<sub>2</sub>O?

**Answer** From the structural formulas, we see that the carbon-bound H/C ratio of hexanoic acid (11/6) is higher than that of glucose (7/6). Hexanoic acid is more reduced and yields more energy upon complete combustion to CO<sub>2</sub> and H<sub>2</sub>O.

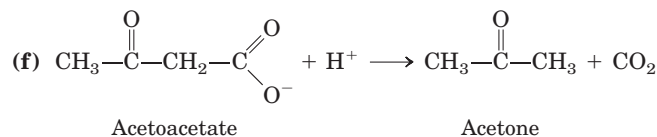
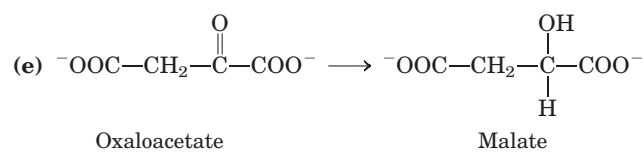
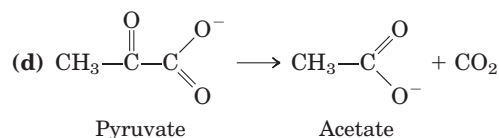
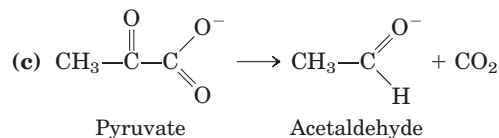
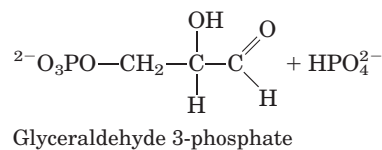
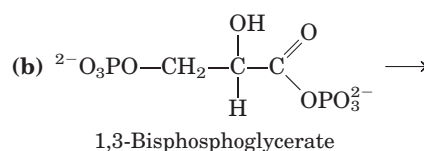
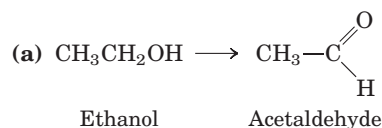
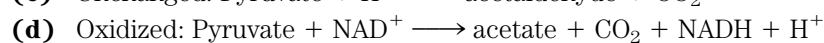
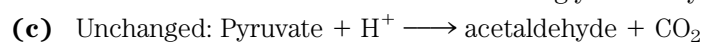
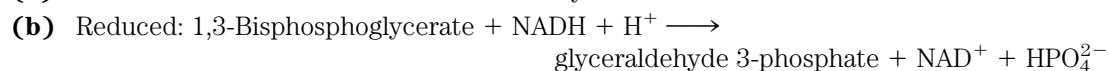
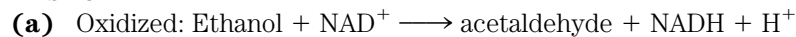
- 5. Nicotinamide Coenzymes as Reversible Redox Carriers** The nicotinamide coenzymes (see Fig. 13-24) can undergo reversible oxidation-reduction reactions with specific substrates in the presence of the appropriate dehydrogenase. In these reactions, NADH + H<sup>+</sup> serves as the hydrogen

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source, as described in Problem 3. Whenever the coenzyme is oxidized, a substrate must be simultaneously reduced:



For each of the reactions in **(a)** through **(f)**, determine whether the substrate has been oxidized or reduced or is unchanged in oxidation state (see Problem 3). If a redox change has occurred, balance the reaction with the necessary amount of  $\text{NAD}^+$ ,  $\text{NADH}$ ,  $\text{H}^+$ , and  $\text{H}_2\text{O}$ . The objective is to recognize when a redox coenzyme is necessary in a metabolic reaction.

**Answer**

- 6. Pyruvate Dehydrogenase Cofactors and Mechanism** Describe the role of each cofactor involved in the reaction catalyzed by the pyruvate dehydrogenase complex.

**Answer** *TPP*: thiazolium ring adds to  $\alpha$  carbon of pyruvate, then stabilizes the resulting carbanion by acting as an electron sink. *Lipoic acid*: oxidizes pyruvate to level of acetate (acetyl-CoA), and activates acetate as a thioester. *CoA-SH*: activates acetate as thioester. *FAD*: oxidizes lipoic acid. *NAD<sup>+</sup>*: oxidizes FAD. (See Fig. 16-6.)

- 7. Thiamine Deficiency** Individuals with a thiamine-deficient diet have relatively high levels of pyruvate in their blood. Explain this in biochemical terms.

**Answer** Thiamine is essential for the formation of thiamine pyrophosphate (TPP), one of the cofactors in the pyruvate dehydrogenase reaction. Without TPP, the pyruvate generated by glycolysis accumulates in cells and enters the blood.

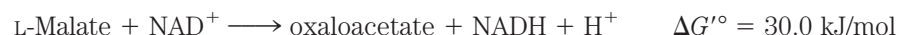
- 8. Isocitrate Dehydrogenase Reaction** What type of chemical reaction is involved in the conversion of isocitrate to  $\alpha$ -ketoglutarate? Name and describe the role of any cofactors. What other reaction(s) of the citric acid cycle are of this same type?

**Answer** Oxidative decarboxylation involving  $\text{NADP}^+$  or  $\text{NAD}^+$  as the electron acceptor; the  $\alpha$ -ketoglutarate dehydrogenase reaction is also an oxidative decarboxylation, but its mechanism is different and involves different cofactors: TPP, lipoate, FAD,  $\text{NAD}^+$ , and CoA-SH.

- 9. Stimulation of Oxygen Consumption by Oxaloacetate and Malate** 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  $\text{CO}_2$  and  $\text{H}_2\text{O}$ ) 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?

**Answer** Oxygen consumption is a measure of the activity of the first two stages of cellular respiration: glycolysis and the citric acid cycle. Initial nutrients being oxidized are carbohydrates and lipids. Because several intermediates of the citric acid cycle can be siphoned off into biosynthetic pathways, the cycle may slow down for lack of oxaloacetate in the citrate synthase reaction, and acetyl-CoA will accumulate. Addition of oxaloacetate or malate (converted to oxaloacetate by malate dehydrogenase) will stimulate the cycle and allow it to use the accumulated acetyl-CoA. This stimulates respiration. Oxaloacetate is regenerated in the cycle, so addition of oxaloacetate (or malate) stimulates the oxidation of a much larger amount of acetyl-CoA.

- 10. Formation of Oxaloacetate in a Mitochondrion** In the last reaction of the citric acid cycle, malate is dehydrogenated to regenerate the oxaloacetate necessary for the entry of acetyl-CoA into the cycle:



- (a) Calculate the equilibrium constant for this reaction at 25 °C.  
 (b) Because  $\Delta G'^{\circ}$  assumes a standard pH of 7, the equilibrium constant calculated in (a) corresponds to

$$K'_{\text{eq}} = \frac{[\text{oxaloacetate}][\text{NADH}]}{[\text{L-malate}][\text{NAD}^+]}$$

The measured concentration of L-malate in rat liver mitochondria is about 0.20 mM when  $[\text{NAD}^+]/[\text{NADH}]$  is 10. Calculate the concentration of oxaloacetate at pH 7 in these mitochondria.

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- (c) To appreciate the magnitude of the mitochondrial oxaloacetate concentration, calculate the number of oxaloacetate molecules in a single rat liver mitochondrion. Assume the mitochondrion is a sphere of diameter 2.0  $\mu\text{m}$ .

**Answer**

$$\begin{aligned} \text{(a)} \quad \Delta G'^{\circ} &= -RT \ln K'_{\text{eq}} \\ \ln K'_{\text{eq}} &= -\Delta G'^{\circ}/RT \\ &= -(30.0 \text{ kJ/mol})/(2.48 \text{ kJ/mol}) \\ &= -12.1 \\ K'_{\text{eq}} &= e^{-12.1} = 5.6 \times 10^{-6} \end{aligned}$$

- (b) Given that

$$K'_{\text{eq}} = ([\text{OAA}]_{\text{eq}}[\text{NADH}]_{\text{eq}})/([\text{malate}]_{\text{eq}}[\text{NAD}^+]_{\text{eq}})$$

if we hold the values of [malate], [NADH], and [NAD<sup>+</sup>] at the values that exist in the cell, we can calculate what [oxaloacetate] must be at equilibrium to give the equilibrium constant calculated in (a):

$$\begin{aligned} [\text{oxaloacetate}] &= K'_{\text{eq}} [\text{malate}][\text{NAD}^+]/[\text{NADH}] \\ &= (5.6 \times 10^{-6})(0.20 \text{ mM})(10) \\ &= 1.1 \times 10^{-5} \text{ mM} = 1.1 \times 10^{-8} \text{ M} \end{aligned}$$

This predicts that [oxaloacetate] at equilibrium would be very low, and the measured concentration is indeed low: less than  $10^{-7}$  M.

- (c) The volume of a sphere is  $\frac{4}{3}\pi r^3$ , thus the volume of a mitochondrion ( $r = 1.0 \times 10^{-3}$  mm) is

$$\frac{4}{3}(3.14)(1.0 \times 10^{-3} \text{ mm})^3 = 4.2 \times 10^{-9} \text{ mm}^3 = 4.2 \times 10^{-15} \text{ L}$$

Given the concentration of oxaloacetate and Avogadro's number, we can calculate the number of molecules in a mitochondrion:

$$(1.1 \times 10^{-8} \text{ mol/L})(6.02 \times 10^{23} \text{ molecules/mol})(4.2 \times 10^{-15} \text{ L}) = 28 \text{ molecules}$$

- 11. Cofactors for the Citric Acid Cycle** Suppose you have prepared a mitochondrial extract that contains all of the soluble enzymes of the matrix but has lost (by dialysis) all the low molecular weight cofactors. What must you add to the extract so that the preparation will oxidize acetyl-CoA to CO<sub>2</sub>?

**Answer** ADP (or GDP), P<sub>i</sub>, CoA-SH, TPP, NAD<sup>+</sup>; *not* lipoic acid, which is covalently attached to the isolated enzymes that use it (see Fig. 16-7).

- 12. Riboflavin Deficiency** How would a riboflavin deficiency affect the functioning of the citric acid cycle? Explain your answer.

**Answer** The flavin nucleotides, FMN and FAD, would not be synthesized. Because FAD is required by the citric acid cycle enzyme succinate dehydrogenase, flavin deficiency would strongly inhibit the cycle.

- 13. Oxaloacetate Pool** What factors might decrease the pool of oxaloacetate available for the activity of the citric acid cycle? How can the pool of oxaloacetate be replenished?

**Answer** Oxaloacetate might be withdrawn for aspartate synthesis or for gluconeogenesis. Oxaloacetate is replenished by the anaplerotic reactions catalyzed by PEP carboxykinase, PEP carboxylase, malic enzyme, or pyruvate carboxylase (see Fig. 16-15, p. 632).

- 14. Energy Yield from the Citric Acid Cycle** The reaction catalyzed by succinyl-CoA synthetase produces the high-energy compound GTP. How is the free energy contained in GTP incorporated into the cellular ATP pool?

**Answer** The terminal phosphoryl group in GTP can be transferred to ADP in a reaction catalyzed by nucleoside diphosphate kinase, with an equilibrium constant of 1.0:



**15. Respiration Studies in Isolated Mitochondria** Cellular respiration can be studied in isolated mitochondria by measuring oxygen consumption under different conditions. If 0.01 M sodium malonate is added to actively respiring mitochondria that are using pyruvate as fuel source, respiration soon stops and a metabolic intermediate accumulates.

- What is the structure of this intermediate?
- Explain why it accumulates.
- Explain why oxygen consumption stops.
- Aside from removal of the malonate, how can this inhibition of respiration be overcome? Explain.

**Answer** Malonate is a structural analog of succinate and a competitive inhibitor of succinate dehydrogenase.

- Succinate:  ${}^{-}\text{OOC}-\text{CH}_2-\text{CH}_2-\text{COO}^{-}$
- When succinate dehydrogenase is inhibited, succinate accumulates.
- Inhibition of any reaction in a pathway causes the substrate of that reaction to accumulate. Because this substrate is also the product of the preceding reaction, its accumulation changes the effective  $\Delta G$  of that reaction, and so on for all the preceding steps in the pathway. The net rate of the pathway (or cycle) slows and eventually becomes almost negligible. In the case of the citric acid cycle, ceasing to produce the primary product, NADH, has the effect of stopping electron transfer and consumption of oxygen, the final acceptor of electrons derived from NADH.
- Because malonate is a competitive inhibitor, the addition of large amounts of succinate will overcome the inhibition.

**16. Labeling Studies in Isolated Mitochondria** The metabolic pathways of organic compounds have often been delineated by using a radioactively labeled substrate and following the fate of the label.

- How can you determine whether glucose added to a suspension of isolated mitochondria is metabolized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ ?
- Suppose you add a brief pulse of  $[\beta\text{-}^{14}\text{C}]$  pyruvate (labeled in the methyl position) to the mitochondria. After one turn of the citric acid cycle, what is the location of the  $^{14}\text{C}$  in the oxaloacetate? Explain by tracing the  $^{14}\text{C}$  label through the pathway. How many turns of the cycle are required to release all the  $[\beta\text{-}^{14}\text{C}]$  pyruvate as  $\text{CO}_2$ ?

**Answer**

- If you added uniformly labeled glucose ( $^{14}\text{C}$  in all carbon atoms), release of labeled  $\text{CO}_2$  would indicate that the glucose is metabolized to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ .
- One turn of the cycle produces oxaloacetate with label equally distributed between C-2 and C-3. The route of the label is from C-3 in pyruvate, to C-2 in acetyl-CoA, to a methylene ( $-\text{CH}_2-$ ) carbon, C-2 or C-4 (see Fig. 16-7), in intermediates to succinate, which is symmetric; from succinate, the label is in C-2 or C-3. The second turn of the cycle releases half the label, and every subsequent turn releases half of what remains, so an infinite number of turns are required to release *all* the labeled carbon.

**17. Pathway of  $\text{CO}_2$  in Gluconeogenesis** In the first bypass step of gluconeogenesis, the conversion of pyruvate to phosphoenolpyruvate (PEP), pyruvate is carboxylated by pyruvate carboxylase to oxaloacetate, which is subsequently decarboxylated to PEP by PEP carboxykinase (Chapter 14). Because the addition of  $\text{CO}_2$  is directly followed by the loss of  $\text{CO}_2$ , you might expect that in tracer experiments, the  $^{14}\text{C}$  of  $^{14}\text{CO}_2$  would not be incorporated into PEP, glucose, or any intermediates in gluconeogenesis.

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However, investigators find that when a rat liver preparation synthesizes glucose in the presence of  $^{14}\text{CO}_2$ ,  $^{14}\text{C}$  slowly appears in PEP and eventually at C-3 and C-4 of glucose. How does the  $^{14}\text{C}$  label get into the PEP and glucose? (Hint: During gluconeogenesis in the presence of  $^{14}\text{CO}_2$ , several of the four-carbon citric acid cycle intermediates also become labeled.)

**Answer** Because pyruvate carboxylase is a mitochondrial enzyme, the [ $^{14}\text{C}$ ]oxaloacetate (OAA) formed by this reaction mixes with the OAA pool of the citric acid cycle. A mixture of [ $1\text{-}^{14}\text{C}$ ] and [ $4\text{-}^{14}\text{C}$ ] OAA eventually forms by randomization of the C-1 and C-4 positions in the reversible conversions  $\text{OAA} \rightarrow \text{malate} \rightarrow \text{succinate}$ . [ $1\text{-}^{14}\text{C}$ ] OAA leads to formation of [ $3,4\text{-}^{14}\text{C}$ ]glucose.

- 18. [ $1\text{-}^{14}\text{C}$ ]Glucose Catabolism** An actively respiring bacterial culture is briefly incubated with [ $1\text{-}^{14}\text{C}$ ] glucose, and the glycolytic and citric acid cycle intermediates are isolated. Where is the  $^{14}\text{C}$  in each of the intermediates listed below? Consider only the initial incorporation of  $^{14}\text{C}$ , in the first pass of labeled glucose through the pathways.
- (a) Fructose 1,6-bisphosphate
  - (b) Glyceraldehyde 3-phosphate
  - (c) Phosphoenolpyruvate
  - (d) Acetyl-CoA
  - (e) Citrate
  - (f)  $\alpha$ -Ketoglutarate
  - (g) Oxaloacetate

**Answer** Figures 14–2, 14–6, and 16–7 and Box 16–3 outline the fate of all the carbon atoms of glucose. In one pass through the pathways, the label appears at:

- (a) C-1
- (b) C-3
- (c) C-3
- (d) C-2 (methyl group)
- (e) C-2 (see Box 16–3)
- (f) C-4
- (g) Equally distributed in C-2 and C-3

- 19. Role of the Vitamin Thiamine** People with beriberi, a disease caused by thiamine deficiency, have elevated levels of blood pyruvate and  $\alpha$ -ketoglutarate, especially after consuming a meal rich in glucose. How are these effects related to a deficiency of thiamine?

**Answer** Thiamine is required for the synthesis of thiamin pyrophosphate (TPP), a prosthetic group in the pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase complexes. A thiamin deficiency reduces the activity of these enzyme complexes and causes the observed accumulation of precursors.

- 20. Synthesis of Oxaloacetate by the Citric Acid Cycle** Oxaloacetate is formed in the last step of the citric acid cycle by the  $\text{NAD}^+$ -dependent oxidation of L-malate. Can a net synthesis of oxaloacetate from acetyl-CoA occur using only the enzymes and cofactors of the citric acid cycle, without depleting the intermediates of the cycle? Explain. How is oxaloacetate that is lost from the cycle (to biosynthetic reactions) replenished?

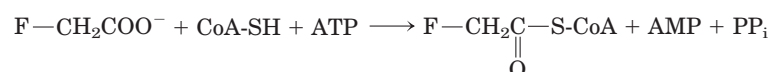
**Answer** In the citric acid cycle, the entering acetyl-CoA combines with oxaloacetate to form citrate. One turn of the cycle regenerates oxaloacetate and produces two  $\text{CO}_2$  molecules. There is *no* net synthesis of oxaloacetate in the cycle. If any cycle intermediates are channeled into biosynthetic reactions, replenishment of oxaloacetate is essential. Four enzymes can

produce oxaloacetate (or malate) from pyruvate or phosphoenolpyruvate. Pyruvate carboxylase (liver, kidney) and PEP carboxykinase (heart, skeletal muscle) are the most important in animals, and PEP carboxylase is most important in plants, yeast and bacteria. Malic enzyme produces malate from pyruvate in many organisms (see Table 16-2).

- 21. Oxaloacetate Depletion** Mammalian liver can carry out gluconeogenesis using oxaloacetate as the starting material (Chapter 14). Would the operation of the citric acid cycle be affected by extensive use of oxaloacetate for gluconeogenesis? Explain your answer.

**Answer** Oxaloacetate depletion would tend to inhibit the citric acid cycle. Oxaloacetate is present at relatively low concentrations in mitochondria, and removing it for gluconeogenesis would tend to shift the equilibrium for the citrate synthase reaction toward oxaloacetate. However, anaplerotic reactions (see Fig. 16-15) counter this effect by replacing oxaloacetate.

- 22. Mode of Action of the Rodenticide Fluoroacetate** Fluoroacetate, prepared commercially for rodent control, is also produced by a South African plant. After entering a cell, fluoroacetate is converted to fluoroacetyl-CoA in a reaction catalyzed by the enzyme acetate thiokinase:



The toxic effect of fluoroacetate was studied in an experiment using intact isolated rat heart. After the heart was perfused with 0.22 mM fluoroacetate, the measured rate of glucose uptake and glycolysis decreased, and glucose 6-phosphate and fructose 6-phosphate accumulated. Examination of the citric acid cycle intermediates revealed that their concentrations were below normal, except for citrate, with a concentration 10 times higher than normal.

- Where did the block in the citric acid cycle occur? What caused citrate to accumulate and the other cycle intermediates to be depleted?
- Fluoroacetyl-CoA is enzymatically transformed in the citric acid cycle. What is the structure of the end product of fluoroacetate metabolism? Why does it block the citric acid cycle? How might the inhibition be overcome?
- In the heart perfusion experiments, why did glucose uptake and glycolysis decrease? Why did hexose monophosphates accumulate?
- Why is fluoroacetate poisoning fatal?

**Answer**

- The block occurs at the aconitase reaction, which normally converts citrate to isocitrate.
- Fluoroacetate, an analog of acetate, can be activated to fluoroacetyl-CoA, which condenses with oxaloacetate to form fluorocitrate—the end product of fluoroacetate metabolism. Fluorocitrate is a structural analog of citrate and a strong competitive inhibitor of aconitase. The inhibition can be overcome by addition of large amounts of citrate.
- Citrate and fluorocitrate are allosteric inhibitors of phosphofructokinase-1, and as their concentration increases, glycolysis and glucose uptake slow down. Inhibition of PFK-1 causes the accumulation of glucose 6-phosphate and fructose 6-phosphate.
- The net effect of fluoroacetate poisoning is to shut down ATP synthesis, aerobic (oxidative) and anaerobic (fermentative).

- 23. Synthesis of L-Malate in Wine Making** The tartness of some wines is due to high concentrations of L-malate. Write a sequence of reactions showing how yeast cells synthesize L-malate from glucose under anaerobic conditions in the presence of dissolved  $\text{CO}_2$  ( $\text{HCO}_3^-$ ). Note that the overall reaction for this fermentation cannot involve the consumption of nicotinamide coenzymes or citric acid cycle intermediates.

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**Answer** The glycolytic reactions



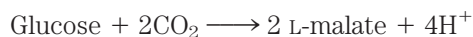
are followed by the pyruvate carboxylase reaction



In the citric acid cycle, the malate dehydrogenase reaction



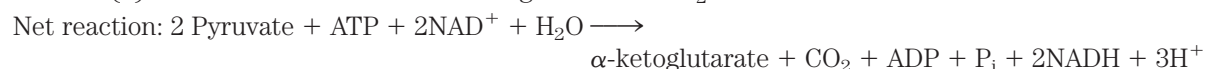
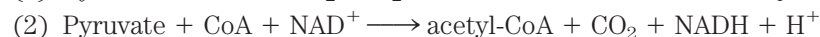
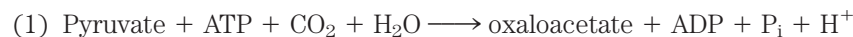
recycles nicotinamide coenzymes under anaerobic conditions. The overall reaction is



which produces four  $\text{H}^+$  per glucose, increasing the acidity and thus the tartness of the wine.

- 24. Net Synthesis of  $\alpha$ -Ketoglutarate**  $\alpha$ -Ketoglutarate plays a central role in the biosynthesis of several amino acids. Write a sequence of enzymatic reactions that could result in the net synthesis of  $\alpha$ -ketoglutarate from pyruvate. Your proposed sequence must not involve the net consumption of other citric acid cycle intermediates. Write an equation for the overall reaction and identify the source of each reactant.

**Answer** Anaplerotic reactions replenish intermediates in the citric acid cycle. Net synthesis of  $\alpha$ -ketoglutarate from pyruvate occurs by the sequential actions of (1) pyruvate carboxylase (which makes extra molecules of oxaloacetate), (2) pyruvate dehydrogenase, and the citric acid cycle enzymes (3) citrate synthase, (4) aconitase, and (5) isocitrate dehydrogenase:



- 25. Amphibolic Pathways** Explain, giving examples, what is meant by the statement that the citric acid cycle is amphibolic.

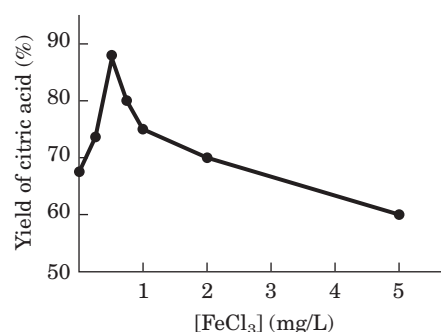
**Answer** Amphibolic pathways can serve either in energy-yielding catabolic or in energy-requiring biosynthetic processes, depending on the cellular circumstances. For example, the citric acid cycle generates NADH and  $\text{FADH}_2$  when functioning catabolically. But it can also provide precursors for the synthesis of such products as glutamate and aspartate (from  $\alpha$ -ketoglutarate and oxaloacetate, respectively), which in turn serve as precursors for other products, such as glutamine, proline, and asparagine (see Fig. 16–15).

- 26. Regulation of the Pyruvate Dehydrogenase Complex** In animal tissues, the rate of conversion of pyruvate to acetyl-CoA is regulated by the ratio of active, phosphorylated to inactive, unphosphorylated PDH complex. Determine what happens to the rate of this reaction when a preparation of rabbit muscle mitochondria containing the PDH complex is treated with **(a)** pyruvate dehydrogenase kinase, ATP, and NADH; **(b)** pyruvate dehydrogenase phosphatase and  $\text{Ca}^{2+}$ ; **(c)** malonate.

**Answer** Pyruvate dehydrogenase is regulated by covalent modification and by allosteric inhibitors. The mitochondrial preparation responds as follows: **(a)** Active pyruvate dehydrogenase (dephosphorylated) is converted to inactive pyruvate dehydrogenase (phosphorylated) and the rate of conversion of pyruvate to acetyl-CoA decreases. **(b)** The phosphoryl group on pyruvate dehydrogenase phosphate is removed enzymatically to yield active pyruvate dehydrogenase, which increases the rate of conversion of pyruvate to acetyl-CoA. **(c)** Malonate inhibits succinate dehydrogenase, and citrate accumulates. The accumulated citrate inhibits citrate synthase, and acetyl-CoA accumulates. High levels of acetyl-CoA inhibit pyruvate dehydrogenase, and the rate of conversion of pyruvate to acetyl-CoA is reduced.

**27. Commercial Synthesis of Citric Acid** Citric acid is used as a flavoring agent in soft drinks, fruit juices, and many other foods. Worldwide, the market for citric acid is valued at hundreds of millions of dollars per year. Commercial production uses the mold *Aspergillus niger*, which metabolizes sucrose under carefully controlled conditions.

- (a)** The yield of citric acid is strongly dependent on the concentration of  $\text{FeCl}_3$  in the culture medium, as indicated in the graph. Why does the yield decrease when the concentration of  $\text{Fe}^{3+}$  is above or below the optimal value of 0.5 mg/L?



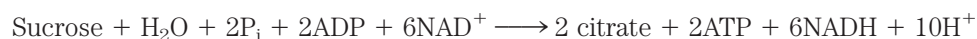
- (b)** Write the sequence of reactions by which *A. niger* synthesizes citric acid from sucrose. Write an equation for the overall reaction.
- (c)** Does the commercial process require the culture medium to be aerated—that is, is this a fermentation or an aerobic process? Explain.

**Answer**

- (a)** Citrate is produced through the action of citrate synthase on oxaloacetate and acetyl-CoA. Although the citric acid cycle does not normally result in an accumulation of intermediates, citrate synthase can be used for net synthesis of citrate when (1) there is a continuous influx of new oxaloacetate and acetyl-CoA, and (2) the transformation of citrate to isocitrate is blocked or at least restricted. *A. niger* grown in a medium rich in sucrose but low in  $\text{Fe}^{3+}$  meets both requirements. Citrate is transformed to isocitrate by aconitase, an  $\text{Fe}^{3+}$ -containing enzyme. In an  $\text{Fe}^{3+}$ -restricted medium, synthesis of aconitase is restricted and thus the breakdown of citrate is partially blocked; citrate accumulates and can be isolated in commercial quantities. Note that *some* aconitase activity is necessary—the mold will not thrive at  $[\text{Fe}^{3+}]$  below 0.5 mg/L. At higher  $[\text{Fe}^{3+}]$ , however, aconitase is synthesized in increasing amounts; this will lead to a decrease in the yield of citrate as it cycles through the citric acid cycle.
- (b)** Sucrose +  $\text{H}_2\text{O}$   $\longrightarrow$  glucose + fructose  
 Glucose +  $2\text{P}_i$  +  $2\text{ADP}$  +  $2\text{NAD}^+$   $\longrightarrow$  2 pyruvate +  $2\text{ATP}$  +  $2\text{NADH}$  +  $2\text{H}^+$  +  $2\text{H}_2\text{O}$   
 Fructose +  $2\text{P}_i$  +  $2\text{ADP}$  +  $2\text{NAD}^+$   $\longrightarrow$  2 pyruvate +  $2\text{ATP}$  +  $2\text{NADH}$  +  $2\text{H}^+$  +  $2\text{H}_2\text{O}$   
 2 Pyruvate +  $2\text{NAD}^+$  +  $2\text{CoA}$   $\longrightarrow$  2 acetyl-CoA +  $2\text{NADH}$  +  $2\text{H}^+$  +  $2\text{CO}_2$   
 2 Pyruvate +  $2\text{CO}_2$  +  $2\text{ATP}$  +  $2\text{H}_2\text{O}$   $\longrightarrow$  2 oxaloacetate +  $2\text{ADP}$  +  $2\text{P}_i$  +  $4\text{H}^+$   
 2 Acetyl-CoA + 2 oxaloacetate +  $2\text{H}_2\text{O}$   $\longrightarrow$  2 citrate +  $2\text{CoA}$

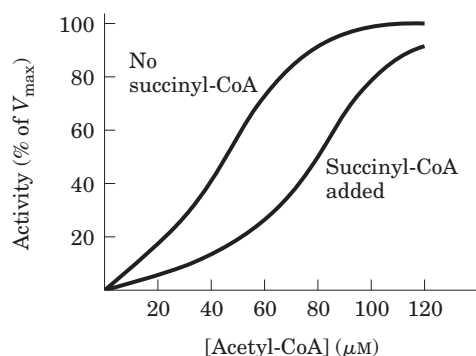
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The overall reaction is



- (c) Note that the overall reaction consumes  $\text{NAD}^+$ . Because the cellular pool of this oxidized coenzyme is limited, it must be recycled by oxidation of  $\text{NADH}$  via the electron-transfer chain, with consumption of oxygen. Consequently, the overall conversion of sucrose to citrate is an aerobic process and requires molecular oxygen.

- 28. Regulation of Citrate Synthase** In the presence of saturating amounts of oxaloacetate, the activity of citrate synthase from pig heart tissue shows a sigmoid dependence on the concentration of acetyl-CoA, as shown in the graph. When succinyl-CoA is added, the curve shifts to the right and the sigmoid dependence is more pronounced.



On the basis of these observations, suggest how succinyl-CoA regulates the activity of citrate synthase. (Hint: see Fig. 6–34) Why is succinyl-CoA an appropriate signal for regulation of the citric acid cycle? How does the regulation of citrate synthase control the rate of cellular respiration in pig heart tissue?

**Answer** Succinyl-CoA is an intermediate of the citric acid cycle—the first four-carbon intermediate, formed in the  $\alpha$ -ketoglutarate dehydrogenase reaction. Its accumulation signals reduced flux through the cycle, and thus the need for reduced entry of acetyl-CoA into the cycle.

As seen in the graph, succinyl-CoA shifts the half-saturation point,  $[\text{S}]_{0.5}$  (or  $K_{0.5}$ ), for acetyl-CoA to the right but does not alter  $V_{\text{max}}$ . This indicates that succinyl-CoA acts as a negative modulator, either directly as a competitive inhibitor with acetyl-CoA or by binding to a site separate from the active site.

Citrate synthase catalyzes the step at which acetyl-CoA enters the cycle, and thus regulation of this enzyme controls the activity of the cycle, the rate of production of reduced coenzymes, and thus the rate of cellular respiration.

- 29. Regulation of Pyruvate Carboxylase** The carboxylation of pyruvate by pyruvate carboxylase occurs at a very low rate unless acetyl-CoA, a positive allosteric modulator, is present. If you have just eaten a meal rich in fatty acids (triacylglycerols) but low in carbohydrates (glucose), how does this regulatory property shut down the oxidation of glucose to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  but increase the oxidation of acetyl-CoA derived from fatty acids?

**Answer** Fatty acid catabolism increases the level of acetyl-CoA, which stimulates pyruvate carboxylase. The resulting increase in oxaloacetate concentration stimulates acetyl-CoA consumption through the citric acid cycle, causing the citrate and ATP concentrations to rise. These metabolites inhibit glycolysis at PFK-1 and inhibit pyruvate dehydrogenase, effectively slowing the utilization of sugars and pyruvate.

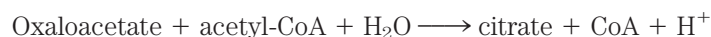
- 30. Relationship between Respiration and the Citric Acid Cycle** Although oxygen does not participate directly in the citric acid cycle, the cycle operates only when  $\text{O}_2$  is present. Why?

**Answer** Oxygen is the terminal electron acceptor in oxidative phosphorylation, and thus is needed to recycle  $\text{NAD}^+$  from NADH. NADH is produced in greatest quantities by the oxidative reactions of the citric acid cycle. In the absence of  $\text{O}_2$ , the supply of  $\text{NAD}^+$  is depleted, and the accumulated NADH allosterically inhibits pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase (see Fig. 16–18).

- 31. Effect of  $[\text{NADH}]/[\text{NAD}^+]$  on the Citric Acid Cycle** How would you expect the operation of the citric acid cycle to respond to a rapid increase in the  $[\text{NADH}]/[\text{NAD}^+]$  ratio in the mitochondrial matrix? Why?

**Answer** Increased  $[\text{NADH}]/[\text{NAD}^+]$  inhibits the citric acid cycle by mass action at each of the three steps that involve reduction of  $\text{NAD}^+$ ; high  $[\text{NADH}]$  shifts the equilibrium toward  $\text{NAD}^+$ . Another way to look at this effect is to consider how an increased ratio of product (NADH) to reactant ( $\text{NAD}^+$ ) affects the free-energy change for any of the three  $\text{NAD}^+$ -dependent steps of the citric acid cycle. Look, for example, at Equation 13–4 (p. 493).

- 32. Thermodynamics of Citrate Synthase Reaction in Cells** Citrate is formed by the condensation of acetyl-CoA with oxaloacetate, catalyzed by citrate synthase:



In rat heart mitochondria at pH 7.0 and  $25^\circ\text{C}$ , the concentrations of reactants and products are: oxaloacetate,  $1\ \mu\text{M}$ ; acetyl-CoA,  $1\ \mu\text{M}$ ; citrate,  $220\ \mu\text{M}$ ; and CoA,  $65\ \mu\text{M}$ . The standard free-energy change for the citrate synthase reaction is  $-32.2\ \text{kJ/mol}$ . What is the direction of metabolite flow through the citrate synthase reaction in rat heart cells? Explain.

**Answer** The free-energy change of the citrate synthase reaction in the cell is

$$\begin{aligned}\Delta G &= \Delta G'^{\circ} + RT \ln \frac{[\text{citrate}][\text{CoA}]}{[\text{OAA}][\text{acetyl-CoA}]} \\ &= -32.2\ \text{kJ/mol} + (2.48\ \text{kJ/mol}) \ln \frac{(220 \times 10^{-6})(65 \times 10^{-6})}{(1 \times 10^{-6})(1 \times 10^{-6})} \\ &= -8\ \text{kJ/mol}\end{aligned}$$

Thus, the citrate synthase reaction is exergonic and proceeds in the direction of citrate formation.

- 33. Reactions of the Pyruvate Dehydrogenase Complex** Two of the steps in the oxidative decarboxylation of pyruvate (steps ④ and ⑤ in Fig. 16–6) do not involve any of the three carbons of pyruvate yet are essential to the operation of the PDH complex. Explain.

**Answer** The pyruvate dehydrogenase complex can be thought of as performing five enzymatic reactions. The first three (see Fig. 16–6) catalyze the oxidation of pyruvate to acetyl-CoA and reduction of the enzyme. The last two reactions are essential to reoxidize the reduced enzyme, reducing  $\text{NAD}^+$  to  $\text{NADH} + \text{H}^+$ . The moiety on the enzyme that is oxidized/reduced is the lipoamide cofactor.

- 34. Citric Acid Cycle Mutants** There are many cases of human disease in which one or another enzyme activity is lacking due to genetic mutation. However, cases in which individuals lack one of the enzymes of the citric acid cycle are extremely rare. Why?

**Answer** The citric acid cycle is so central to metabolism that a serious defect in any cycle enzyme would probably be lethal to the embryo.

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- 35. Partitioning between the Citric Acid and Glyoxylate Cycles** In an organism (such as *E. coli*) that has both the citric acid cycle and the glyoxylate cycle, what determines which of these pathways isocitrate will enter?

**Answer** Isocitrate can be metabolized by the citric acid cycle or by the glyoxylate cycle. The first enzyme in each pathway is allosterically regulated, so that the accumulation of citric acid cycle intermediates stimulate that cycle while inhibiting the glyoxylate cycle. AMP and ADP, which signal an inadequate reserve of ATP, inhibit the glyoxylate cycle, shifting the use of isocitrate to the energy-producing citric acid cycle. This reciprocal regulation of the two enzymes at the branch point determine which pathway isocitrate will enter.

## Data Analysis Problem

- 36. How the Citric Acid Cycle Was Determined** 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  $\mu\text{L}$ ) 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. Addition of *citrate* increased  $\text{O}_2$  consumption by 893  $\mu\text{L}$  compared with samples without added citrate. They calculated, based on the  $\text{O}_2$  consumed during respiration of other carbon-containing compounds, that the expected  $\text{O}_2$  consumption for complete respiration of this quantity of citrate was only 302  $\mu\text{L}$ .

*Experiment II.* They measured  $\text{O}_2$  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	$\mu\text{L O}_2$ 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  $\text{O}_2$  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* ( $\text{AsO}_4^{3-}$ ) inhibits  $\alpha$ -ketoglutarate 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 pathway in Figure 16-7, 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  $\alpha$ -ketoglutarate. (2) In the presence of malonate, citrate was quantitatively converted to large amounts of succinate and small amounts of  $\alpha$ -ketoglutarate. (3) 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 workers had found the following pathway in similar muscle tissue preparations:



- (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 Figure 16-7? Explain your reasoning.
- (h) Why was it important to show the *quantitative* conversion of citrate to  $\alpha$ -ketoglutarate?

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.

### Answer

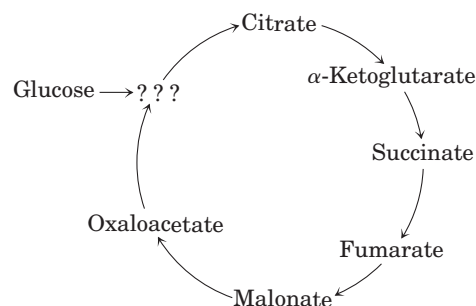
- (a) The only reaction in muscle tissue that consumes significant amounts of oxygen is cellular respiration, so  $\text{O}_2$  consumption is a good proxy for respiration.
- (b) Freshly prepared muscle tissue contains some residual glucose;  $\text{O}_2$  consumption is due to oxidation of this glucose.
- (c) Yes. Because the amount of  $\text{O}_2$  consumed increased when citrate or 1-phosphoglycerol was added, both can serve as substrate for cellular respiration in this system.
- (d) *Experiment I:* citrate is causing much more  $\text{O}_2$  consumption than would be expected from its complete oxidation. Each molecule of citrate seems to be acting as though it were more than one molecule. The only possible explanation is that each molecule of citrate functions more than once in the reaction—which is how a catalyst operates. *Experiment II:* the key is to calculate the excess  $\text{O}_2$  consumed by each sample compared with the control (sample 1).

Sample	Substrate(s) added	$\mu\text{L O}_2$ absorbed	Excess $\mu\text{L O}_2$ consumed
1	No extra	342	0
2	0.3 mL 0.2 M 1-phosphoglycerol	757	415
3	0.15 mL 0.02 M citrate	431	89
4	0.3 mL 0.2 M 1-phosphoglycerol + 0.15 mL 0.02 M citrate	1,385	1,043

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If both citrate and 1-phosphoglycerol were simply substrates for the reaction, you would expect the excess  $O_2$  consumption by sample 4 to be the sum of the individual excess consumptions by samples 2 and 3 ( $415 \mu\text{L} + 89 \mu\text{L} = 504 \mu\text{L}$ ). However, the excess consumption when both substrates are present is roughly twice this amount ( $1,043 \mu\text{L}$ ). Thus citrate increases the ability of the tissue to metabolize 1-phosphoglycerol. This behavior is typical of a catalyst. Both experiments (I and II) are required to make this case convincing. Based on experiment I only, citrate is somehow accelerating the reaction, but it is not clear whether it acts by helping substrate metabolism or by some other mechanism. Based on experiment II only, it is not clear which molecule is the catalyst, citrate or 1-phosphoglycerol. Together, the experiments show that citrate is acting as a “catalyst” for the oxidation of 1-phosphoglycerol.

- (e) Given that the pathway can consume citrate (see sample 3), if citrate is to act as a catalyst it must be regenerated. If the set of reactions first consumes then regenerates citrate, it must be a circular rather than a linear pathway.
- (f) When the pathway is blocked at  $\alpha$ -ketoglutarate dehydrogenase, citrate is converted to  $\alpha$ -ketoglutarate but the pathway goes no further. Oxygen is consumed by reoxidation of the NADH produced by isocitrate dehydrogenase.
- (g)



This differs from Figure 16–7 in that it does not include *cis*-aconitate and isocitrate (between citrate and  $\alpha$ -ketoglutarate), or succinyl-CoA, or acetyl-CoA.

- (h) Establishing a quantitative conversion was essential to rule out a branched or other, more complex pathway.

## 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.]