Chapter 8
Bacterial Fermentations

Large areas in the soil, in rivers, lakes, and oceans are devoid of oxygen. Nevertheless, numerous microorganisms live in these anaerobic environments. We have already discussed the dissimilatory reduction of nitrate, which takes place under anaerobic conditions. Besides this process, two other microbiological processes account to a large extent for the biological activities in environments devoid of oxygen: bacterial fermentations and bacterial photosynthesis. The term fermentation was first defined by Pasteur, to whom we owe the pioneering work in this field; he described fermentations as life in the absence of oxygen. Today fermentations can be defined as those biological processes that occur in the dark and that do not involve respiratory chains with oxygen or nitrate as electron acceptors. In many fermentations ATP is formed only by substrate-level phosphorylation. However, in a number of fermentations electron transport phosphorylation is also involved in ATP synthesis.

The bacteria carrying out fermentations are either facultative or obligate anaerobes. Facultative anaerobes such as the enterobacteria grow as aerobic heterotrophs in the presence of oxygen; under anaerobic conditions they carry out a fermentative metabolism. In contrast, **obligate anaerobes** are not able to synthesize the components of an oxygen-linked respiratory chain. Consequently, they cannot grow as aerobes. Moreover, many of the obligate anaerobes do not even tolerate oxygen and are killed in air. These organisms are referred to as **strict anaerobes**.

When reduced flavoproteins or reduced iron-sulfur proteins come together with oxygen, two toxic compounds are formed: hydrogen peroxide and the superoxide radical (see Chapter 2, Section V). Aerobes contain catalase and superoxide dismutase for destruction of these compounds. Table 8.1 shows the activity of these enzymes as found in aerobes,

aerotolerant anaerobes, and strict anaerobes. It is evident that aerobes contain high levels of catalase and superoxide dismutase. Most aerotolerant anaerobes are devoid of catalase but contain superoxide dismutase. Strict anaerobes lack both enzymes. Although other factors might be involved, it can be concluded that aerointolerant species die in the presence of oxygen because of the deleterious effects of the superoxide radical.

For growth of most strict anaerobes it is not sufficient to exclude molecular oxygen. A low redox potential is required and usually the growth media have to be supplemented with SH-containing compounds such as thioglycolate, cysteine, or sodium sulfide. These compounds establish reducing conditions. Methanogenic bacteria grow only in media with a redox potential lower than $E_0' = -0.3$ V.

A comparison of aerobic heterotrophic metabolism with fermentative metabolism reveals one major difference: aerobic heterotrophs couple the oxidation of organic substrates to the reduction of oxygen or nitrate. This involves respiratory chains with high ATP yields. With the exception of

Table 8.1. Activity of superoxide dismutase and catalase in a variety of microorganisms[a]

	specific activity (U/mg)	
organism	superoxide dismutase	catalase
Aerobes		
Escherichia coli	1.8	6.1
Salmonella typhimurium	1.4	2.4
Rhizobium japonicum	2.6	0.7
Micrococcus radiodurans	7.0	289
Pseudomonas species	2.0	22.5
Aerotolerant anaerobes		
Eubacterium limosum	1.6	0
Streptococcus faecalis	0.8	0
Streptococcus lactis	1.4	0
Clostridium oroticum	0.6	0
Lactobacillus plantarum	0	0
Strict anaerobes		
Veillonella alcalescens	0	0
Clostridium pasteurianum	0	0
Clostridium butyricum	0	0
Clostridium sticklandii	0	0
Butyrivibrio fibrisolvens	0	0.1

[a]J. M. McCord, B. B. Keele, and I. Fridovich, *Proc. Natl. Acad. Sci.* **68**, 1024–1027 (1971).

some incomplete oxidizers, the substrate is converted into cell material, carbon dioxide, and water.

Fermentative anaerobes carry out a variety of oxidation-reduction reactions involving organic compounds, carbon dioxide, molecular hydrogen, and sulfur compounds. These reactions are coupled to substrate-level and/or electron transport phosphorylation. The ATP yield (mol ATP per mol substrate consumed), however, is very low. Therefore, the amount of cells obtained per mol of substrate is much smaller than with aerobes and, in addition to cell material, large amounts of "fermentation end products" are formed.

There are two fermentative processes that at first appear to be quite similar to oxygen- and nitrate-dependent respirations: the reduction of CO_2 to methane and of sulfate to sulfide. Together with the nitrate-dependent respiration these processes are frequently called **anaerobic respirations**. However, they bear little resemblance to the process of denitrification. The reduction of CO_2 and of sulfate is carried out by strict anaerobes whereas nitrate reduction is carried out predominantly by aerobes if oxygen is not available. Furthermore, the energetics of these processes is very different. Table 8.2 shows that the free energy change of O_2 and nitrate reduction per two electrons is about the same; the values are much lower for CO_2 and sulfate reduction. In fact, the values are so low that the formation of one ATP per H_2 or NADH oxidized cannot be expected (ATP synthesis from ADP + P_i requires more than 35.6 kJ (8.5 kcal) per mol. Consequently, not all the reduction steps in methane and sulfide formation can be coupled to ATP synthesis. Only the reduction of one or two intermediates may yield ATP by electron transport phosphorylation, and the ATP gain is therefore small. In conclusion, it seems appropriate to reserve the term anaerobic respiration for denitrification and nitrate/nitrite respiration and to consider CO_2 reduction and sulfate reduction as fermentations.

Fermentations are usually classified according to the main fermentation end products, and we distinguish alcohol, lactate, propionate, butyrate, mixed acid, acetate, methane, and sulfide fermentations.

I. Alcohol Fermentation

A. Ethanol fermentation by yeasts

Alcohol fermentation is the domain of yeasts, notably of *Saccharomyces* species, and most of the ethanol formed in nature and produced by the fermentation industry comes from the anaerobic breakdown of glucose and other hexoses by these organisms. Gay-Lussac had already established in 1815 that hexoses are converted into ethanol and CO_2 according to the

Table 8.2. Free energy changes in aerobic and anaerobic respiration and in sulfide and methane fermentation

redox reaction	$\Delta G^{0\prime}$ (kJ/mol acceptor)	$\Delta G^{0\prime}$ (kJ/$2e^-$)	
		H_2	NADH
$2H_2 + O_2 \rightarrow 2H_2O$	−474.38 (−113.38)	−237.19 (−56.69)	−219.07 (−52.36)
$\frac{5}{2}H_2 + NO_3^- + H^+ \rightarrow \frac{1}{2}N_2 + 3H_2O$	−560.61 (−133.99)	−224.24 (−53.60)	−206.12 (−49.27)
$4H_2 + SO_4^{2-} + \frac{3}{2}H^+ \rightarrow \frac{1}{2}H_2S + \frac{1}{2}HS^- + 4H_2O$	−153.46 (−36.68)	−38.36 (−9.17)	−20.24 (−4.84)
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−130.79 (−31.26)	−32.70 (−7.81)	−14.58 (−3.48)

[a]kcal values in parentheses

following equation:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

Figure 8.1 summarizes the alcohol fermentation as carried out by yeasts. It is apparent that yeasts employ the Embden–Meyerhof–Parnas pathway for glucose degradation. Thus, 2 mol of pyruvate are formed from 1 mol of glucose. Pyruvate, however, is not converted to acetyl-CoA as in aerobic metabolism, but is decarboxylated to acetaldehyde. The enzyme that catalyzes this reaction is **pyruvate decarboxylase**; it can be regarded as the key enzyme of alcohol fermentation. Pyruvate decarboxylase contains bound thiamine pyrophosphate; in fact, the function of thiamine pyrophosphate was first studied using this enzyme. As in the pyruvate dehydrogenase reaction, hydroxyethyl-thiamine pyrophosphate enzyme is an intermediate. The hydroxyethyl group, however, is not oxidized but is released as free acetaldehyde. The latter is then reduced to ethanol by alcohol dehydrogenase.

One important feature of fermentations is apparent from Fig. 8.1: an even hydrogen balance. Since there is no external H-acceptor such as

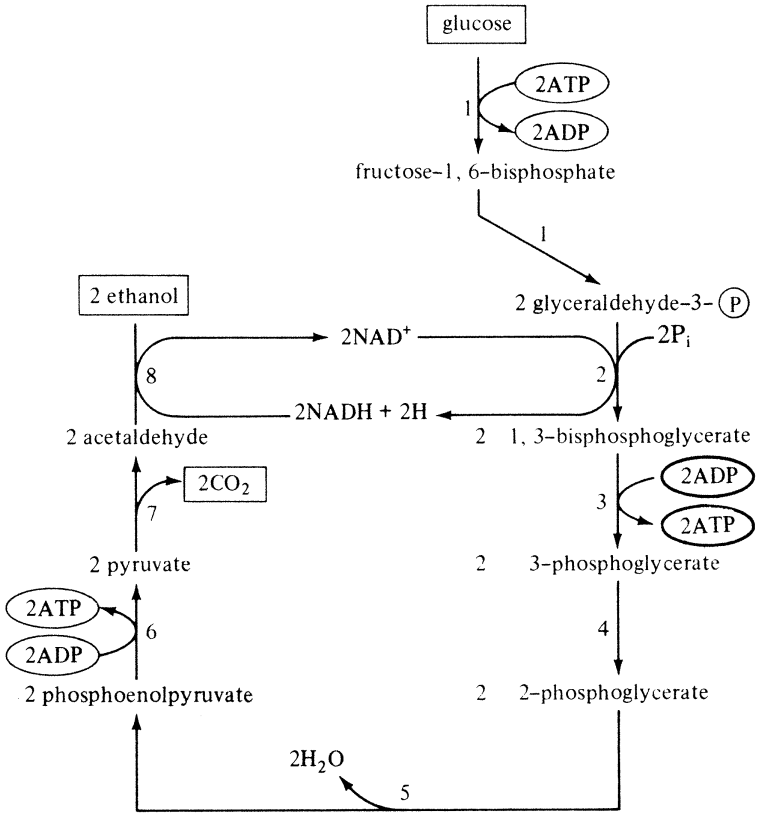
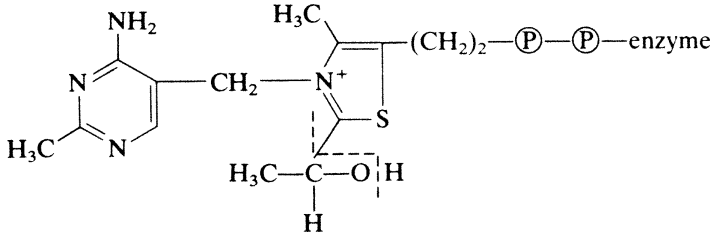


Figure 8.1. Fermentation of glucose to ethanol and CO_2 by yeasts. 1, Initial enzymes of the Embden–Meyerhof–Parnas pathway; 2, glyceraldehyde-3-phosphate dehydrogenase; 3, 3-phosphoglycerate kinase; 4, phosphoglycerate mutase; 5, enolase; 6, pyruvate kinase; 7, pyruvate decarboxylase; 8, alcohol dehydrogenase.

oxygen, NADH-producing and NADH-consuming reactions have to be balanced out. This also implies that NADH formation is avoided by anaerobes if possible. Therefore, they do not oxidize acetyl-CoA via the tricarboxylic acid cycle. The cycle is usually interrupted between α-oxoglutarate and succinyl-CoA so that glutamate can still be formed from oxaloacetate and acetyl-CoA via citrate.



hydroxyethyl-thiamine pyrophosphate enzyme

B. The Pasteur effect

The net ATP yield of the alcohol fermentation is 2 mol ATP/mol glucose (Fig. 8.1)—much lower than the ATP yield of aerobic metabolism. Yeast cells respond to this large difference. When they are transferred from aerobic to anaerobic conditions they increase the rate of glucose breakdown by a factor of 3 to 4; a change from anaerobic to aerobic metabolism is accompanied by a reduction of this rate and a stoppage of alcohol formation. This phenomenon is called the **Pasteur effect**. Apparently, the Pasteur effect is the result of differences in the cell's energy charge under aerobic and anaerobic conditions. In the presence of oxygen the respiratory chain and the sites of substrate-level phosphorylation in the glycolytic pathway compete for ADP. Furthermore, phosphofructokinase activity is controlled by the level of ATP and citrate. Under anaerobic conditions the activity of phosphofructokinase increases because it is activated by ADP and AMP; also, more ADP is available for the enzymes catalyzing substrate-level phosphorylation reactions. All this allows a greater substrate flow through the glycolytic pathway.

C. Alcohol fermentation by bacteria

It should be mentioned that yeasts are not truly facultatively anaerobic organisms. They grow only for some generations under these conditions. There are, however, some bacterial species that carry out an alcohol fermentation and grow very well under anaerobic conditions. *Zymomonas mobilis* isolated from Mexican pulque and the closely related *Zymomonas anaerobica* degrade glucose to pyruvate via the Entner–Doudoroff pathway; they contain pyruvate decarboxylase and form nearly 2 mol each of ethanol and carbon dioxide from 1 mol glucose. *Sarcina ventriculi*, a strict anaerobe capable of growth under extremely acidic conditions, and

Erwinia amylovora, a facultatively anaerobic enterobacterium, ferment glucose to ethanol and CO_2 via the Embden–Meyerhof–Parnas pathway and the pyruvate decarboxylase and alcohol dehydrogenase reactions. Both organisms, however, form small quantities of other products in addition: acetate and molecular hydrogen (*S. ventriculi*) or lactate (*E. amylovora*). In general, pyruvate decarboxylase is rare in bacteria. Many lactic acid bacteria, enterobacteria, and clostridia form considerable amounts of ethanol but do not employ pyruvate decarboxylase for acetaldehyde synthesis. In these organisms acetyl-CoA functions as ultimate precursor of acetaldehyde; it is reduced by acetaldehyde dehydrogenase:

$$CH_3—CO—SCoA + NADH + H^+ \xrightarrow{\text{acetaldehyde dehydrogenase}} CH_3—CHO + CoASH + NAD^+$$

The investigation of the alcohol fermentation has been of inestimable importance for the development of biological sciences. In 1897 the Buchner brothers discovered that an extract of macerated yeast fermented glucose to ethanol. This demonstrated that a complex biological process could function outside the cell.

In 1905 Harden and Young discovered the importance of phosphate esters in metabolism; they identified a hexose bisphosphate (fructose 1,6-bisphosphate) as an intermediate in sugar fermentation. Later, the path of glucose breakdown and the function of the coenzymes in this process were unraveled by Neuberg, Embden, Meyerhof, Parnas, and Warburg.

It should be mentioned here that ethanol is now produced on a large scale by fermentation for industrial purposes. Bargasse (from sugar cane), molasses (from sugar beets), and corn starch are used as substrates; yeasts and to a small extent *Zymomonas mobilis* are employed as organisms. Processes are currently being developed that allow ethanol production from various substrates by thermophiles, such as *Clostridium thermohydrosulfuricum*, *Thermoanaerobium brockii*, or *Thermoanaerobacter ethanolicus*. These organisms, however, do not contain pyruvate decarboxylase; they form ethanol via acetyl-CoA and excrete in addition to ethanol considerable amounts of acetate and lactate.

II. Lactate Fermentation

Lactate is a very common end product of bacterial fermentations. Some genera—often referred to as **lactic acid bacteria**—form large amounts of this product. These microorganisms have in common that they are highly saccharolytic and that they lack most anabolic pathways. So they exhibit very complex nutritional requirements, which are met by their environment such as plant materials, milk, and the intestinal tract of animals. Most

lactic acid bacteria are strictly fermentative but are aerotolerant. Some streptococci, however, can use oxygen as H-acceptor and even form cytochromes under certain conditions.

In Table 8.3 species of the genera *Lactobacillus*, *Sporolactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Bifidobacterium* are listed. Either of three pathways is employed by these microorganisms for the fermentation of carbohydrates to lactate.

The **homofermentative pathway** yields 2 mol of lactate per mol of glucose:

$$\text{glucose} \xrightarrow{\text{homofermentative pathway}} 2 \text{ lactate}$$

The **heterofermentative pathway** yields 1 mol each of lactate, ethanol, and CO_2 per mol of glucose:

$$\text{glucose} \xrightarrow{\text{heterofermentative pathway}} \text{lactate} + \text{ethanol} + CO_2$$

Table 8.3. Homo- and heterofermentative lactic acid bacteria and configuration of lactic acid produced

genera and species	homofermentative	heterofermentative	configuration of lactic acid
Lactobacillus			
L. delbrueckii	+	−	D (−)
L. lactis	+	−	D (−)
L. bulgaricus	+	−	D (−)
L. casei	+	−	L (+)
L. plantarum	+	−	DL
L. curvatus	+	−	DL
L. brevis	−	+	DL
L. fermentum	−	+	DL
Sporolactobacillus			
S. inulinus	+	−	D (−)
Streptococcus			
S. faecalis	+	−	L (+)
S. cremoris	+	−	L (+)
S. lactis	+	−	L (+)
Leuconostoc			
L. mesenteroides	−	+	D (−)
L. dextranicum	−	+	D (−)
Pediococcus			
P. damnosus	+	−	DL
Bifidobacterium			
B. bifidum	−	+	L (+)

The **bifidum pathway** yields acetate and lactate in a ratio of 3 to 2:

$$2 \text{ glucose} \xrightarrow{\text{bifidum pathway}} 3 \text{ acetate} + 2 \text{ lactate}$$

These equations are, of course, not followed completely by the lactic acid bacteria but in homofermenting organisms the yield is frequently 1.8 mol lactate/mol glucose. The heterofermenting bacteria produce 0.8 mol lactate and some acetate in addition to ethanol and CO_2.

A. Homofermentative pathway

The homofermentative pathway is illustrated in Fig. 8.2. A close relationship to the alcohol fermentation is apparent. Glucose is degraded via the Embden–Meyerhof–Parnas pathway to pyruvate. The latter is, however, not decarboxylated to acetaldehyde as in the alcohol fermentation but used directly as H-acceptor. The ATP yield in both fermentations is the same, 2ATP/glucose.

B. Heterofermentative pathway

The heterofermentative pathway is illustrated in Fig. 8.3. As in the oxidative pentose phosphate cycle, ribulose-5-phosphate is formed via 6-phosphogluconate. Epimerization yields xylulose-5-phosphate, which is cleaved into glyceraldehyde-3-phosphate and acetyl phosphate by an

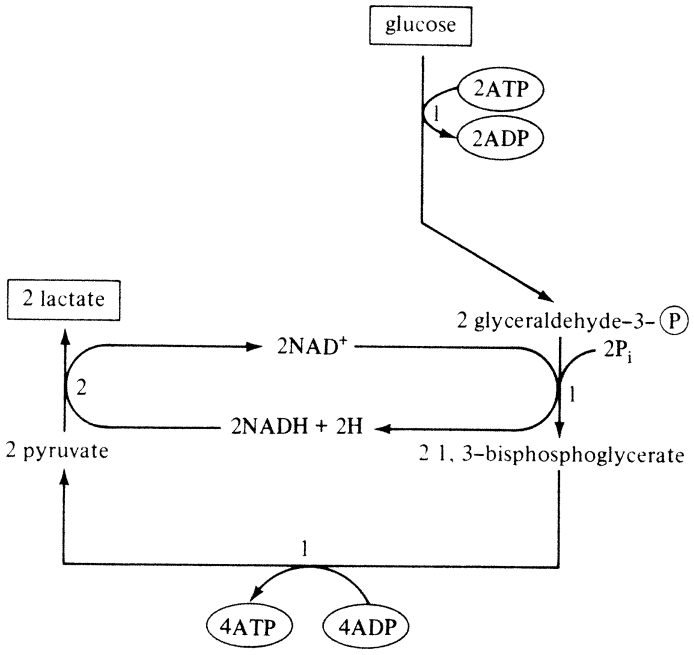


Figure 8.2. Formation of lactate from glucose by the homofermentative pathway. 1, Enzymes of the Embden–Meyerhof–Parnas pathway; 2, lactate dehydrogenase.

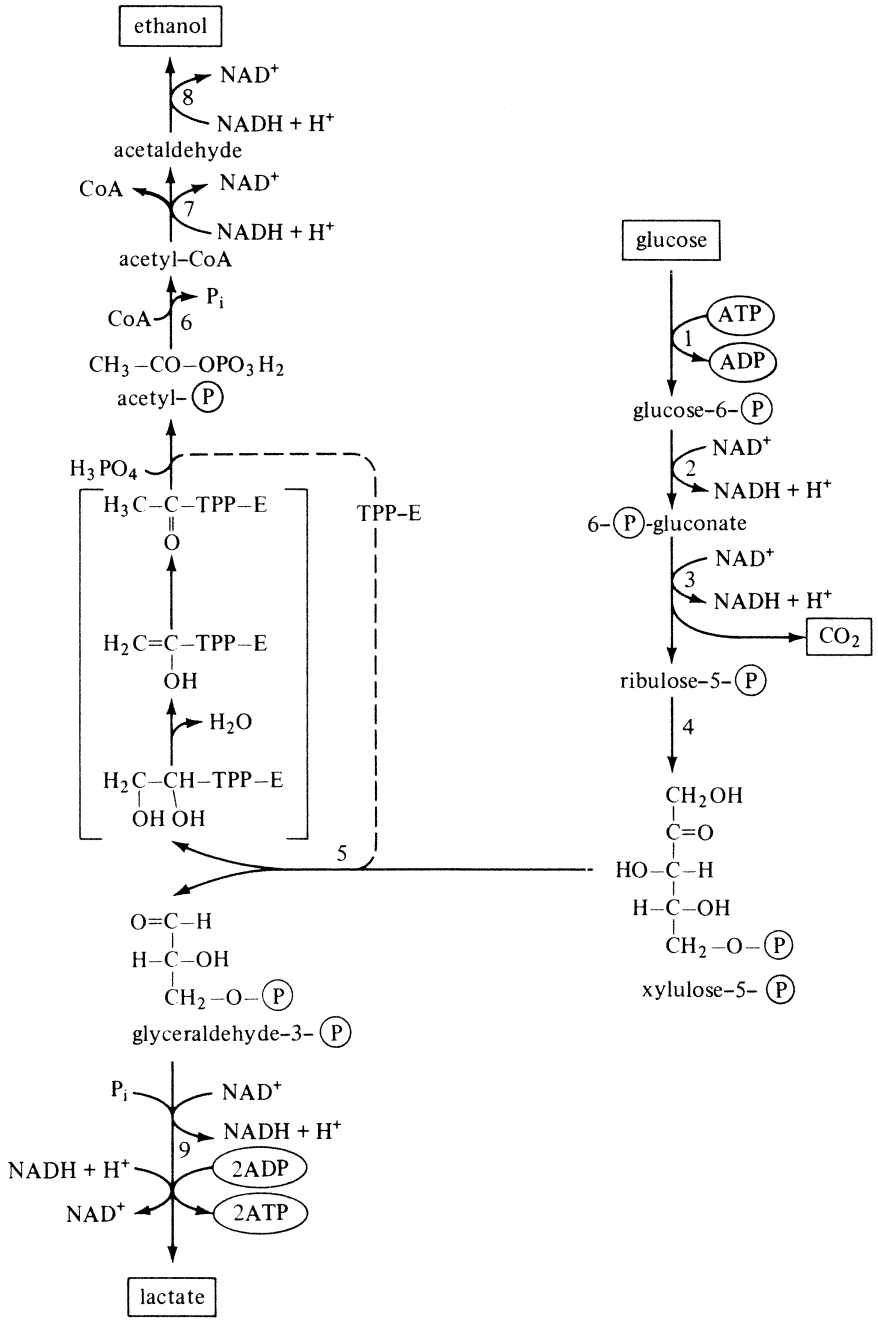


Figure 8.3. Formation of CO_2, lactate, and ethanol from glucose by the heterofermentative pathway. 1, Hexokinase; 2, glucose-6-phosphate dehydrogenase; 3, 6-phosphogluconate dehydrogenase; 4, ribulose-5-phosphate 3-epimerase; 5, phosphoketolase. The cleavage reaction yields glyceraldehyde-3-phosphate and enzyme-bound α,β-dihydroxyethyl-thiamine pyrophosphate. This is converted to acetyl-TPP-E via the α-hydroxyvinyl derivative; phosphorylytic cleavage results in acetyl phosphate formation. 6, phosphotransacetylase; 7, acetaldehyde dehydrogenase; 8, alcohol dehydrogenase; 9, enzymes as in homofermentative pathway.

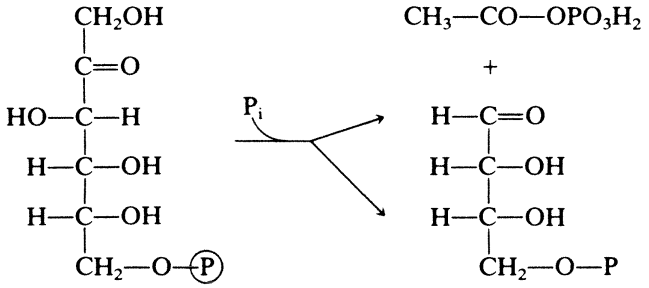
enzyme not mentioned thus far, **phosphoketolase**. It contains thiamine pyrophosphate and the formation of acetyl phosphate can be understood if enzyme-bound α-hydroxyvinyl-thiamine pyrophosphate is assumed to occur as an intermediate.

Acetyl phosphate is converted into acetyl-CoA by phosphotransacetylase. Subsequent reduction by acetaldehyde and alcohol dehydrogenases yields ethanol. The glyceraldehyde-3-phosphate formed in the phosphoketolase reaction is converted to lactate as in the homofermentative pathway.

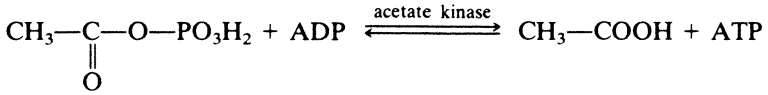
In the course of this fermentation 2NADH are formed and consumed; the ATP yield is 1 per mol of glucose–i.e., half of that of the homofermentative pathway.

C. Bifidum pathway

In glucose breakdown by *Bifidobacterium bifidum* two phosphoketolases are involved: one specific for fructose-6-phosphate and one specific for xylulose-5-phosphate. The mechanism of both reactions is similar; fructose-6-phosphate phosphoketolase splits fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate.



The bifidum pathway is illustrated in Fig. 8.4. It exhibits a very interesting sequence of reactions. Without the participation of hydrogenation and dehydrogenation reactions 2 mol of glucose are converted into 3 mol of acetate and 2 mol of glyceraldehyde-3-phosphate. The conversion of the latter to lactate involves then glyceraldehyde-3-phosphate and lactate dehydrogenases. The formation of acetate from acetyl phosphate is coupled to the formation of ATP from ADP.



This reaction, which is catalyzed by acetate kinase, is of great importance for all anaerobes that form acetate because it effects ATP synthesis by substrate-level phosphorylation. With 2.5 mol of ATP per mol of glucose the ATP yield of the bifidum pathway is higher than that of the homo- and heterofermentative pathway.

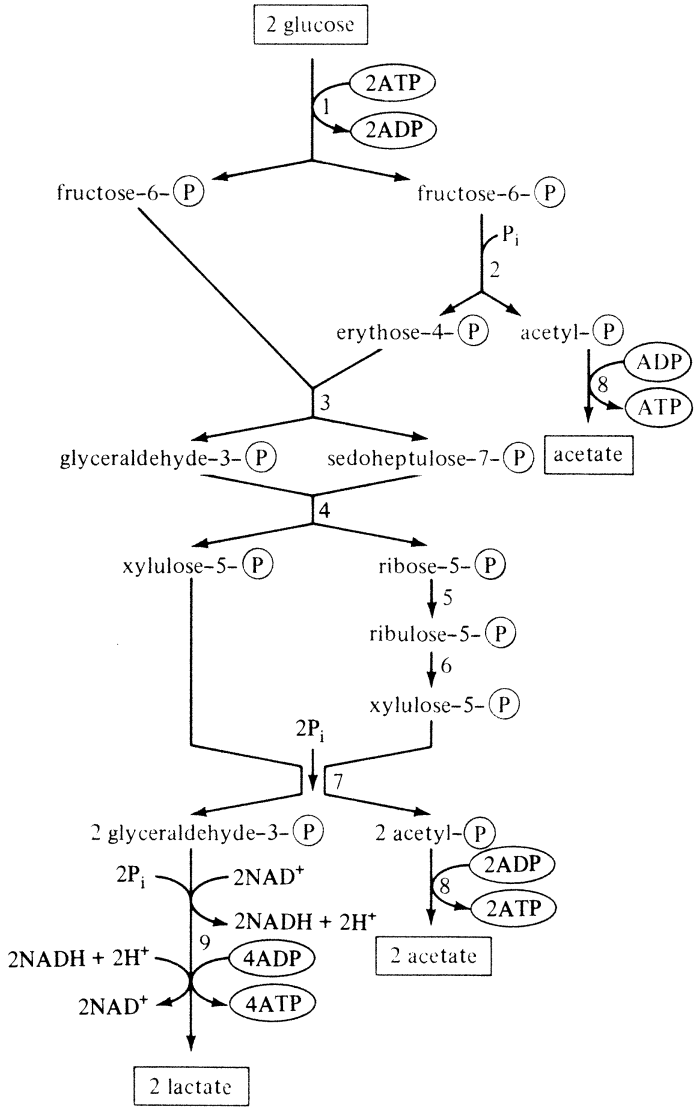


Figure 8.4. Formation of acetate and lactate from glucose by the bifidum pathway. 1, Hexokinase and glucose-6-phosphate isomerase; 2, fructose-6-phosphate phosphoketolase; 3, transaldolase; 4, transketolase; 5, ribose-5-phosphate isomerase; 6, ribulose-5-phosphate 3-epimerase; 7, xylulose-5-phosphate phosphoketolase; 8, acetate kinase; 9, enzymes as in homofermentative pathway.

D. Stereospecificity of lactate dehydrogenases

Table 8.3 has shown that lactic acid bacteria form either D(−)-, L(+)-, or DL-lactic acid. D(−)-Lactic acid-formers produce this enantiomer exclusively whereas L(+)-lactic acid-formers always produce some D(−)-enantiomer. Most organisms that excrete DL-lactate contain two lactate dehydrogenases that differ in their stereospecificity. Some lactobacilli, however, produce first L(+)-lactic acid, which—while accumulating—induces a racemase. This enzyme then converts the L(+)-form into the

D(−)-form until equilibrium is reached. *L. curvatus* belongs to this rather small group of lactobacilli.

The lactate dehydrogenases of streptococci, of *Bifidobacterium bifidum*, and of *L. casei* have an absolute and specific requirement for fructose 1,6-bisphosphate and manganese ions. The bisphosphate brings about the association of inactive protein dimers to enzymatically active tetramers.

E. Fermentation of other saccharides

A large number of other sugars besides glucose are fermented by lactic acid bacteria; they include fructose, galactose, mannose, saccharose, lactose, maltose, and pentoses. Certain variations of the normal fermentation schemes have been observed with some of these substrates. Pentoses are, for instance, fermented by some homofermentative lactobacilli (e.g., *L. casei*) using phosphoketolase—the key enzyme of the heterofermentative pathway. Fructose is fermented by *Leuconostoc mesenteroides*, but part of the NADH formed is not used to reduce acetyl-CoA to ethanol; instead fructose is reduced to mannitol. Thus, the products of fructose fermentation are lactate, acetate, CO_2, and mannitol.

F. Malo-lactate fermentation

The decrease of the acidity of wine is partly due to the conversion of L-malate to L-lactate. This process is called malo-lactate fermentation, and it is carried out by some lactic acid bacteria, e.g., *L. plantarum*, *L. casei*, and *Lc. mesenteroides*. In the presence of L-malate and a fermentable sugar these organisms synthesize a special **malic enzyme**, which converts L-malate to L-lactate:

$$\text{L-malate} \xrightarrow{Mn^{2+},\ NAD^+} \text{L-lactate} + CO_2$$

The purified enzyme does not possess lactate dehydrogenase activity so that free pyruvate cannot be an intermediate. Presumably enzyme-bound oxaloacetate and pyruvate function as intermediates in this reaction. It is not known whether or not this decarboxylation reaction is coupled to the generation of a chemical gradient across the membrane.

G. Formation of diacetyl and acetoin

In addition to the usual end products of the lactate fermentation *Streptococcus lactis* subsp. *diacetylactis* and *Leuconostoc cremoris* form acetoin and diacetyl—the characteristic flavor of butter. Citrate, which occurs in milk in concentrations up to 1.5 g/liter, is the preferred substrate for acetoin and diacetyl formation by the above organisms. As illustrated in Fig. 8.5 citrate is cleaved by citrate lyase into acetate and oxaloacetate. The enzyme **citrate lyase** is the key enzyme for the anaerobic breakdown of

citrate. It occurs in lactic acid bacteria, in enterobacteria, *Veillonella* species, *Clostridium sphenoides*, and *Rhodopseudomonas gelatinosa* but is not involved in citrate degradation under aerobic conditions; its interesting reaction mechanism and its regulation in *R. gelatinosa* have been discussed in Chapter 7.

The acetate formed in the citrate lyase reaction is excreted, and oxaloacetate is decarboxylated to yield pyruvate. Diacetyl synthesis does not proceed via α-acetolactate as in bacilli (see Fig. 6.26) or in enterobacteria (Fig. 8.15), but is accomplished by reaction of acetyl-CoA with "active acetaldehyde" (enzyme-bound hydroxyethyl-thiamine pyrophosphate). Diacetyl is usually only produced in small amounts (2 mg/l); most of it is reduced to acetoin by acetoin dehydrogenase:

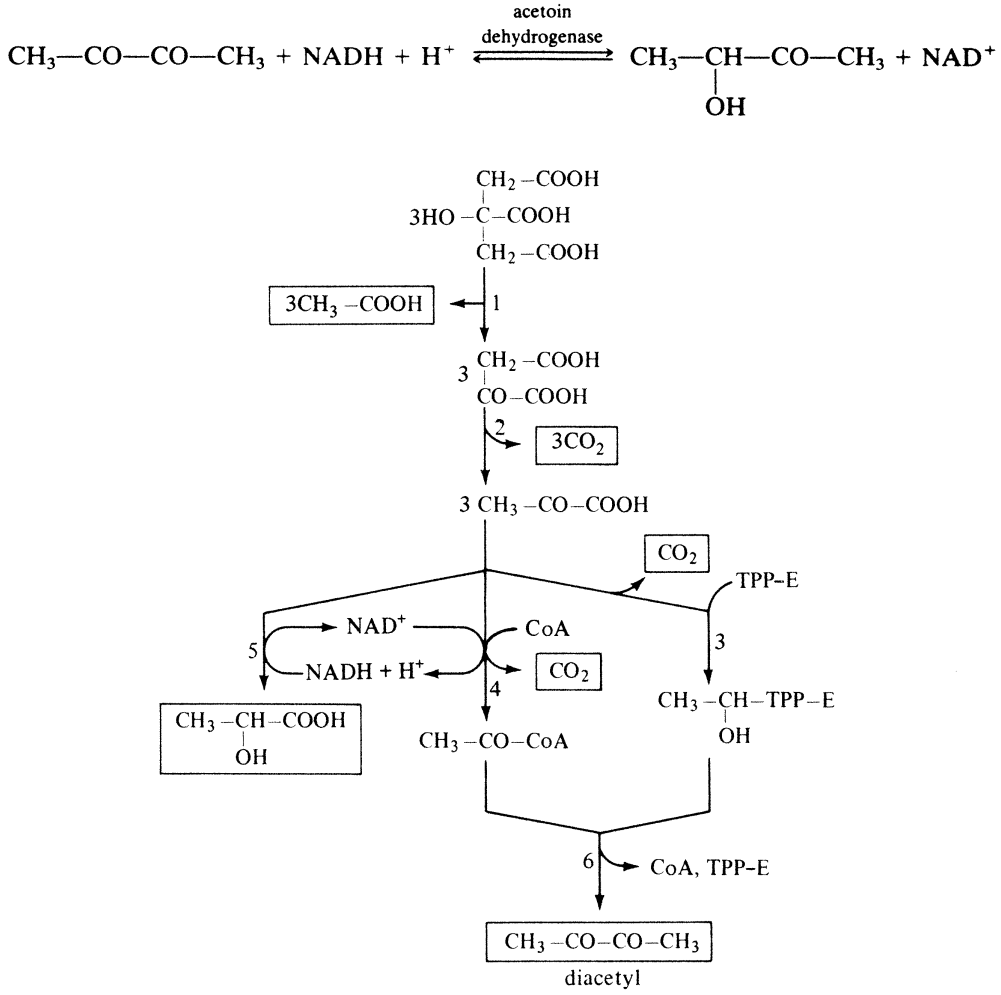


Figure 8.5. Formation of diacetyl from citrate by lactic acid bacteria. 1, Citrate lyase; 2, oxaloacetate decarboxylase; 3, enzyme not characterized; 4, pyruvate dehydrogenase complex; 5, lactate dehydrogenase; 6, diacetyl synthase. Instead of pyruvate dehydrogenase the other pyruvate-degrading enzymes mentioned in the text may be involved as well.

Whereas diacetyl synthesis according to Fig. 8.5 does not yield any ATP, some ATP can be formed in the formation of acetoin. Because of the NADH consumption in the last step only 0.5 lactate, but additional 0.5 acetate (via acetyl-CoA), are produced.

Diacetyl synthesis requires the conversion of pyruvate into C_2 compounds. Moreover, all homofermentative lactic acid bacteria have to synthesize acetyl-CoA for biosynthetic purposes from pyruvate. The occurrence of three enzyme systems that yield acetyl-CoA or acetyl phosphate from pyruvate have been demonstrated in lactic acid bacteria. Streptococci contain the pyruvate dehydrogenase multienzyme complex. In addition, the pyruvate-formate lyase system, which will be discussed in Section IV of this chapter, has been detected in *S. faecalis*, in *Bifidobacterium bifidum*, and in some lactobacilli (e.g., *L. casei*). Finally, *L. delbrückii* and *L. plantarum* carry out a dismutation of pyruvate to lactate and acetyl phosphate:

$$\text{pyruvate} + P_i + \text{FAD} \xrightarrow[\text{oxidase}]{\text{pyruvate}} \text{acetyl phosphate} + CO_2 + FADH_2$$

$$FADH_2 + \text{pyruvate} \xrightarrow[\text{oxidase}]{\text{lactate}} \text{lactate} + \text{FAD}$$

Pyruvate oxidase is a flavoprotein that contains thiamine pyrophosphate. Neither lipoate nor CoA is involved in acetyl phosphate formation. Lactate oxidase is also a flavoprotein, and the hydrogen transfer from one flavoprotein to the other is mediated by riboflavin.

H. Growth yields and growth in air

It has been outlined in Chapter 3 (see Table 3.2) that approximately 35 mmol of ATP are necessary for the synthesis of 1 g of cells from glucose and inorganic salts. Thus, about 30 g of cells can be formed per mol of ATP. This value does not account for the energy of maintenance and for other ATP expenditures of the living cell. However, if the amount of ATP required for these processes is constant or if the variations are small, a given amount of ATP should result in the formation of the same amount of cells independent of the species. That this is true was shown by Bauchop and Elsden. They determined a yield of 22 g *S. faecalis* cells per mol of glucose fermented (all monomers required for macromolecule synthesis were supplied with the medium). Since the homofermentative pathway yields 2ATP per glucose fermented, 11 g of cells were produced per mol of ATP:

S. faecalis growth yield: $Y_m = 22$ g/mol glucose

$Y_{ATP} = 11$ g/mol ATP

Zymomonas mobilis, which ferments glucose to ethanol and CO_2 via the Entner-Doudoroff pathway (1ATP per glucose; see Chapter 8, Section I),

yielded 8.3 g of cells per mol of glucose:

Z. mobilis growth yield: $Y_m = 8.3$ g/mol glucose

$Y_{ATP} = 8.3$ g/mol ATP

Thus, an organism gaining half the amount of ATP from glucose (compared to *S. faecalis*) formed approximately half the amount of cells. A Y_{ATP} value of 10.5 for growth with all the monomers available for biosynthesis is now generally accepted. It should be indicated, however, that growth yield determinations in continuous culture are much more reliable and allow more precise calculations of the amount of ATP synthesized per mol of substrate consumed.

It has already been mentioned that many lactic acid bacteria are aerotolerant. These organisms contain superoxide dismutase but lack a true catalase. Instead, peroxidases are present, which catalyze the oxidation of organic compounds (alcohols, aldehydes) or of NADH with H_2O_2:

$$NADH + H^+ + H_2O_2 \xrightarrow{\text{NADH peroxidase}} NAD^+ + 2H_2O$$

However, some lactic acid bacteria, notably *S. faecalis*, *L. brevis*, and *L. plantarum* form a true catalase when they are grown in the presence of hemin. Thus, they are only unable to synthesize the heme prosthetic group of catalase.

When aerotolerant lactic acid bacteria are grown in air, the growth yield is usually much higher than under anaerobic conditions. For *S. faecalis* Y_m increases from 22 to 52. This increase is partly due to a change of the fermentation pattern:NADH is oxidized with oxygen; pyruvate can be converted to acetyl-CoA and additional ATP can be formed by the acetate kinase reaction. Thus, oxygen changes the lactate fermentation into an acetate fermentation. There are, however, also indications that *S. faecalis* is capable of oxidative phosphorylation to a limited extent. In the presence of hemin and in air cytochromes are formed by this organism and by some other streptococci. So it appears that some lactic acid bacteria are not truly obligately anaerobic bacteria.

I. Energetics of lactate export

Lactic acid is a stronger acid than acetic and butyric acids. The pK values of these three acids are 3.86 (lactic), 4.75 (acetic), and 4.82 (butyric), respectively. This difference is one reason why lactic acid bacteria reach lower pH values during growth than anaerobes producing acetic or butyric acid. This can be explained as follows: The membrane of obligate anaerobes has to be energized like that of aerobes. This means that a protonmotive force has to be continuously generated. In lactic acid bacteria which lack membrane-bound electron transport chains, ATP hydrolysis as catalyzed by the proton-translocating ATPase is the principal

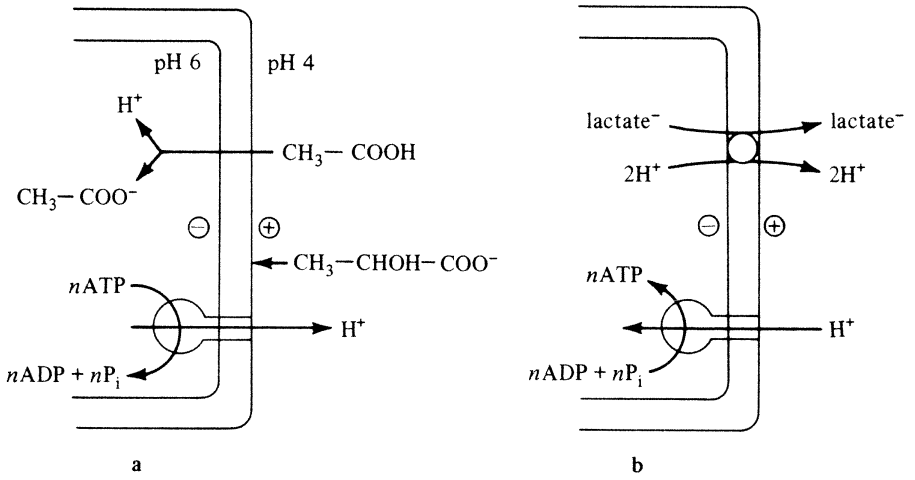


Figure 8.6. Function of weak acids as uncouplers at low pH (**a**), coupling of lactate efflux with ΔP generation (**b**).

mechanism for ΔP generation (see Chapter 2). At a pH of, e.g., 4, acetic and butyric acids are present in the medium in the undissociated forms. These undissociated acids are lipophilic and can penetrate the membrane. At an intracellular pH of, e.g., 6, the acids will dissociate again; more acid diffuses in, and the ΔpH between outside and inside will finally be evened (Fig. 8.6a). Thus, undissociated organic acids function as uncouplers at pH values around or below their pK values. Since lactic acid is a stronger acid than acetic or butyric acids, its uncoupling function comes into play only at a very low pH (pH $\sim$ 3.5).

Recently, Konings and co-workers have demonstrated that at least some lactic acid bacteria (shown for *Streptococcus cremoris*) have a second mechanism for ΔP generation at their disposal. As depicted in Fig. 8.6b, lactate export is electrogenic; two protons are excreted per one molecule of lactate, and a ΔpH is generated that can be used to drive symport processes or ATP synthesis. This systems's driving force is the inside/outside lactate gradient. Thus, it can be employed only at low extracellular lactate concentrations. Such conditions exist when lactic acid bacteria coexist with organisms consuming lactate.

III. Butyrate and Butanol-Acetone Fermentation

The fermentation of sugars to butyric acid was discovered by Pasteur in 1861. Soon after, microorganisms responsible for the formation of butyrate were isolated, and it was found that several clostridial species carried out this type of fermentation. Generally, only obligate anaerobes form butyrate as a main fermentation product; they belong to the four genera *Clostridium*, *Butyrivibrio*, *Eubacterium*, and *Fusobacterium* (Table 8.4).

Table 8.4. Some species forming butyrate as a major fermentation end product

Clostridium butyricum
C. kluyveri
C. pasteurianum
Butyrivibrio fibrisolvens
Eubacterium limosum
Fusobacterium nucleatum

The mechanism of butyrate formation was not well understood until Barker and collaborators did their pioneering studies on *C. kluyveri* and until ferredoxin was discovered in *C. pasteurianum*. The clostridia employ phosphotransferase systems for sugar uptake and the Embden–Meyerhof–Parnas pathway for degradation of hexose phosphates to pyruvate. The conversion of pyruvate to acetyl-CoA involves an enzyme system not discussed thus far: pyruvate-ferredoxin oxidoreductase.

A. Ferredoxin and pyruvate-ferredoxin oxidoreductase

When a cell extract of *C. pasteurianum* is incubated with pyruvate under anaerobic conditions the decomposition of pyruvate according to the following equation can be observed:

$$CH_3\text{–}CO\text{–}COOH + H_3PO_4 \longrightarrow CH_3\text{–}CO\text{–}OPO_3H_2 + CO_2 + H_2$$

Acetyl phosphate is formed and hydrogen gas is evolved. This reaction is known as the **phosphoroclastic reaction**. A thorough investigation of the enzymes involved resulted in the finding that this phosphorylytic cleavage is the sum of several reactions; they are summarized in Fig. 8.7. Pyruvate is first decarboxylated by pyruvate-ferredoxin oxidoreductase; the remaining C_2-moiety is covalently bound to the TPP-containing enzyme as in the pyruvate decarboxylase and the pyruvate dehydrogenase reactions. In the next step acetyl-CoA is formed; in contrast to pyruvate dehydrogenase the two hydrogens are not transferred to NAD^+, but are used to reduce ferredoxin. This difference is important because ferredoxin has a very low redox potential; at pH 7.0 it is about the same as that of the hydrogen electrode:

$H_2/2H^+$	$E'_0 = -0.42$ V
red. ferredoxin/ox. ferredoxin	$E'_0 = -0.41$ V
$NADH + H^+/NAD^+$	$E'_0 = -0.32$ V

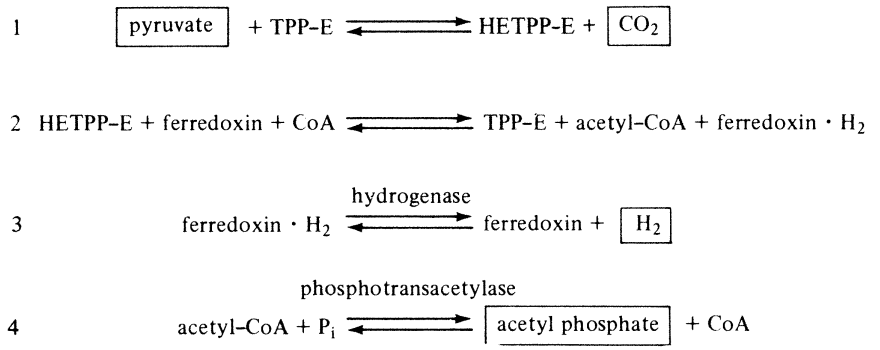


Figure 8.7. Steps of phosphoroclastic reaction. Steps 1 and 2 involve the enzyme pyruvate-ferredoxin oxidoreductase and ferredoxin. TPP-E, thiamine pyrophosphate-containing oxidoreductase; HETPP-E, hydroxyethyl-TPP-E. Steps 3 and 4 are catalyzed by hydrogenase and phosphotransacetylase, respectively.

Consequently, even in an environment saturated with hydrogen gas, reduced ferredoxin can transfer electrons to hydrogenase and hydrogen can be evolved. This is what is observed when clostridia ferment carbohydrates. Moreover, the pyruvate-ferredoxin oxidoreductase is reversible and under appropriate conditions pyruvate can be synthesized from acetyl-CoA, reduced ferredoxin, and CO_2 (see *C. kluyveri*).

NADH is a much weaker reducing agent than reduced ferredoxin. This is one reason why the pyruvate dehydrogenase reaction is not reversible.

Ferredoxin was discovered in *C. pasteurianum* by Mortenson, Valentine, and Carnahan in 1962. It belongs to the **iron-sulfur proteins** (until recently called nonheme-iron proteins) and it should be remembered that this class of proteins plays an important role in the respiratory chain (see Figs. 2.7 and 2.11). Clostridial ferredoxins have a molecular weight of 6000 and contain eight iron atoms per molecule, which are arranged in the form of two cubanelike iron-sulfur clusters, [4Fe-4S]-clusters. One of these clusters is shown in Fig. 8.8. The iron atoms are bound to cysteine residues of the peptide chain and they are interconnected by sulfide bridges. So ferredoxin contains eight sulfide bridges; upon acidification of ferredoxin solutions they are liberated as H_2S. This sulfur is therefore called labile sulfur. Clostridial ferredoxins are 2-electron carriers (one electron per

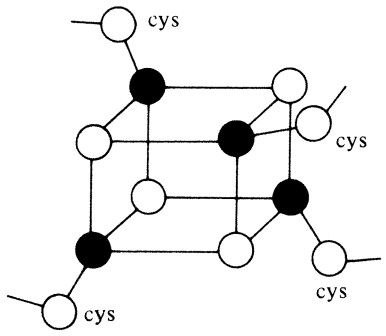


Figure 8.8. [4Fe-4S]-cluster of ferredoxin. ●, Fe atoms; ○, sulfur atoms.

cluster). Because of their brownish color they are easily recognized in cell extracts and during fractionation of such extracts.

It should be emphasized that not all ferredoxins have the same general composition. The span of variation is apparent from Table 8.5. It can be seen that the *C. thermoaceticum* ferredoxin differs from the typical clostridial ones in that it contains only one [4Fe-4S] cluster. The *Chromatium* ferredoxin is larger and the one of *D. gigas* harbors even three clusters. In *E. coli*, in *A. vinelandii*, and in many other aerobes we find iron-sulfur proteins with [2Fe-2S] clusters. The occurrence of even [3Fe-3S] clusters in sulfate-reducing bacteria has been reported.

All the [4Fe-4S]-ferredoxins mentioned have in common that they are low-potential electron carriers. The redox state of their reduced form is $3Fe^{2+} + 1Fe^{3+}$ and that of their oxidized form is correspondingly $2Fe^{2+} + 2Fe^{3+}$. Another class of iron-sulfur protein is present in many bacteria, the high-potential iron proteins called HiPiP. Their redox potential is in the order of +350 mV; the HiPiP's reduced form contains $2Fe^{2+} + 2Fe^{3+}$ and the oxidized form $1Fe^{2+} + 3Fe^{3+}$. This difference makes the difference in redox potential understandable.

The investigation of composition and structure of purified enzymes in recent years has shown that many of them contain iron-sulfur centers as well. Examples are hydrogenase, formate dehydrogenase, sulfite reductase, pyruvate: ferredoxin oxidoreductase, CO_2 reductase, nitrogenase, CO dehydrogenase, CO oxidase, trimethylamine dehydrogenase, dihydroorotate dehydrogenase, glutamate synthase, and xanthine dehydrogenase.

One other protein is known that is also a low-potential electron carrier but differs in other aspects: **flavodoxin**. It is formed by several obligate anaerobic bacteria when they grow in iron-limited media. Iron and labile sulfide are absent and flavin mononucleotide functions as redox group. The

Table 8.5. Properties of various ferredoxins

Organism	molecular weight	cluster	No. of clusters	E_0' (mV)
Clostridium pasteurianum	6000	4Fe-4S	2	−390
C. acidiurici	6000	4Fe-4S	2	−430
C. thermoaceticum	7500	4Fe-4S	1	ND
Chromatium vinosum	10,000	4Fe-4S	2	−480
Desulfovibrio gigas	18,000	4Fe-4S	3	−455
Escherichia coli	12,000	2Fe-2S	1	−380
Azotobacter vinelandii	21,000	2Fe-2S	1	−225
Chromatium vinosum HiPiP[a]	10,000	4Fe-4S	1	+35

[a] HiPiP, High-potential iron protein; ND, not determined

redox potential lies around −0.4 V. Finally it should be mentioned that **rubredoxin** occurs in many anaerobes. This redox carrier contains per redox center one iron, which is bound to four cysteine residues of the polypeptide chain. Thus, rubredoxin does not contain labile sulfide. Its redox potential lies around −0.06 V. The function of rubredoxin in anaerobes is unknown; in some aerobes it is involved in alkane oxidation (see Fig. 6.11).

B. The path of butyrate formation

The reactions involved in butyrate formation from glucose are summarized in Fig. 8.9. The Embden–Meyerhof–Parnas pathway and pyruvate-ferredoxin oxidoreductase yield 2 acetyl-CoA, $2CO_2$, and 2 reduced ferredoxins, which are reconverted to the oxidized form by hydrogenase under hydrogen evolution. Furthermore, $2NAD^+$ are reduced and 2ATP are formed. The advantage of hydrogen evolution in the course of pyruvate breakdown is apparent; only 2NADH are formed in the degradation of glucose to acetyl-CoA.

The NADH formed in the glyceraldehyde-3-phosphate dehydrogenase reaction is oxidized by the conversion of 2 acetyl-CoA into butyrate. In this pathway acetoacetyl-CoA, L(+)-β-hydroxybutyryl-CoA, and crotonyl-CoA are intermediates. It should be remembered that the storage material poly-β-hydroxybutyric acid is a polymer of the D(−)-form (see Fig. 5.20) and that the thioester of the D(−)enantiomer is an intermediate in long-chain fatty acid synthesis.

Butyrate is not formed from butyryl-CoA by simple hydrolysis; this would be a waste of energy. Instead butyryl-CoA is converted to butyryl phosphate, and finally the phosphate group is transferred to ADP. These reactions proceed under catalysis of the enzymes phosphotransbutyrylase and butyrate kinase, which are analogous to phosphotransacetylase and acetate kinase.

Figure 8.9 shows that the ATP yield of the butyrate fermentation is 3ATP/glucose; this is more than was gained in the fermentations discussed thus far.

C. The formation of acetone and butanol

A number of butyrate producing clostridia form small amounts of *n*-butanol. With a few species, however, a real shift from butyrate production to solvent production (*n*-butanol and acetone or isopropanol) can be observed under certain conditions. These species include *C. acetobutylicum*, *C. beijerinckii*, *C. tetanomorphum*, and *C. aurantibutyricum*. The most prominent species among these is *C. acetobutylicum*, which has been used on an industrial scale for the synthesis of *n*-butanol and acetone from molasses. Since *n*-butanol is very toxic (it interferes with membrane

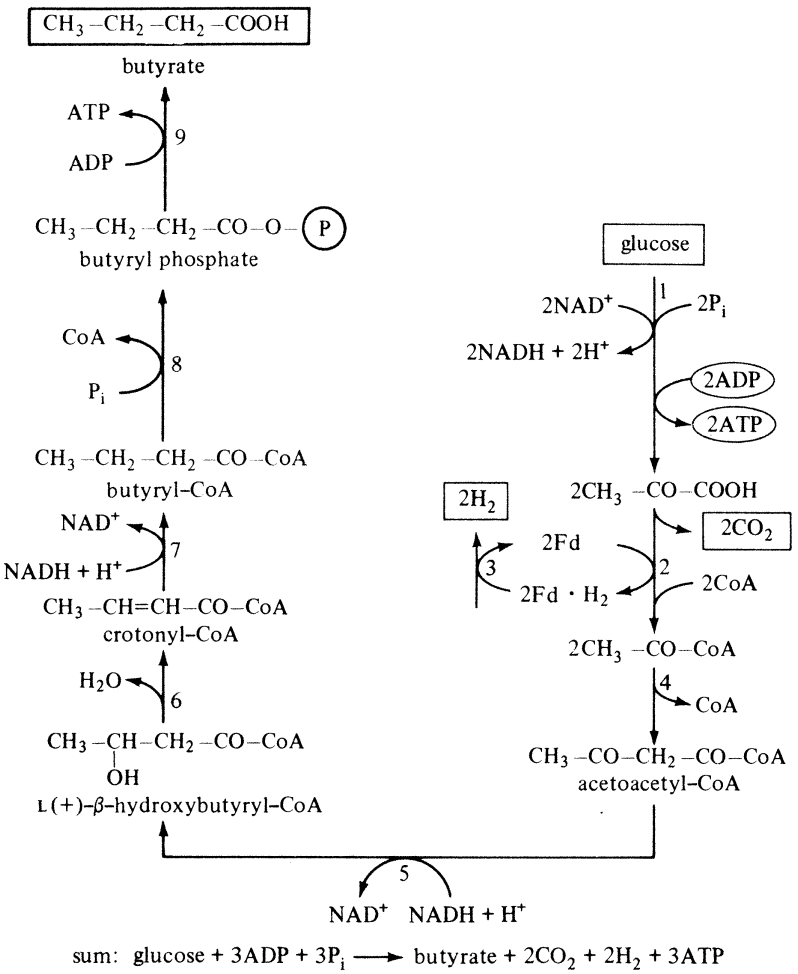


Figure 8.9. Path of butyrate formation from glucose. 1, Phosphotransferase system and Embden–Meyerhof–Parnas pathway; 2, pyruvate-ferredoxin oxidoreductase; 3, hydrogenase; 4, acetyl-CoA-acetyltransferase (thiolase); 5, L(+)-β-hydroxybutyryl-CoA dehydrogenase; 6, L-3-hydroxyacyl-CoA hydrolyase (crotonase); 7, butyryl-CoA dehydrogenase; 8, phosphotransbutyrylase; 9, butyrate kinase.

functions) the final solvent concentration reached is relatively low, in the order of 2%.

Figure 8.10 illustrates the course of product formation during growth of *C. acetobutylicum*. Towards the middle of the fermentation when the pH had dropped below 5, acids are no longer produced, and *n*-butanol and acetone appear as new products. During the solvent phase the butyrate concentration decreases again and an increase of the pH is recorded. Acetate stays relatively constant. The shift from acid to solvent production is not only a response of the cells to the low pH. Cultures growing very rapidly often "miss" the shift and produce acids only. Recent results indicate that the shift and the initiation of sporulation are connected to one another.

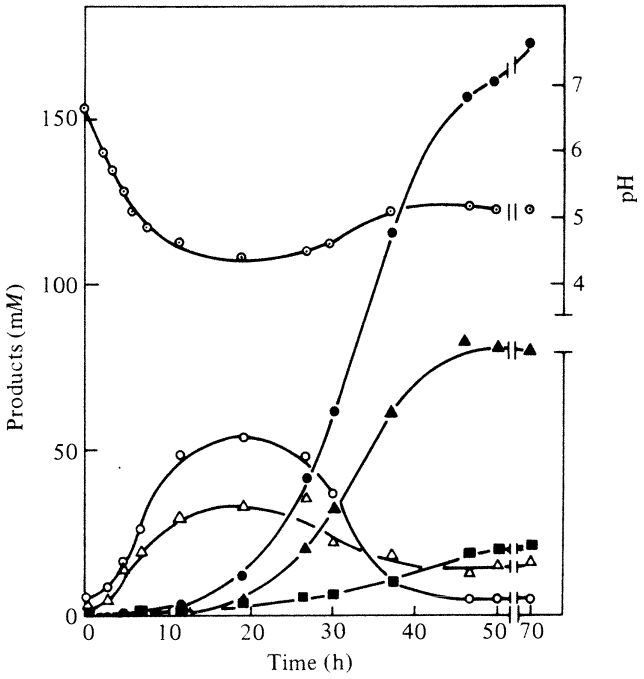


Figure 8.10. Course of fermentation in a batch culture of *C. acetobutylicum* at low phosphate concentration (0.62 mM). Butanol, ●; acetone, ▲; ethanol, ■; butyrate, ○; acetate, △; pH, ⊙. [H. Bahl, W. Andersch, G. Gottschalk. *Eur. J. Appl. Microbiol. Biotechnol.* **15**, 201–205 (1982).]

The reactions involved in acetone and butanol formation are summarized in Fig. 8.11. Under shift conditions two enzyme activities appear in the cells that convert acetoacetyl-CoA into acetone: a coenzyme A transferase and acetoacetate decarboxylase. Likewise, two enzymes are formed that reduce butyryl-CoA to *n*-butanol. The ratio in which butanol and acetone are formed lies in the order of 2:1.

Table 8.6. Fermentation balances of clostridia[a]

products	*C. butyricum*	*C. perfringens*	*C. acetobutylicum*
	(amounts formed in mol/100 mol glucose fermented)		
butyrate	76	34	4
acetate	42	60	14
lactate	—	33	—
CO_2	188	176	221
H_2	235	214	135
ethanol	—	26	7
butanol	—	—	56
acetone	—	—	22

[a] W. A. Wood, In: *The Bacteria*, I. C. Gunsalus and R. Y. Stanier (eds.). Academic Press, New York and London, 1961, vol. 2, pp. 59–149.

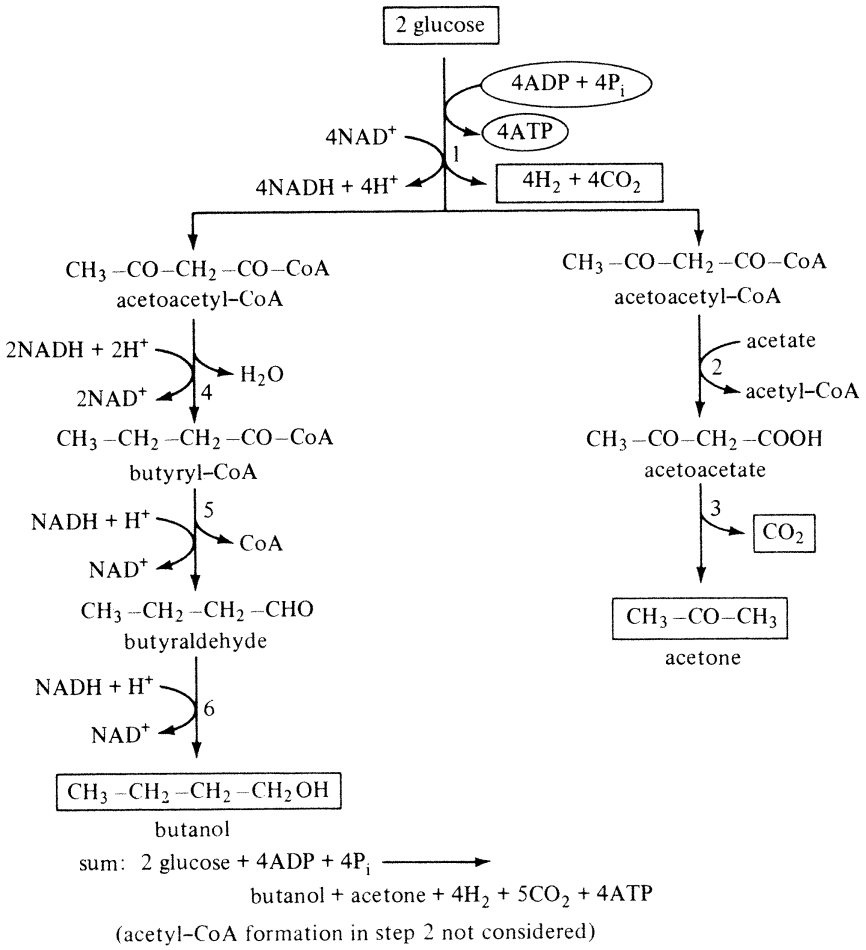


Figure 8.11. Formation of acetone and butanol by *C. acetobutylicum*. 1, Reactions as in Figure 8.9; 2, acetoacetyl-CoA: acetate coenzyme A transferase; 3, acetoacetate decarboxylase; 4, L(+)-β-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase; 5, butyraldehyde dehydrogenase; 6, butanol dehydrogenase.

The acetone-butanol fermentation is an interesting process, and it may become of economic importance again in the future.

D. Fermentation balances

Table 8.6 gives fermentation balances for *C. butyricum*, *C. perfringens*, and *C. acetobutylicum*. It can be seen that the fermentation schemes depicted in Figs. 8.9 and 8.11 are not exactly followed. *C. butyricum* forms acetate and some extra hydrogen; *C. perfringens* forms lactate and ethanol as do many saccharolytic clostridia. In the *C. acetobutylicum* fermentation the amount of H_2 evolved is largely diminished because of the reutilization of butyrate for butanol synthesis.

In complex fermentations such as the one carried out by *C. acetobutylicum* it is difficult to judge whether the hydrogen balance is even or not.

Therefore, the **oxidation/reduction (O/R) balance** of complex fermentations is determined, as illustrated in Table 8.7. Arbitrarily the O/R value of formaldehyde and multiples thereof are taken as zero. Each 2H in excess is expressed as −1, and a lack of 2H is expressed as +1. Ethanol has the sum formula C_2H_6O; addition of H_2O gives $C_2H_8O_2$. In comparison to $C_2H_4O_2$, 4H are in excess (O/R value = −2). From CO_2 water is subtracted; in C(−2H)O four hydrogens are missing as compared to CH_2O (O/R value = +2).

Alternatively, such calculations can be done on the basis of the **number of available hydrogen** in the substrate and in the products. This number is determined by oxidizing the compound to CO_2 with water ($C_6H_{12}O_6 + 6H_2O \rightarrow$ **24H** $+ 6CO_2$; $C_3H_6O + 5H_2O \rightarrow$ **16H** $+ 3CO_2$).

E. Linear and branched fermentative pathways

Product formation in the alcohol or the homolactate fermentation can be described by linear pathways. The substrate:product ratios and also the thermodynamic efficiencies of these fermentations are invariable. Thauer and colleagues distinguished from them the branched fermentative pathways. Here, either a more oxidized product resulting in a comparatively

Table 8.7. Carbon recovery, O/R balance, and balance of available hydrogen of an acetone-butanol fermentation[a]

			O/R balance		balance of available hydrogen	
substrate and products	mol/100 mol substrate	mol carbon	O/R value	O/R value (mol/100 mol)	available H	available H (mol/100 mol)
glucose	100	600	0	–	24	2400
butyrate	4	16	−2	−8	20	80
acetate	14	28	0	–	8	112
CO_2	221	221	+2	+442	0	—
H_2	135	—	−1	−135	2	270
ethanol	7	14	−2	−14	12	84
butanol	56	224	−4	−224	24	1344
acetone	22	66	−2	−44	16	352
total		569		−425 +442		2242

[a] Carbon recovered: 569/600 × 100 = 94.8%; O/R balance: 442/425 = 1.04; balance of available H: 2400/2242 = 1.07. The latter two values are different because the carbon recovery affects the balance of available H but not the O/R balance (O/R value of glucose = 0).

high ATP yield or a reduced product resulting in a low ATP yield can be formed. The ratio in which the two products are formed depends on the portion of NADH that can be oxidized by the organisms to NAD^+ under H_2 evolution. A good example for a branched pathway is the formation of butyrate and acetate by *C. butyricum*. According to the pathway depicted in Fig. 8.9, the fermentation balance is even if butyrate only is produced. Table 8.6, however, teaches us that acetate is among the products and that more than 2 mol H_2 per mol of glucose are produced. The explanation for this is depicted in Fig. 8.12: H_2 evolution from NADH makes acetate excretion possible. Since 1ATP can be synthesized from 1 acetyl-CoA through conversion to acetate and only 1/2ATP through conversion to butyrate, the evolution of more H_2 and the excretion of acetate increase the thermodynamic efficiency of the pathway.

Since the redox potential of NADH is more positive than the one of H_2 and of ferredoxin, the enzyme NADH:ferredoxin oxidoreductase can work only in the direction of ferredoxin reduction at a low partial pressure of H_2 (see section IX of this chapter) or when driven by some type of reverse electron transport. It has been shown that the reverse direction (reduction of NAD^+ by reduced ferredoxin) is strongly inhibited by NADH. In other words, the system functions like a valve. H_2 formation from NADH is allowed; NAD^+ reduction by H_2 is prevented:

$$\text{NADH} \underset{\text{NADH}}{\rightleftharpoons} \text{red Fd} \rightleftharpoons H_2$$

Branched fermentative pathways provide organisms with more flexibility and allow them to adjust the thermodynamic efficiency of their catabolism according to the possibilities to oxidize reduced coenzymes under H_2 evolution.

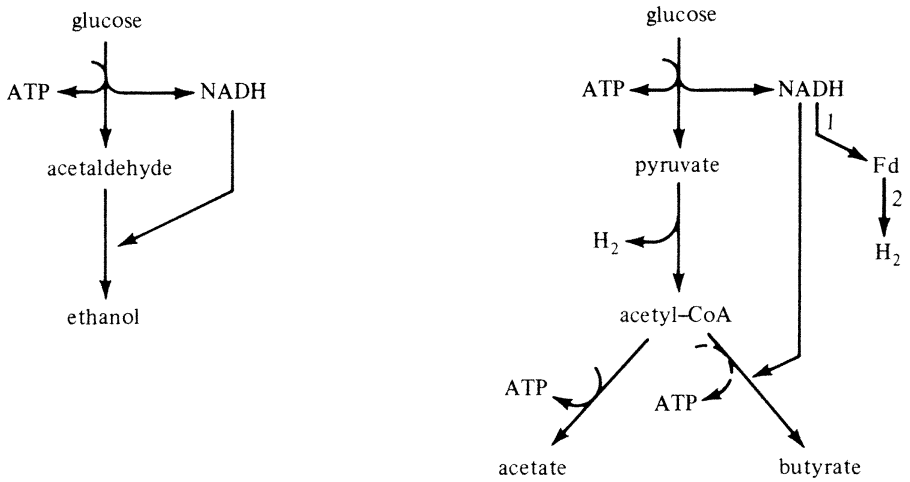


Figure 8.12. Alcohol fermentation and butyrate-acetate fermentation as examples of linear and branched pathways. 1, Reaction catalyzed by NADH:ferredoxin oxidoreductase; 2, hydrogenase reaction. [Modified from R. K. Thauer, K. Jungermann, K. Decker; *Bacteriol. Rev.* **41**, 100–180 (1977)].

F. Not all saccharolytic clostridia form butyrate

Clostridia are always brought into close connection with the butyrate fermentation; and it is, therefore, important to stress that several saccharolytic clostridia do not produce butyrate at all. *C. sphenoides*, for instance, ferments glucose to ethanol, acetate, CO_2, hydrogen and small amounts of lactate. Some additional clostridial species represent the same fermentation type: *C. glycolicum*, *C. indolis*, *C. thermohydrosulfuricum*, and the cellulolytic species *C. cellobioparum* and *C. thermocellum*. Furthermore, a few clostridial species (*C. thermoaceticum* and *C. formicoaceticum*) ferment hexose to almost three acetate; they will be discussed in a separate section of this chapter.

G. The ethanol-acetate fermentation of *Clostridium kluyveri*

C. kluyveri carries out a very interesting fermentation. It produces butyrate, caproate, and hydrogen from ethanol and acetate. Ethanol alone is not fermented, but acetate can be replaced by propionate. The ratio in which butyrate and caproate are formed can vary; an increase of the ethanol concentration of the medium favors caproate formation. A typical fermentation balance is:

$$6\ \text{ethanol} + 3\ \text{acetate} \longrightarrow 3\ \text{butyrate} + 1\ \text{caproate} + 2H_2 + 4H_2O + H^+$$

Approximately 0.3 mol of hydrogen is evolved per mol of ethanol fermented.

How *C. kluyveri* gains ATP for growth is difficult to see. The principle is that per mol of hydrogen evolved, 0.5 mol of acetyl-CoA is not required as H-acceptor as has been shown by Decker and collaborators. This acetyl-CoA serves to form ATP. The reactions involved are summarized in Fig. 8.13. For the sake of simplicity only the formation of butyrate and H_2 is considered. Reactions involved in caproate formation are analogous (butyryl-CoA condenses with acetyl-CoA, 3-oxocaproyl-CoA is reduced, water is removed, and subsequent reduction yields caproyl-CoA).

Alcohol and acetaldehyde dehydrogenases are associated with one another and are particle bound. The first enzyme is NAD^+-specific and the second one reacts with either NAD^+ and/or $NADP^+$. Hydrogen is evolved in the course of these reactions by a mechanism not fully understood. Presumably electrons are transferred from NADH to hydrogenase via ferredoxin as outlined in section E. A NADH:ferredoxin oxidoreductase has been demonstrated in cell extracts of *C. kluyveri*. L(+)-β-Hydroxybutyryl-CoA dehydrogenase is $NADP^+$-specific, which is unique among butyrate-forming organisms. It indicates a close coupling between acetaldehyde oxidation and acetoacetyl-CoA reduction.

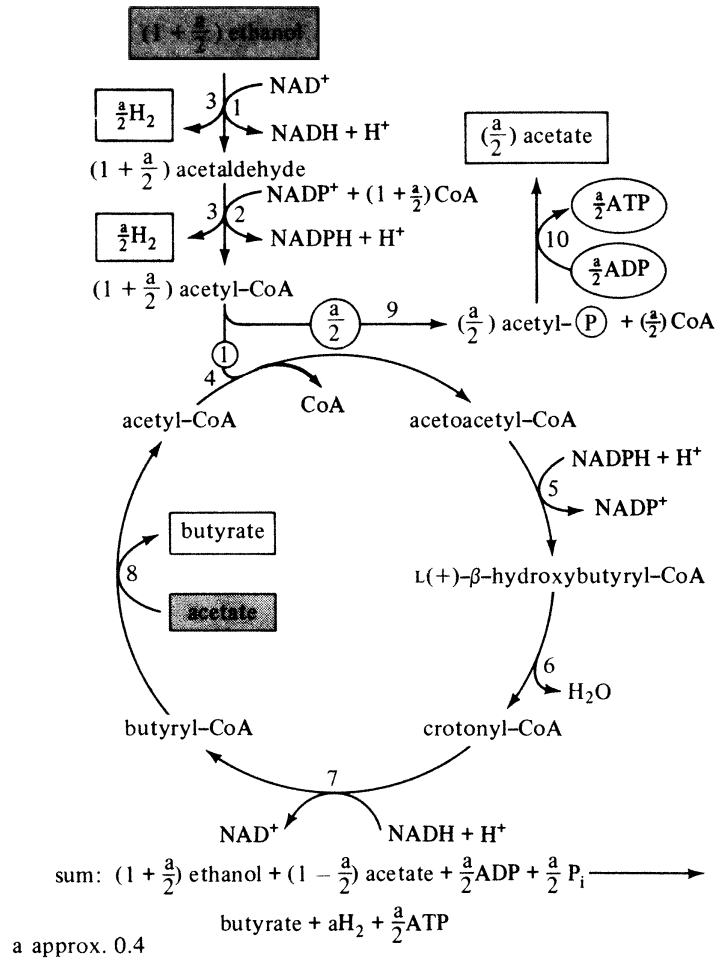


Figure 8.13. The ethanol-acetate fermentation of *C. kluyveri*. 1, Alcohol dehydrogenase; 2, acetaldehyde dehydrogenase; 3, H_2-evolving enzyme system; 4, thiolase; 5, L(+)-β-hydroxybutyryl-CoA dehydrogenase (NADP-specific); 6, crotonase; 7, butyryl-CoA dehydrogenase; 8, CoA transferase; 9, phosphotransacetylase; 10, acetate kinase.

In Figure 8.13 "a" has a value of about 0.4 so that approximately 0.4 mol H_2 are evolved per 1.2 mol ethanol consumed. From the 1.2 mol of acetyl-CoA formed, 1 mol is required for NADH and NADPH oxidation in the butyric acid cycle; 0.2 mol is available for ATP formation. Thus, the ATP yield is 0.2/1.2 or 1ATP per 6 ethanol.

H. Aspects of biosynthetic metabolism of *Clostridium kluyveri*

Although *C. kluyveri* grows on C_2 compounds, it does not contain a glyoxylate cycle for the synthesis of C_4 dicarboxylic acids. Instead, it takes advantage of the reversibility of the pyruvate-ferredoxin oxidoreductase

reaction. Under the reducing conditions of the ethanol-acetate fermentation the reductive carboxylation of acetyl-CoA is feasible:

$$CH_3CO\text{–}SCoA + CO_2 + Fd \cdot H_2 \rightleftharpoons CH_3\text{–}CO\text{–}COOH + HSCoA + Fd$$

Pyruvate is trapped by formation of alanine or by further carboxylation to oxaloacetate by pyruvate carboxylase. The importance of carboxylation reactions in biosynthetic metabolism of *C. kluyveri* is underlined by the fact that *C. kluyveri* requires CO_2 for growth. About 30% of its cellular material is derived from CO_2. In agreement with the above-mentioned carboxylation reactions, the alanine carboxyl group and both carboxyl groups of aspartate originate quantitatively from CO_2. Since *C. kluyveri* contains also a pyruvate-formate lyase, the carboxyl group of pyruvate is also the precursor of formate, which is the starting material for C_1 units in biosynthesis.

Interestingly, *C. kluyveri* uses enzymes of the tricarboxylic acid cycle to synthesize glutamate; it contains citrate synthase, *cis*-aconitase, isocitrate dehydrogenase, and glutamate dehydrogenase, and so do many other obligate anaerobic bacteria. This emphasizes the dual function of the tricarboxylic acid portion of the cycle: provision of NADH as part of the complete cycle (aerobes) and provision of α-oxoglutarate (aerobes and anaerobes).

The citrate synthase of *C. kluyveri* and a few other anaerobes (*C. acidiurici*, *C. cylindrosporum*, some sulfate-reducing bacteria) differs in two respects from all other citrate synthases. It has a specific requirement for Mn^{2+} whereas all other citrate synthases do not require metal ions at all. Moreover, it differs in its stereospecificity (Fig. 8.14). Thus, a very small group of anaerobic bacteria contain a special enzyme, *re*-citrate synthase. All other organisms contain—as far as tested—the *si*-type synthase.

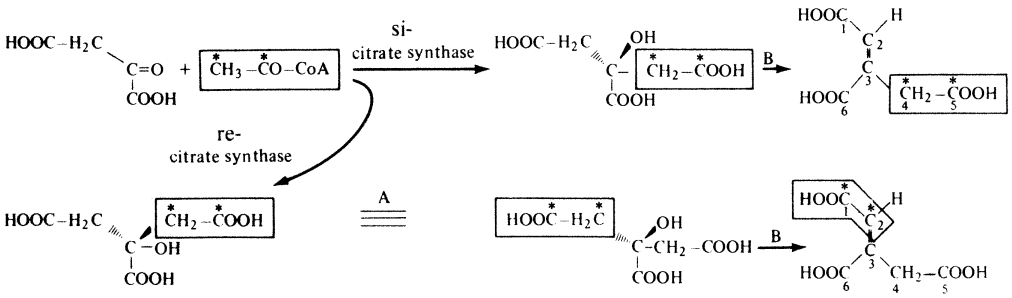


Figure 8.14. Formation of citrate by *si*-citrate synthase and by *re*-citrate synthase. Note that the carboxymethyl residue derived from acetyl-CoA comes into different positions. A, Molecule turned 120°; B, stereospecific removal of water by *cis*-aconitase allows distinction between the two carboxymethyl groups of radioactive citrate prepared. With ^{14}C-labeled acetyl-CoA, 4,5-^{14}C-*cis*-aconitate and 1,2-^{14}C-*cis*-aconitate are formed, respectively.

IV. Mixed Acid and Butanediol Fermentation

This type of fermentation is carried out by the enterobacteria. Organisms belonging to the genera *Escherichia*, *Salmonella*, and *Shigella* ferment sugars to lactic, acetic, succinic, and formic acids. In addition CO_2, H_2, and ethanol are formed. Species of the genera *Enterobacter*, *Serratia*, and *Erwinia* produce less acids but more gas (CO_2), ethanol, and above all large amounts of 2,3-butanediol. Two typical fermentation balances are given in Table 8.8, and the pathways leading to all these products are summarized in Fig. 8.15.

Enterobacteria employ the Embden–Meyerhof–Parnas pathway for hexose breakdown. The pathway leading to succinate branches off at phosphoenolpyruvate; all other end products are derived from pyruvate. Three enzyme systems act on pyruvate, and the amounts in which the fermentation products are formed depend very much on the activity of these enzyme systems. In the mixed acid fermentation large amounts of lactate are formed by the action of lactate dehydrogenase. Little lactate only is produced in the butanediol fermentation. The two other enzyme systems—pyruvate-formate lyase and α-acetolactate synthase–deserve special attention.

A. Pyruvate-formate lyase

The enterobacteria are able to synthesize two enzyme systems for pyruvate breakdown to acetyl-CoA. The pyruvate dehydrogenase multienzyme complex is involved in aerobic metabolism. Under anaerobic conditions it is no longer synthesized, and the enzyme still present is inhibited

Table 8.8. Products formed in the mixed acid and butanediol fermentation[a]

product	*Escherichia coli* (mol formed/100 mol glucose fermented)	*Enterobacter aerogenes* (mol formed/100 mol glucose fermented)
formate	2.4	17.0
acetate	36.5	0.5
lactate	79.5	2.9
succinate	10.7	—
ethanol	49.8	69.5
2,3-butanediol	0.3	66.4
CO_2	88.0	172.0
hydrogen	75.0	35.4

[a] W.A . Wood, In: *The Bacteria*, I. C. Gunsalus and R. Y. Stanier (eds.). Academic Press, New York and London, 1961, vol. 2, pp. 59–149.

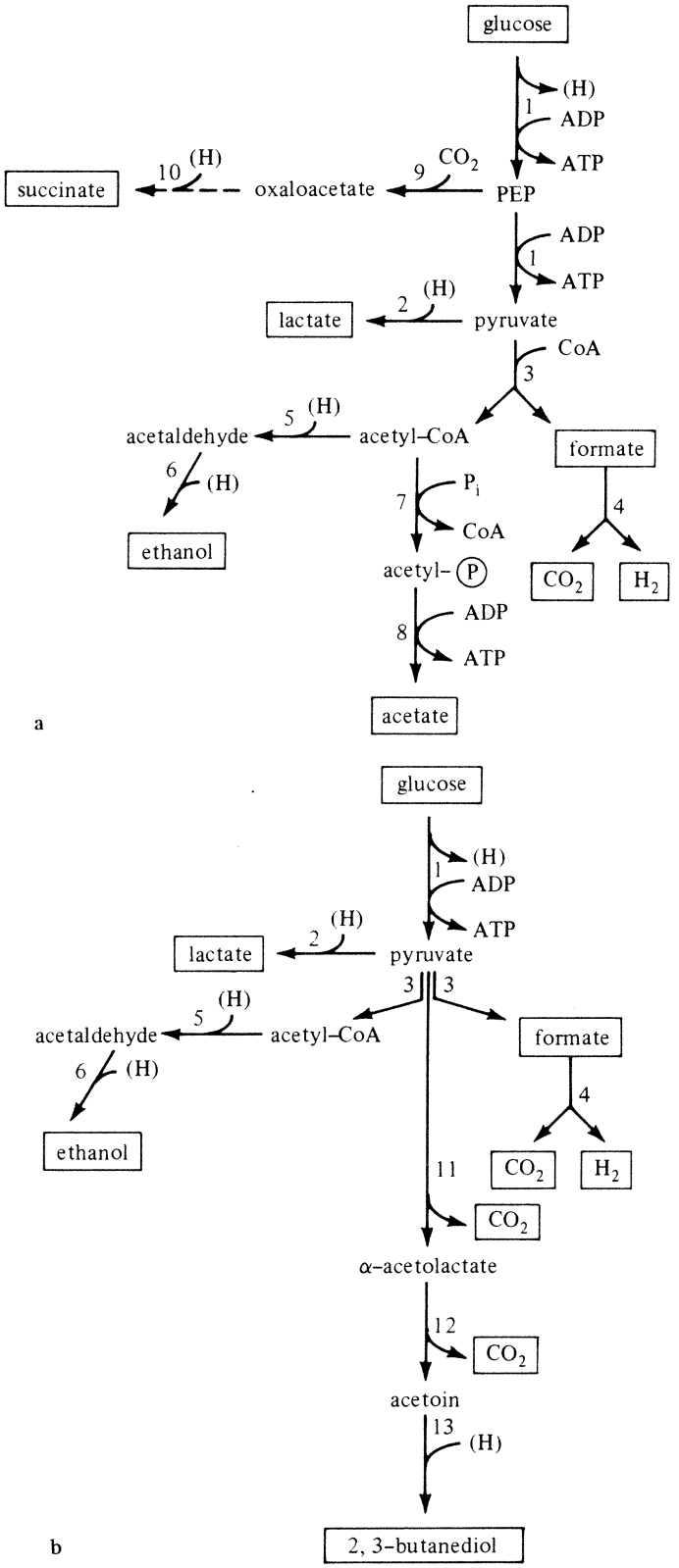


Figure 8.15. Mixed acid (**a**) and butanediol (**b**) fermentation. 1, Enzymes of the Embden–Meyerhof–Parnas pathway; 2, lactate dehydrogenase; 3, pyruvate-formate lyase; 4, formate-hydrogen lyase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, phosphotransacetylase; 8, acetate kinase; 9, PEP carboxylase; 10, malate dehydrogenase, fumarase, and fumarate reductase; 11, α-acetolactate synthase; 12, α-acetolactate decarboxylase; 13, 2,3-butanediol dehydrogenase.

by NADH. Instead, the synthesis of pyruvate-formate lyase is induced under anaerobic conditions. The reaction catalyzed by this enzyme proceeds in two steps with an acetyl-enzyme as intermediate and formate and acetyl-CoA as products:

$$CH_3\text{–}CO\text{–}COOH + \text{enzyme} \longrightarrow CH_3\text{–}CO\text{-enzyme} + HCOOH$$
$$CH_3\text{–}CO\text{-enzyme} + CoASH \longrightarrow \text{enzyme} + CH_3\text{–}CO\text{–}SCoA$$

Pyruvate-formate lyase is irreversibly and rapidly inactivated under air so that it functions only in fermentative metabolism of the enterobacteria. Apparently even under anaerobic conditions the active enzyme is not very stable; at low concentrations of pyruvate it changes over to an inactive form which can again be reactivated. This reactivation requires the presence of four components; reduced flavodoxin, activating enzyme (enzyme II), *S*-adenosylmethionine, and pyruvate. The latter is not consumed in the activation reaction; thus, it functions as positive effector.

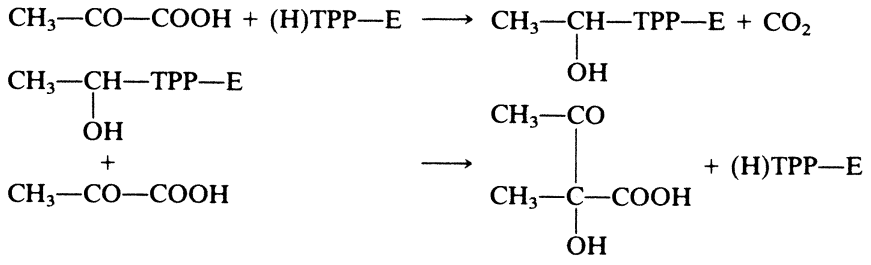
$$\begin{array}{c}\text{pyruvate-formate lyase}\\ \text{(inactive)}\\ +\\ \text{reduced flavodoxin}\\ +\\ \textit{S}\text{-adenosylmethionine}\end{array} \xrightarrow[\text{(pyruvate)}]{\text{enzyme II}} \begin{array}{c}\text{pyruvate-formate lyase}\\ \text{(active)}\\ +\\ \text{flavodoxin}\\ +\\ \text{methionine} + 5'\text{-deoxyadenosine}\end{array}$$

S-adenosylmethionine is reductively cleaved in this activation reaction. How and where the lyase actually is modified is not known.

The advantage of the pyruvate-formate lyase over the pyruvate dehydrogenase complex in fermentative metabolism is apparent: the formation of acetyl-CoA is not accompanied by the reduction of NAD^+.

B. α-Acetolactate synthase

The third enzyme system acting upon pyruvate is α-acetolactate synthase. This enzyme is also involved in 2,3-butanediol formation by bacilli (see Fig. 6.26). It contains thiamine pyrophosphate. First, enzyme-bound hydroxyethyl-thiamine pyrophosphate and CO_2 are formed from pyruvate. Active acetaldehyde is then transferred to a second molecule of pyruvate:



This synthase is formed and is active under slightly acidic conditions, and it is referred to as the pH 6 enzyme. Thus, a decrease of pH in the

environment of *Enterobacter aerogenes* leads to an increase of 2,3-butanediol formation. Consequently less acids can be produced from pyruvate.

The pH 6 enzyme is distinct from the anabolic α-acetolactate synthase, which is involved in valine synthesis. This enzyme is most active at pH 8 (pH 8 enzyme) and is subject to feedback inhibition of L-valine.

C. Formate-hydrogen lyase

Species belonging to the genera *Shigella* and *Erwinia* do not contain formate-hydrogen lyase; they produce considerable amounts of formate. *Escherichia coli* and *Enterobacter aerogenes* contain this activity when grown on sugars under anaerobic conditions, and formate can be cleaved into CO_2 and H_2.

Formate-hydrogen lyase is not one single enzyme entity. The formation of H_2 and CO_2 from formate is the result of the combined activity of a special formate dehydrogenase (FDH_H) and a hydrogenase. FDH_H is under redox control, and formate cleavage is observed only, if this compound cannot function as electron donor for the nitrate reductase or fumarate reductase. In other words, if nitrate or fumarate is present, formate is oxidized by another formate dehydrogenase (FDH_I), and from there the electrons are channelled to nitrate or fumarate, but not to H^+ (Fig. 8.16). Thus, H_2 evolution is not observed in the presence of nitrate or fumarate.

All the enzyme systems mentioned above are membrane-bound. The formate dehydrogenases are selenoproteins containing iron-sulfur centers, molybdenum in the form of Moco-factor and cytochrome *b*.

D. Decarboxylations coupled to membrane energization

Like several lactic acid bacteria, some enterobacteria are able to grow with citrate under anaerobic conditions. Citrate is cleaved by citrate lyase, and the oxaloacetate formed is decarboxylated to yield pyruvate (see section II.G of this chapter). Stern and collaborators showed a number of years ago that growth of *Enterobacter aerogenes* on citrate depended on the presence of sodium ions. Dimroth demonstrated that Na^+ was required by the oxaloacetate decarboxylase which is a biotin-containing and membrane-associated enzyme. Interestingly enough this enzyme manages to couple the decarboxylation reaction with the generation of an electrochemical gradient of sodium ions as depicted in Fig. 8.17. This gradient can be transformed into a pH gradient that can be taken advantage of by the ATP synthase. The decarboxylation of oxaloacetate is associated with a free energy change of $\Delta G^{0\prime} = -30$ kJ (-7.2 kcal) mol^{-1}, and one could expect synthesis of 1/3 ATP per 1 CO_2 formed. The uptake of 3 H^+ per

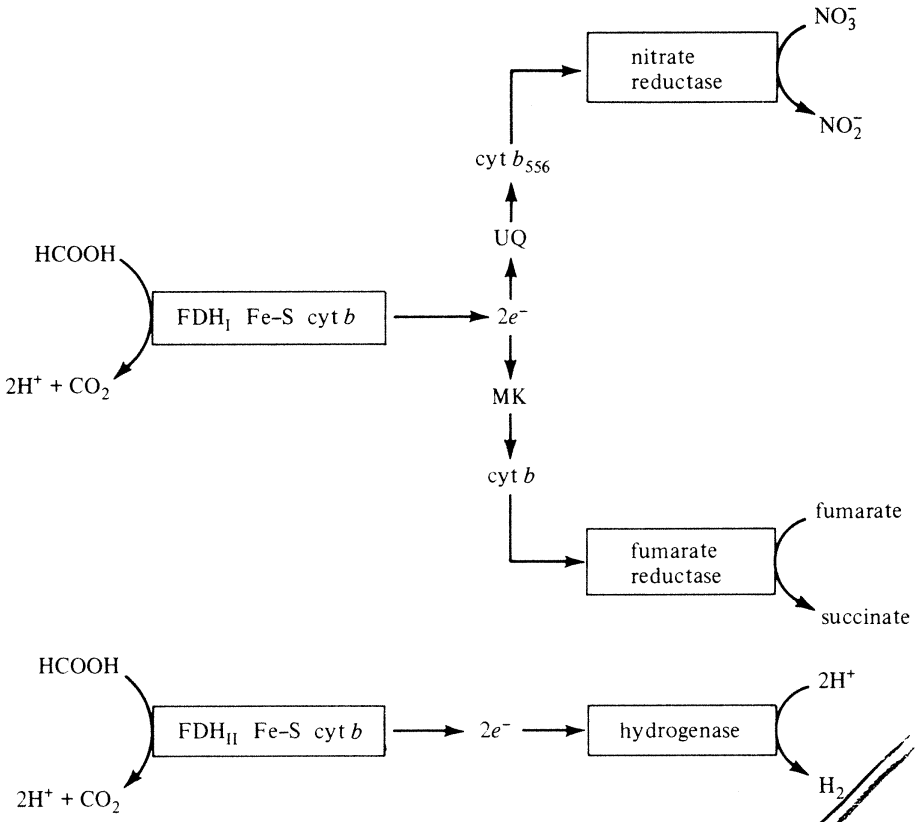


Figure 8.16. Formate-hydrogen lyase reaction and relationship to nitrate and fumarate reductases. Only when NO_3^- or fumarate is not available is H_2 formed.

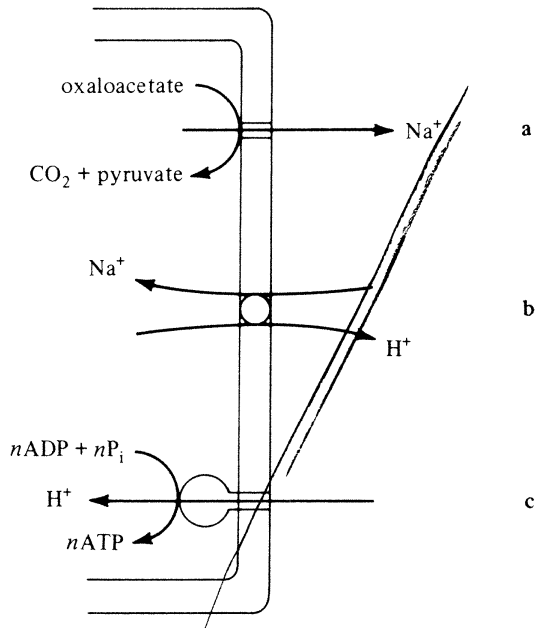


Figure 8.17. Sodium-dependent oxaloacetate decarboxylase. **a**: Sodium translocation as coupled to the decarboxylation reaction. **b**: $Na^+ - H^+$ antiporter. **c**: Proton-translocating ATP synthase; *n* may be in the order of 1/3.

ATP synthesized would be in agreement with this figure. Three remarks are necessary in this context:

1. Anaerobic citrate degradation in most organisms is not sodium-dependent. This is true for lactic acid bacteria, phototrophs, and clostridia. Here, oxaloacetate decarboxylase is a soluble enzyme not containing biotin. *E. coli* is able to utilize citrate anaerobically in the presence of a cosubstrate such as glucose; it does not contain oxaloacetate decarboxylase, and the oxaloacetate formed is reduced to succinate (with the reducing power from the degradation of glucose to acetate). Thus, the sodium-dependent oxaloacetate decarboxylase does not seem to be widespread among the anaerobes.
2. Other decarboxylation reactions are coupled to Na^+ translocation as well. This has been shown for methylmalonyl-CoA decarboxylase of *Veillonella alcalescens* and glutaconyl-CoA decarboxylase of *Acidaminococcus fermentans* and *Clostridium symbosium*.

$$\text{methylmalonyl-CoA} \xrightarrow{Na^+} \text{propionyl-CoA} + CO_2$$

$$\text{glutaconyl-CoA} \xrightarrow{Na^+} \text{crotonyl-CoA} + CO_2$$

The enzyme mentioned first participates in propionate fermentation, the second one in a pathway for the anaerobic breakdown of L-glutamate.

Decarboxylation of methylmalonyl-CoA is the only energy-yielding reaction, when *Propiogenium modestum* grows on succinate:

$$\text{succinate} + H^+ \longrightarrow \text{propionate} + CO_2 \quad \Delta G^{0\prime} = -20.6 \text{ kJ/mol}$$

3. We have seen that not only substrate level phosphorylation is used by anaerobes for ATP synthesis and not only ATP hydrolysis for energization of the membrane. Some lactic acid bacteria take advantage of product efflux for the generation of an electrochemical gradient; here, anaerobes employ decarboxylation reactions for this purpose.

The next sections of this chapter will show that electron transport is also used by several groups of obligate anaerobes for the generation of a protonmotive force across the membrane.

V. Propionate and Succinate Fermentation

Propionate is a major end product of fermentations carried out by a variety of anaerobic bacteria. Many of them ferment glucose to propionate, acetate, and CO_2:

$$1.5 \text{ glucose} \longrightarrow 2 \text{ propionate} + \text{acetate} + CO_2$$

A preferred substrate of propionate-forming bacteria is lactate, so that

these organisms can grow with the major end product of the lactate fermentation:

$$3 \text{ lactate} \longrightarrow 2 \text{ propionate} + \text{acetate} + CO_2$$

There are two pathways for propionate formation from lactate; in the acrylate pathway lactate is reduced stepwise to propionate; in the succinate-propionate pathway lactate is converted to propionate via pyruvate and succinate.

A. The acrylate pathway

This pathway seems to occur only in a few microorganisms, e.g., in *Clostridium propionicum* and in *Megasphaera* (*Peptostreptococcus*) *elsdenii*. It is shown in Fig. 8.18; L-, D, or DL-lactate may serve as substrate; a racemase is present which interconverts the enantiomers. L-Lactate is converted to L-lactyl-CoA in a CoA transferase reaction. By reactions not yet established in detail acrylyl-CoA is formed. It is reduced to propionyl-CoA, and propionate is produced by the above-mentioned CoA transferase.

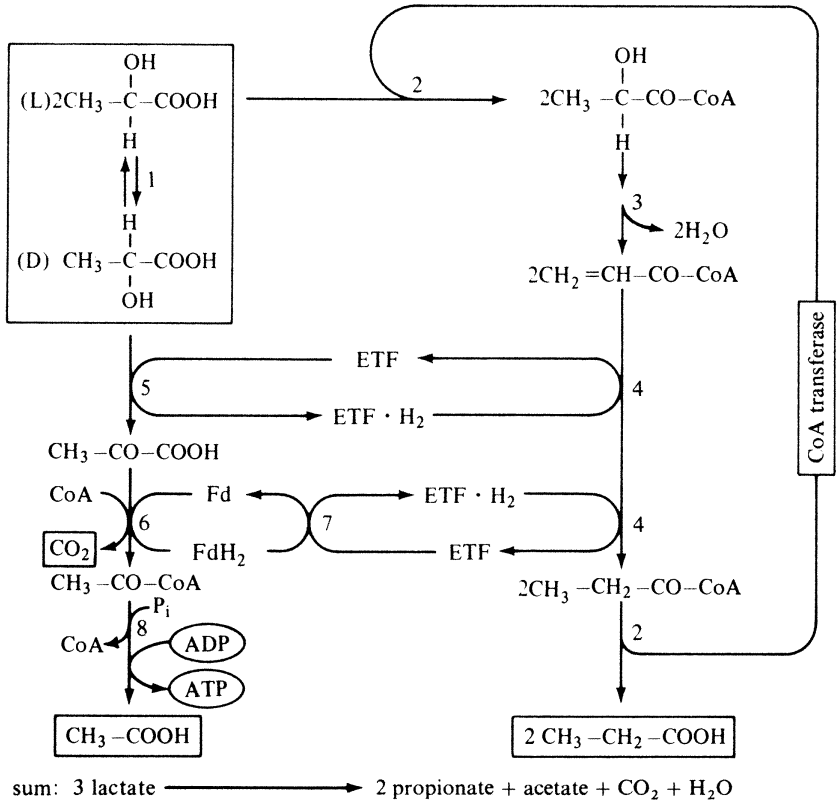


Figure 8.18. Formation of propionate, acetate, and CO_2 from DL-lactate by *Megasphaera elsdenii* and *Clostridium propionicum*. 1, Lactate racemase; 2, CoA transferase; 3, reaction not established; 4, dehydrogenase, which employs reduced electron-transferring flavoprotein ($ETF \cdot H_2$) as H-donor; 5, D-lactate dehydrogenase; 6, pyruvate-ferredoxin oxidoreductase; 7, transhydrogenase; 8, phosphotransacetylase + acetate kinase.

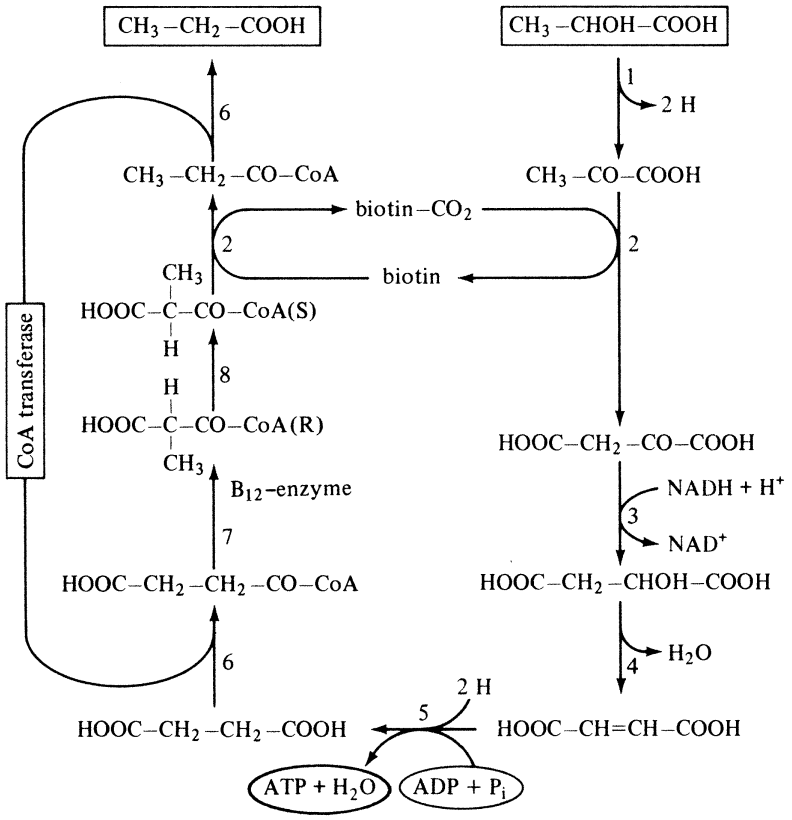
The H-donor for acrylyl-CoA reduction is reduced electron-transferring flavoprotein. It is formed from D-lactate and from reduced ferredoxin (or flavodoxin). The ATP yield of this fermentation is 1 mol/3 mol of lactate.

C. propionicum also ferments alanine and acrylate to propionate.

B. The succinate-propionate pathway

This pathway is employed by most propionate-producing organisms. Succinate is an intermediate but is also produced as end product in small or large amounts. On the other hand, organisms using the acrylate pathway do not excrete significant amounts of succinate.

The establishment of the succinate-propionate pathway was a rather difficult task. As is shown in Fig. 8.19 several enzymes are involved. First, lactate is oxidized to pyruvate in a reaction requiring a flavoprotein as



sum: lactate + NADH + H^+ + ADP + P_i ⟶ propionate + NAD^+ + ATP + $2H_2O$

Figure 8.19. Fermentation of lactate via the succinate-propionate pathway by propionibacteria. 1, Lactate dehydrogenase (the H-acceptor is probably a flavoprotein); 2, (*S*)-methylmalonyl-CoA-pyruvate transcarboxylase; 3, malate dehydrogenase; 4, fumarase; 5, fumarate reductase; 6, CoA transferase; 7, (*R*)-methylmalonyl-CoA mutase; 8, methylmalonyl-Co-A racemase.

H-acceptor. Oxaloacetate is then formed in a transcarboxylation reaction with (*S*)-methylmalonyl-CoA as CO_2-donor and biotin as CO_2-carrier. The action of malate dehydrogenase and fumarase yields fumarate, which is reduced to succinate by fumarate reductase. This reduction reaction is coupled to ATP formation by electron transport phosphorylation. Succinyl-CoA is then formed in a CoA transferase reaction and the rearrangement as catalyzed by the coenzyme B_{12}-containing methylmalonyl-CoA mutase leads to (*R*)-methylmalonyl-CoA, which is not a substrate for the transcarboxylase. Rather, the (*S*)-enantiomer is formed by a specific racemase. Then transcarboxylation yields propionyl-CoA and CoA transfer to succinate finally yields propionate.

One NADH is consumed in propionate formation from lactate; it comes from lactate oxidation to acetate according to the overall fermentation equation given above.

Besides the transcarboxylase—a biotin-containing enzyme with a high molecular weight (approximately 800,000 daltons) and a very complex quarternary structure—two enzymes of the succinate-propionate pathway deserve special attention: methylmalonyl-CoA mutase and fumarate reductase.

C. Methylmalonyl-CoA mutase and other coenzyme B_{12}-dependent rearrangement reactions

Rearrangements of this type were discovered by Barker and collaborators when they investigated the fermentation of glutamate by *Clostridium tetanomorphum*. As is apparent from Fig. 8.20 glutamate and succinyl-CoA are rearranged in analogous reactions to yield β-methylaspartate and methylmalonyl-CoA, respectively. The principle of these reactions is that a substituent group is moved between two adjacent positions of the carbon skeleton while a hydrogen is moved in the opposite direction. Not only carbon-carbon bonds are rearranged in coenzyme B_{12}–dependent reactions. A number of dehydratases, deaminases, and amino mutases are also B_{12}-enzymes and catalyze analogous reactions (Fig. 8.20c–e).Glycerol dehydrase, which is present in some lactobacilli, converts glycerol into β-hydroxypropionaldehyde. Ethanolamine deaminase is present in choline-fermenting clostridia and β-lysine mutase is the second enzyme in clostridial L-lysine fermentation (see Chapter 8, Section X).

Coenzyme B_{12} is not identical with **vitamin B_{12}**. The latter is a corrin ring system with cobalt^{2+} as central metal atom and 5,6-dimethylbenzimidazole ribonucleotide as characteristic component (Fig. 8.21). The sixth coordination position of Co^{2+} is occupied by hydroxyl or cyanide (hydroxy- or cyanocobalamin). Coenzyme B_{12} contains in addition a 5'-deoxyadenosyl group, which is covalently bound to cobalt replacing cyanide (5'-deoxyadenosylcobalamin). In the rearrangement reactions the hydrogen is

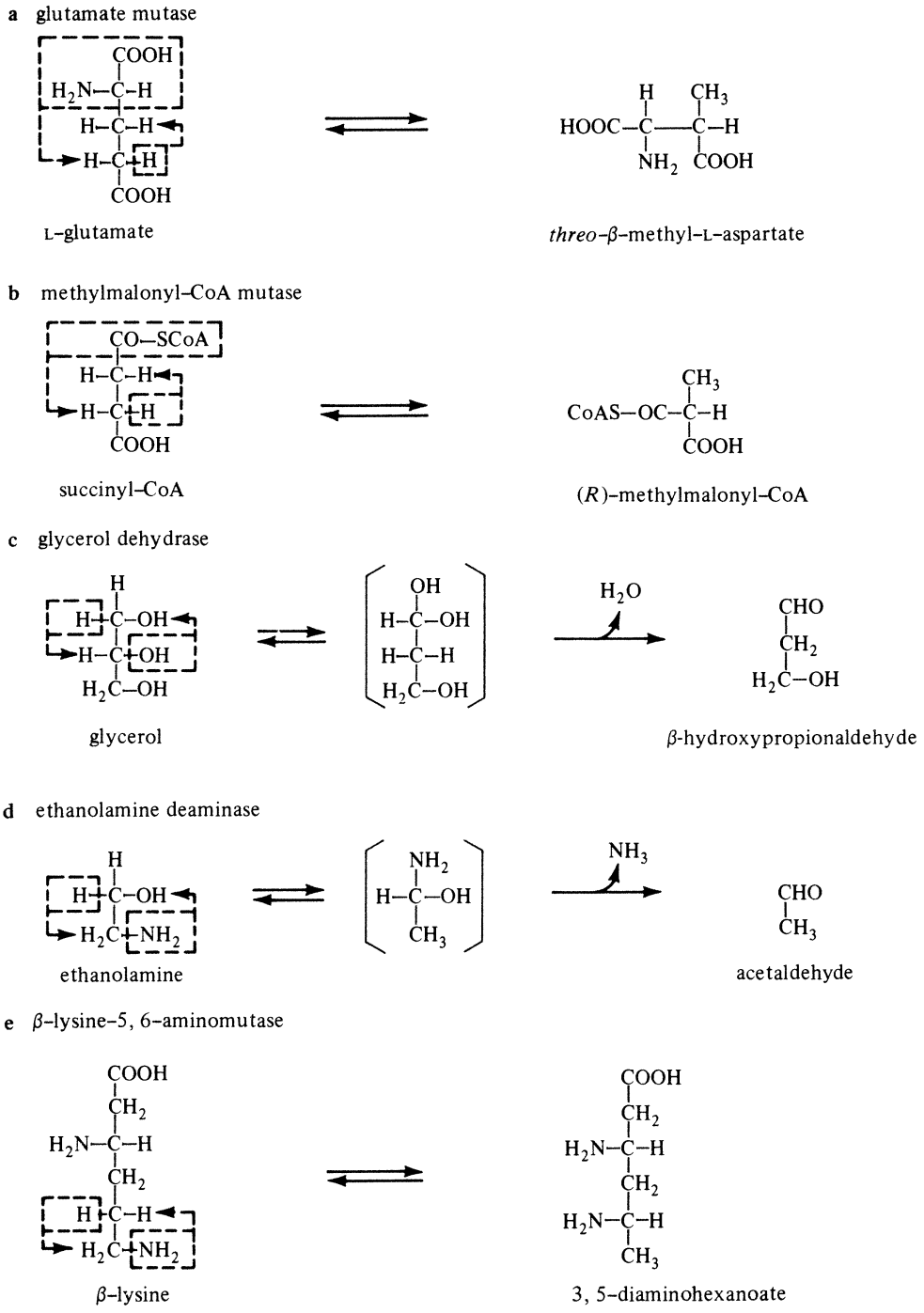


Figure 8.20. Coenzyme B_{12}-dependent rearrangement reactions.

transferred from the substrate to coenzyme B_{12} (to the C-5' methylene group) and from there to the product.

D. Fumarate reductase

The reduction of fumarate to succinate is a process that can be coupled by anaerobes with the generation of an electrochemical proton gradient and subsequently with ATP synthesis. That ATP is formed in this reaction is

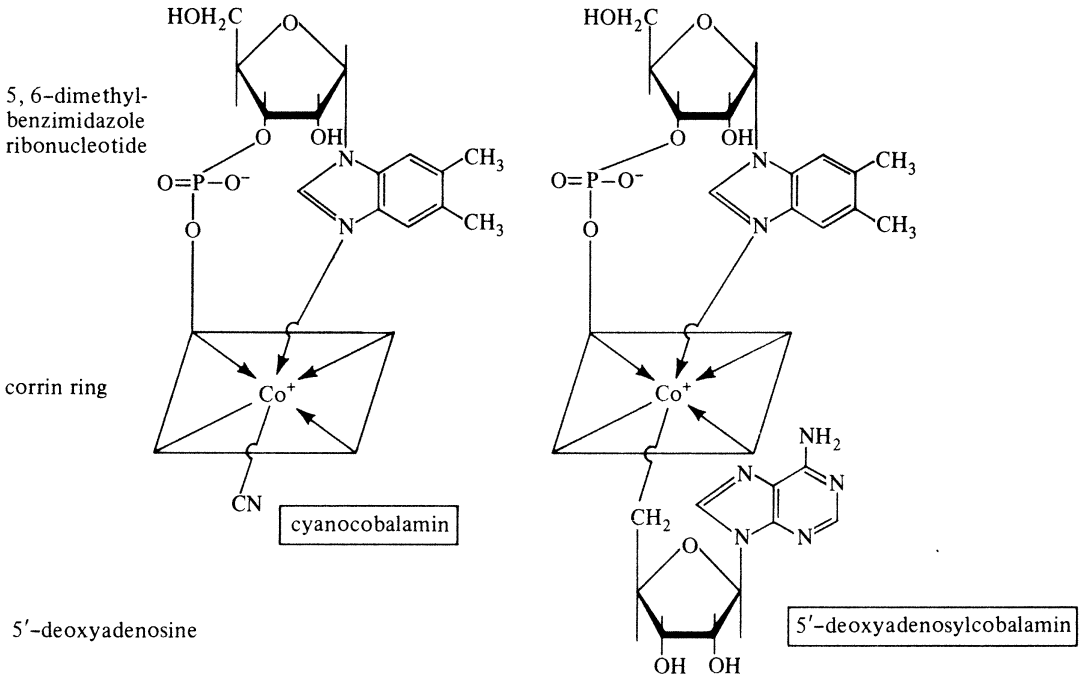


Figure 8.21. Structure of vitamin B_{12} and coenzyme B_{12}.

indicated by the high growth yields of propionate-succinate-producing organisms. Moreover, an ATP synthesis coupled to the reduction of fumarate could be demonstrated in cell-free systems of some organisms, first with extracts of *Desulfovibrio gigas* by Peck, Le Gall, and Barton. Fumarate reductase is membrane-bound, and it is associated with the electron carriers menaquinone and cytochrome *b*. *Bacteroides fragilis* grows only slowly in the absence of hemin (precursor in cytochrome synthesis) and ferments glucose to lactate, acetate, and malate. In the presence of hemin, however, a cytochrome *b* is formed and glucose is rapidly fermented to propionate and succinate indicating that cytochrome *b* is an essential component of the fumarate reductase system. *Bacteroides amylophilus*, is the only known exception; it contains a membrane-bound fumarate reductase but no cytochromes.

The fumarate reductase system is present not only in the classic propionate-forming microorganisms, the propionibacteria, but also in several enterobacteria and in various species of the genera *Bacteroides*, *Veillonella*, *Peptostreptococcus*, *Ruminococcus*, *Succinivibrio*, and *Selenomonas*. Even a clostridium (*C. formicoaceticum*) contains fumarate reductase. Some of these bacteria (such as *E. coli* and other enterobacteria) form only succinate but no propionate.

The redox potential of the fumarate/succinate couple is $E'_0 = +33$ mV. Thus, the potential span between donors such as H_2, formate, and NADH is large enough to be coupled to proton translocation and ATP synthesis. In addition, α-glycerol phosphate (*E. coli*) and lactate (most propionic acid bacteria) have been shown to function as NAD^+-independent donors for fumarate reductase. The entire system—the H-donating enzymes

(hydrogenase; NADH dehydrogenase, α-glycerol phosphate dehydrogenase, etc.), carriers, and the fumarate reductase—is membrane-bound. The reductase is a flavoprotein containing iron-sulfur centers. The arrangement of the components in the membrane, as unravelled for *Wolinella* (Vibrio) *succinogenes* by Kröger, is depicted in Fig. 8.22.

In correspondence with the discussed energetics of the fumarate reductase system, organisms, such as *W. succinogenes*, *E. coli*, *Citrobacter freundii*, and *D. gigas* are able to grow with fumarate plus H_2 or formate.

$$\text{fumarate} + H_2 \longrightarrow \text{succinate}$$
$$\text{fumarate} + \text{formate} \longrightarrow \text{succinate} + CO_2$$

E. PEP carboxytransphosphorylase of propionibacteria

Since propionibacteria produce succinate in addition to propionate, the transcarboxylase alone cannot be responsible for the formation of C_4-dicarboxylic acids; an enzyme system must be present in these microorganisms that catalyzes the synthesis of C_4-dicarboxylic acids from a C_3 compound and CO_2. It was discovered by Wood and Werkman in 1936. Since then carboxylating reactions that yield oxaloacetate are called **Wood—Werkman reactions**. We have already discussed the role of

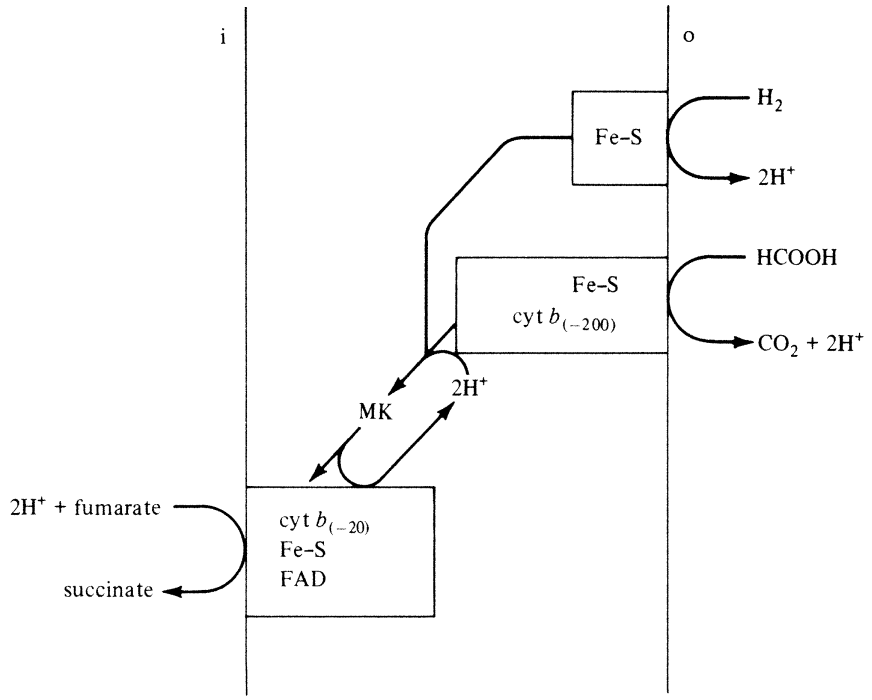


Figure 8.22. Electron flow from H_2 or HCOOH to fumarate. Boxes represent hydrogenase, formate dehydrogenase, and fumarate reductase, respectively. Numbers in parentheses give redox potentials of the two *b*-type cytochromes; MK, menaquinone.

pyruvate carboxylase and PEP carboxylase as C_3-carboxylating enzymes. In some *Bacteroides* species the energy-conserving PEP carboxykinase reaction is involved in oxaloacetate formation (this enzyme functions normally in the direction of PEP synthesis; see Table 5.4):

$$\text{PEP} + CO_2 + \text{ADP} \xrightleftharpoons{\text{PEP carboxykinase}} \text{oxaloacetate} + \text{ATP}$$

Propionibacteria contain an enzyme that catalyzes an analogous reaction:

$$\text{PEP} + CO_2 + P_i \xrightarrow{\text{PEP carboxytransphosphorylase}} \text{oxaloacetate} + PP_i$$

Here, the phosphoryl group is transferred to inorganic phosphate to form pyrophosphate. Thus, propionibacteria contain two enzyme systems that yield pyrophosphate: PEP carboxytransphosphorylase and pyruvate-phosphate dikinase (see Table 5.4). The pyrophosphate formed is used to phosphorylate fructose-6-phosphate to fructose-1,6-bisphosphate and serine to phosphoserine.

It is apparent from the discussion of the enzyme systems in this and the previous section that a number of variations exist in the propionate-succinate pathway as depicted in Fig. 8.19. In some organisms (e.g., *Veillonella alcalescens*) the transcarboxylase is replaced by sodium-dependent decarboxylase. Both enzymes yield propionyl-CoA from methylmalonyl-CoA.

With respect to oxaloacetate formation from an intermediate of the glycolytic pathway, one out of three enzymes might be involved: the transcarboxylase using pyruvate as acceptor or the two enzymes discussed above that use PEP as acceptor.

VI. Acetate Fermentation

We have seen that acetate is an important product in a number of fermentations. There is a group of organisms, however, by which acetate is formed as the predominant nongaseous product. Butyrate is usually not detectable in the fermentation broth; ethanol and lactate in very small amounts at the most. This formerly very small group of *acetogenic organisms* has grown very much in recent years. Representative species are given in Table 8.9. It is obvious that most species are able to live at the expense of acetate formation from H_2 and CO_2 according to the following equation:

$$4H_2 + 2CO_2 \longrightarrow CH_3\text{–}COOH + 2H_2O$$

$$\Delta G^{0\prime} = -107.1 \text{ kJ } (-25.6 \text{ kcal}) \text{ per mol acetate}$$

This type of fermentation was discovered in 1936 by Wieringa who isolated *Clostridium aceticum*. Several acetogens grow with carbon monoxide and

Table 8.9. Acetogenic bacteria and some of their properties

organism	thermophilic	growth on		cytochromes present
		$H_2 + CO_2$	sugars	
Clostridium aceticum	−	+	+	+
C. thermoautotrophicum	+	+	+	+
C. formicoaceticum	−	−	+	+
C. thermoaceticum	+	−, +[a]	+	+
Acetobacterium woodii	−	+	+	−
A. wieringae	−	+	+	−
Acetogenium kivui	+	+	+	?
Sporomusa sphaeroides	−	+	−	+

[a] Some strains are positive.
Eubacterium limosum is able to produce acetate from $CO_2 + H_2$, but it forms considerable amounts of butyrate when growing on other substrates.

with methanol plus CO_2:

$$4CO + 2H_2O \longrightarrow CH_3-COOH + 2CO_2$$
$$\Delta G^{0\prime} = -484.4 \text{ kJ } (-115.9 \text{ kcal}) \text{ per reaction}$$

$$4CH_3OH + 2CO_2 \longrightarrow 3CH_3-COOH + 2H_2O$$
$$\Delta G^{0\prime} = -706.3 \text{ kJ } (-168.9 \text{ kcal}) \text{ per reaction}$$

Hexoses are converted by acetogenic bacteria to almost 3 mol acetate per mol:

$$C_6H_{12}O_6 \longrightarrow 3CH_3-COOH$$

The recently described genus *Sporomusa* represents the first genus of Gram-negative endospore-formers. In addition to $H_2 + 2CO_2$, its members utilize *N*-methyl compounds (e.g., betaine) and primary alcohols (ethanol, *n*-butanol).

How is it possible for the acetogens to make three molecules of acetate from one molecule of hexose? The pathway is depicted in Fig. 8.23. First, the hexose is degraded to two pyruvates via the Embden–Meyerhof-Parnas pathway. Their subsequent degradation by the action of pyruvate; ferredoxin oxidoreductase and the typical enzymes acting on acetyl-CoA yield 2 acetates. In addition, CO_2 is produced, and NADH and reduced ferredoxin are generated from the corresponding oxidized forms. Synthesis of the third molecule of acetate is now initiated by the reduction of CO_2 all the way to 5-methyltetrahydrofolate. The first enzyme of this pathway is a remarkable formate dehydrogenase. It is a tungsten-selenoprotein and is structured so as to make formate and not only to oxidize it like the membrane-bound formate dehydrogenases of other organisms (e.g., enterobacteria). The electron donor is known for only some acetogenic organisms; in *C. thermoaceticum* it is NADPH. Formyltetrahydrofolate is

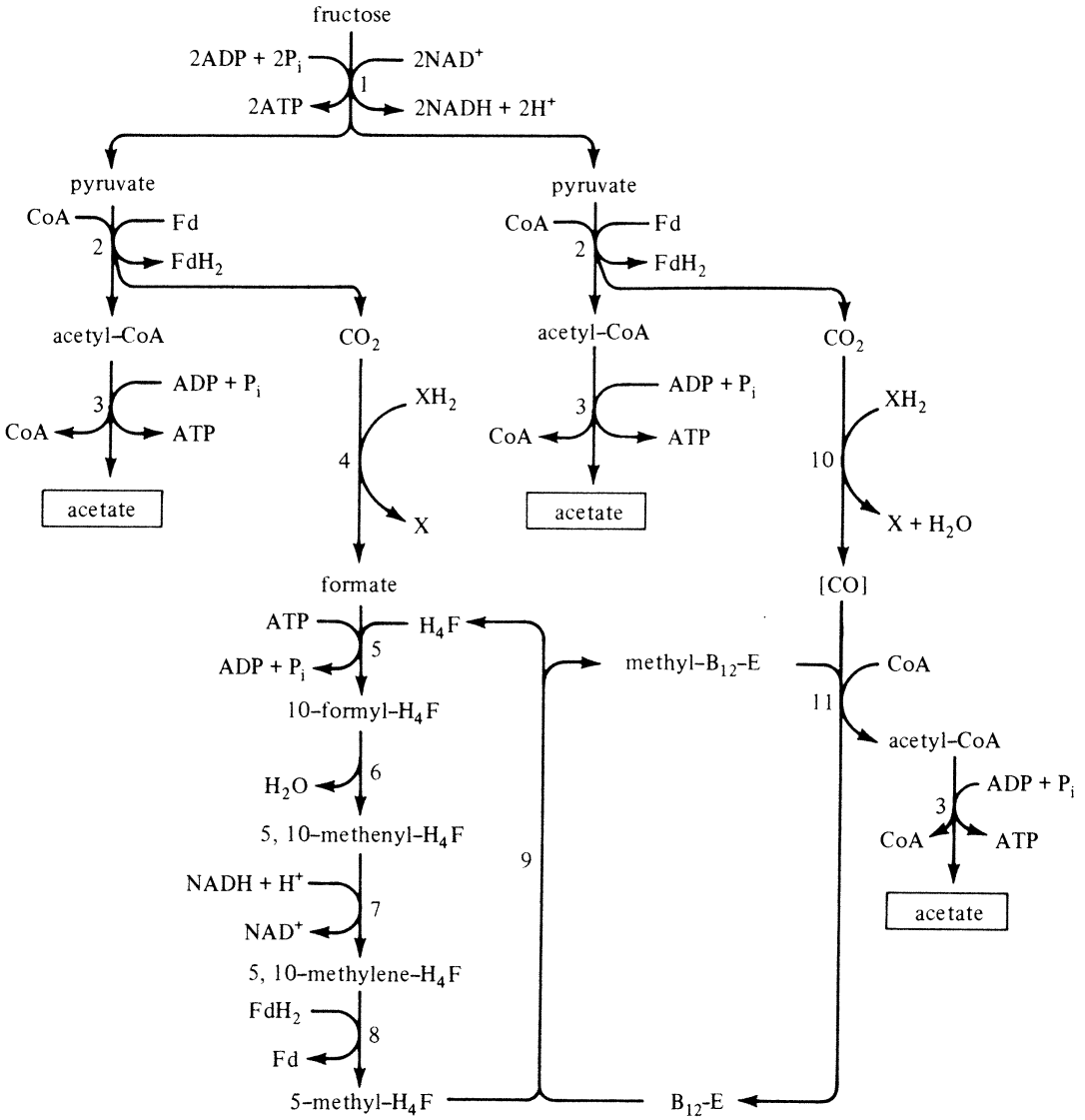


Figure 8.23. Pathway of the acetate fermentation. 1, Degradation of fructose via the Embden–Meyerhof–Parnas pathway; 2, pyruvate-ferredoxin oxidoreductase; 3, phosphotransacetylase plus acetate kinase; 4, formate dehydrogenase; 5, formyl-tetrahydrofolate synthetase; 6, methenyl-tetrahydrofolate cyclohydrolase; 7, methylene-tetrahydrofolate dehydrogenase; 8, methylene-tetrahydrofolate reductase; 9, tetrahydrofolate: B_{12} methyltransferase; 10, CO dehydrogenase; 11, acetyl-CoA-synthesizing enzyme (probably ATP-requiring); [CO], enzyme-bound.

then formed from formate in an ATP-consuming reaction, and subsequent reduction leads to the previously mentioned methyltetrahydrofolate.

How acetate is synthesized from methyltetrahydrofolate was the subject to intense research in the laboratories of Wood and of Thauer. The breakthrough came with the discovery of carbon monoxide dehydrogenase—a nickel-containing enzyme. It is different from CO oxidase, the characteristic enzyme of the carboxydobacteria, in that the enzyme-specific electron carrier has a low redox potential. That reaction is,

therefore, reversible under physiological conditions:

$$CO_2 + X\text{-}H_2 \rightleftharpoons CO + X + H_2O$$

In the pathway, CO_2 is now reduced to CO, and it is the latter which finally gives the carboxyl group of acetate: methyltetrahydrofolate is carbonylated, and acetyl-CoA and finally acetate are formed.

The pathway as depicted in Fig. 8.23 also allows to outline the routes used for acetate formation from methanol + CO_2 and from H_2 + CO_2. The strategy is to make CO from CO_2 and to make methyltetrahydrofolate from H_2 + CO_2 or methanol. Thus, part of the methanol has to be oxidized to provide the reducing power for CO_2 reduction to CO; methyltetrahydrofolate and CO finally yield acetate. The energetics of the latter fermentations is not clear yet. Likewise, the function of the cytochromes in acetogens is unknown.

VII. Methane Fermentation

Methane is the most reduced organic compound and its formation is the terminal step of the anaerobic food chain that will be discussed in Section IX of this chapter. Methanogenesis is a domain of the Archaebacteria; in other words, all known methanogenic bacteria belong to this kingdom of organisms. Some of their characteristic features have already been outlined in Chapter 5.

A. Substrate utilization

Methanogenic bacteria are extremely oxygen-sensitive. This is not a great disadvantage to them in nature. In habitats rich in degradable organic compounds, oxygen is consumed rapidly and trapped by organisms in surface layers. Thus the methanogenic bacteria are particularly abundant in all sorts of mud and sediments. Other important habitats of these bacteria are the rumen and the (man-made) anaerobic digesters in sewage plants. The oxygen sensitivity of the methanogens creates problems when pure cultures are to be isolated and experiments with pure cultures are to be carried out. Appropriate methods (the so-called Hungate technique) have been worked out for this purpose.

Representatives of various genera of methanogenic bacteria, the substrates utilized by them, and the distribution of cytochromes are summarized in Table 8.10. It is apparent that complex organic compounds cannot be utilized by the methanogens. Substrates are C_1 compounds and as the only C_2 compound: acetate. Two nutritional groups of organisms can be envisaged:

Table 8.10. Representative species of the methanogenic bacteria, the substrates utilized by them, and the presence of cytochromes

organism	substrates utilized	presence of cytochromes
Methanobacterium thermoautotrophicum	$H_2 + CO_2, CO^a$	–
Methanobrevibacter arboriphilus	$H_2 + CO_2$	–
Methanococcus vanniellii	$H_2 + CO_2$, HCOOH	–
Methanospirillum hungatei	$H_2 + CO_2$, HCOOH	–
Methanosarcina barkeri	$H_2 + CO_2$, CH_3OH, CH_3COOH, methylamines	+
Methanosarcina mazei	CH_3OH, CH_3COOH, methylamines	+
Methanothrix soehngenii	CH_3COOH	+
Methanolobus tindarius	CH_3OH, methylamines	+
Methanococcoides methylutens	CH_3OH, methylamines	+
Methanoplanus limicola	$H_2 + CO_2$, HCOOH	–

[a] CO allows only slow growth; it may also be used by other methanogens listed.

1. Obligate chemolithotrophic methanogens that grow with $CO_2 + H_2$ according to the equation:

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \quad \Delta G^{0\prime} = -136 \text{ kJ } (-32.4 \text{ kcal})$$

Some of these organisms also grow with the "quasi-chemolithotrophic" substrates HCOOH and CO. This term is adequate here because both substrates are utilized via $CO_2 + H_2$:

$$\begin{aligned} 4HCOOH &\longrightarrow 4CO_2 + 4H_2 \text{ (carrier-bound)} \\ CO_2 + 4H_2 &\longrightarrow CH_4 + 2H_2O \\ \hline 4HCOOH &\longrightarrow CH_4 + 3CO_2 + 2H_2O \end{aligned}$$

$$\Delta G^{0\prime} = -144.2 \text{ kJ } (-34.5 \text{ kcal}) \text{ per mol methane}$$

correspondingly:

$$4CO + 2H_2O \longrightarrow CH_4 + 3CO_2$$

$$\Delta G^{0\prime} = -211 \text{ kJ } (-50.4 \text{ kcal}) \text{ per mol methane}$$

2. Methylotrophic methanogens that grow with methyl-group-containing substrates (methanol, methylamines, acetate). The fermentation equation for acetate is very simple:

$$CH_3\text{–}COOH \longrightarrow CH_4 + CO_2$$

$$\Delta G^{0\prime} = -37 \text{ kJ } (-8.9 \text{ kcal}) \text{ per mol methane}$$

Organisms such as *Methanosarcina barkeri* grow on methanol or methylamines. Here, one-fourth of the substrate has to be oxidized to CO_2 for reducing power generation:

$$\begin{array}{r} CH_3OH + H_2O \longrightarrow CO_2 + 6H \\ 3CH_3OH + 6H \longrightarrow 3CH_4 + 3H_2O \\ \hline 4CH_3OH \longrightarrow 3CH_4 + CO_2 + 2H_2O \end{array}$$

$\Delta G^{0\prime} = -319.5$ kJ (-76.4 kcal) per reaction

$\Delta G^{0\prime}$ (per mol methane) $= -106.5$ kJ (-25.5 kcal)

or

$$4(CH_3)_3{-}N + 6H_2O \longrightarrow 9CH_4 + 3CO_2 + 4NH_3$$

$\Delta G^{0\prime} = -683.2$ kJ (-163.4 kcal) per reaction

$\Delta G^{0\prime}$ (per mol methane) $= -75.9$ kJ (-18.1 kcal)

Group 2 organisms produce methane directly from the methyl groups and not via CO_2. This has been demonstrated with deuterated substrates that gave methane with three deuteriums in it:

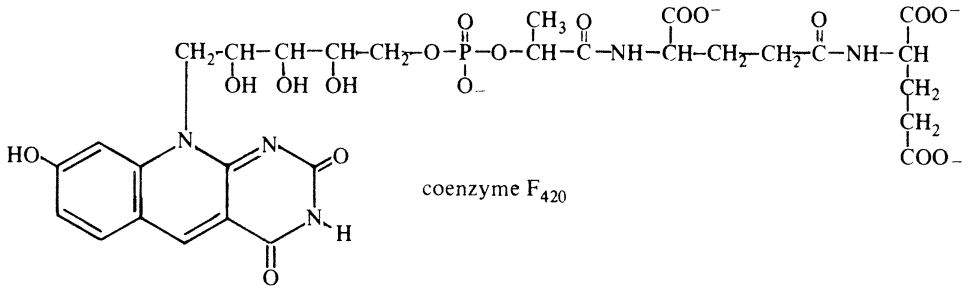
$$CD_3OH \xrightarrow{\text{fermentation}} CD_3H$$

Obligate chemolithotrophic methanogens do not contain cytochromes, which are, however, present in methylotrophic methanogens. The methanogenic bacteria require Na^+ in concentrations of about 5 m*M* for growth; its function is still unknown. A number of rather unique redox carriers and coenzymes have been found to occur in all of these organisms; they do not occur in other organisms (few exceptions) and are specifically involved in the pathway from CO_2 to CH_4. It should be mentioned that most of these were first isolated from *Methanobacterium thermoautotrophicum*, an organism that was described by Zeikus and Wolfe and that grows in a mineral medium with $H_2 + CO_2$ at 55°C.

B. Novel coenzymes

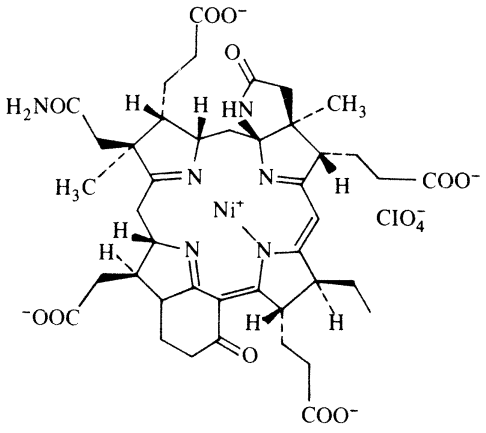
The first two novel coenzymes discovered in methanogens by Wolfe and co-workers were coenzyme M and coenzyme F_{420}. Their chemical structures are given in Fig. 8.24. Coenzyme M is a simple chemical compound. Its reactive group is the mercapto group which can be methylated and methyl-coenzyme M is the ultimate precursor of methane. Coenzyme F_{420} is a deazaflavin (ring-N in position 5 of the flavin skeleton is missing); it is a redox carrier with an $E_0' = -370$ mV, and its role is analogous to the one of ferredoxin in other anaerobes. It functions as electron acceptor of hydrogenase and as electron donor in several reduction reactions.

The structure of factor F_{430} which is involved in the final step of methane formation was elucidated by Thauer, Eschenmoser, and co-workers as a tetrapyrrol with nickel as central metal ion. In addition to hydrogenase and CO dehydrogenase, both of which are nickel proteins, factor F_{430} repre-

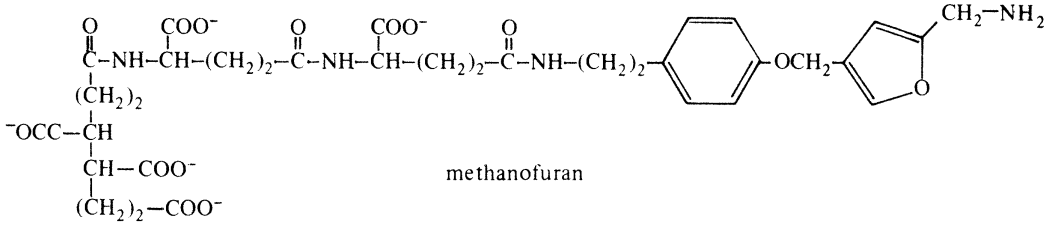


coenzyme F_{420}

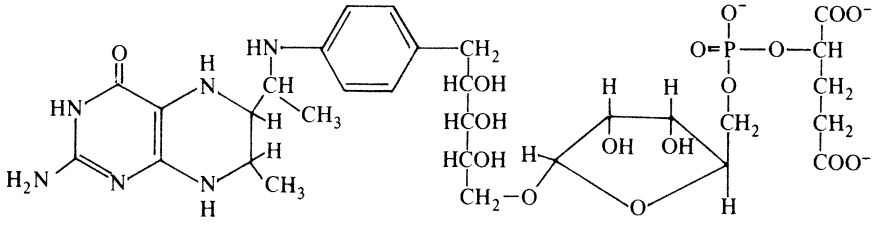
coenzyme M



factor F_{430}



methanofuran



5, 6, 7, 8-tetrahydromethanopterin

Figure 8.24. Novel coenzymes in methanogenic bacteria.

sents the third component of methanogens containing this metal. Two additional structures are given in Fig. 8.24: that of tetrahydromethanopterin determined by Vogels and co-workers, and of methanofuran. Both are involved in CO_2 reduction. Methanofuran functions as the primary CO_2 acceptor.

C. Pathway of methane formation

First we shall look at methane formation from $CO_2 + H_2$. A sequence based on Barker's scheme of methane formation from CO_2 is given in Fig. 8.25. The first carrier molecule known to be involved is methanofuran. In a reaction that requires CO_2 and reducing equivalents it is converted to formylmethanofuran with the formyl group residing at the aminomethyl group of the furan ring. Transfer of the C_1 moiety to tetrahydromethanopterin and reduction of the formyl to the methyl group follows. In analogy to the tetrahydrofolate biochemistry the methyl group resides at N^5 of the ring skeleton. It finally is transferred to coenzyme M and reduced to methane. The methyl-coenzyme M methylreductase reaction has been studied in detail; the responsible enzyme is of a very complex structure; it contains several proteins, factor F_{430} and an as yet unidentified factor B.

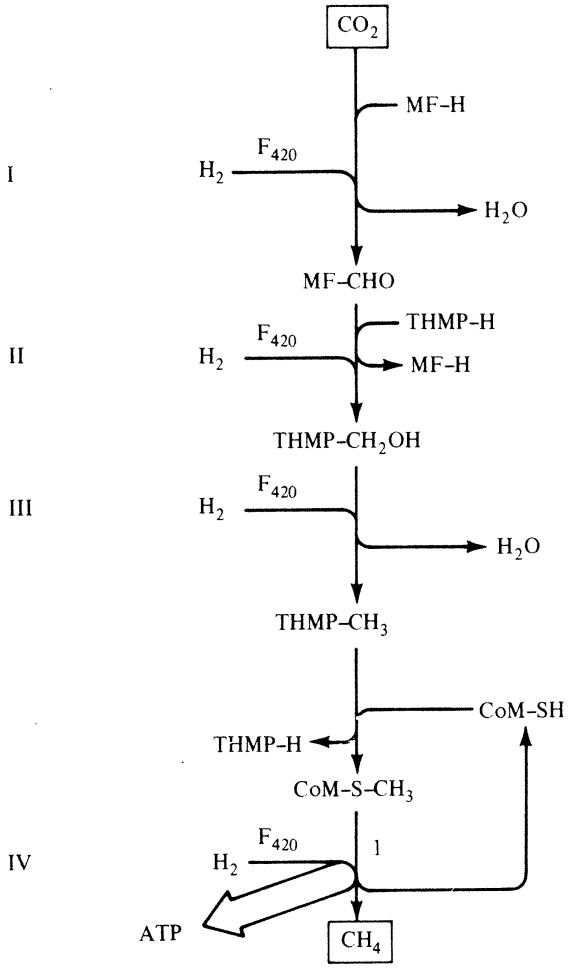


Figure 8.25. Scheme for the reduction of CO_2 to CH_4 and site of ATP synthesis. MF-H, Methanofuran; THMP-H, tetrahydromethanopterin; 1, methyl-coenzyme M methylreductase.

The free energy changes and the redox potentials of the four reactions leading from bicarbonate to methane are as follows:

		$\Delta G^{0\prime}$	$\Delta E_0'$
$HCO_3^- + H_2$	$\longrightarrow HCOO^- + H_2O$	−1.3 kJ (−0.3 kcal)	−432 mV
$HCOO^- + H_2 + H^+$	$\longrightarrow CH_2O + H_2O$	+23.0 kJ (+5.5 kcal)	−535 mV
$CH_2O + H_2$	$\longrightarrow CH_3OH$	−44.8 kJ (−10.7 kcal)	−182 mV
$CH_3OH + H_2$	$\longrightarrow CH_4 + H_2O$	−112.5 kJ (−26.9 kcal)	+169 mV
$HCO_3^- + H^+ + 4H_2$	$\longrightarrow CH_4 + 3H_2O$	−135.6 kJ (−32.4 kcal) (values per mol product)	

It is obvious that the first two steps are not very favorable. The third step is distinctly exergonic; most of the energy, however, is released in the fourth step. The values given are for the free redox couples (e.g., CH_2O/CH_3OH). They might be slighly different for the carrier-bound compounds.

It can be stated now that the fourth reduction step is coupled to the generation of a protonmotive force at the membrane which in turn is used by an ATP synthase for the phosphorylation of ADP. The evidence comes from experiments with *Methanosarcina barkeri*. Reduction of CH_3OH to CH_4 by H_2 is coupled in this organism to an increase of the intracellular ATP concentration. This ATP synthesis and also methane formation are inhibited by dicyclohexylcarbodiimide (DCCD)—the classic inhibitor of the ATP synthase (Fig. 8.26). The protonmotive force, ΔP, however, remains unaffected (because it cannot be utilized for ATP synthesis in the presence of DCCD). The two processes—methane formation and ATP synthesis—are apparently coupled via ΔP. As expected, this coupling can be abolished by an uncoupler. In its presence, the protonmotive force is dissipated and methane is produced again from $CH_3OH + H_2$, but ATP is not synthesized. The sequence of effects is therefore: methane formation → protonmotive force → ATP synthesis and not: methane formation → ATP synthesis → protonmotive force. It excludes that ATP is formed by substrate-level phosphorylation. Since methane is produced from methanol + H_2 in one reduction step—in the methylreductase reaction—this step must be the site of energy conservation by a chemiosmotic mechanism.

Methanosarcina barkeri is the classic, and representative species for those methanogenic bacteria that utilize acetate, methanol, and methylamines as substrates. Growth on acetate is much slower as compared to that on the other substrates. Nevertheless, it is the most important methanogenic substrate in nature (see Section IX). Looking at the fermentation equation may give the false impression that methanogenesis from acetate is a simple decarboxylation. After the discovery of high levels of CO

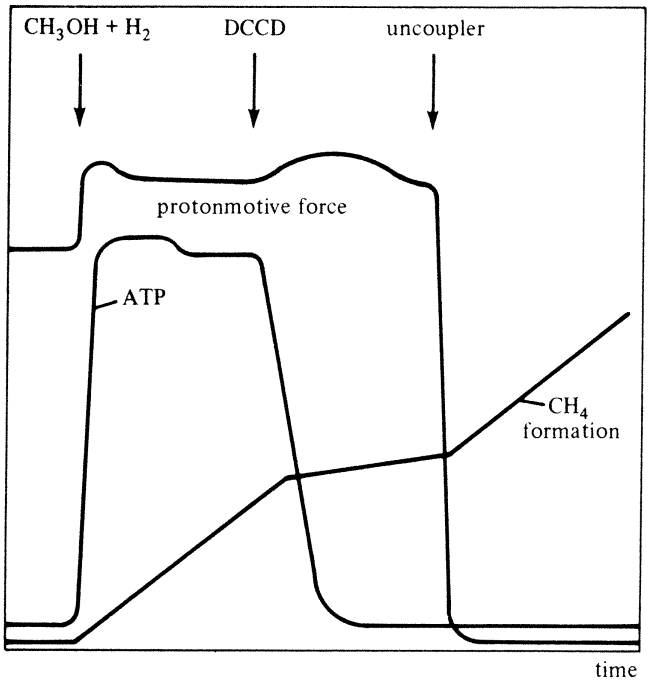


Figure 8.26. Coupling of ATP synthesis to methane formation from $CH_3OH + H_2$ in *M. barkeri* by a chemiosmotic mechanism [Redrawn from M. Blaut and G. Gottschalk; *Eur. J. Biochem.* **141**, 217–222 (1984)].

dehydrogenase in acetate-grown cells of *M. barkeri* by Zeikus and co-workers, it became more and more clear that CO is the primary cleavage product and not CO_2. The fermentation scheme in Fig. 8.27 shows that the fermentation can then be written as an oxidoreduction process: first CO and methyl-coenzyme M are produced, the oxidation of the former provides the reducing equivalents for the reduction of the latter to methane. From the free energy change (−31 kJ) it is apparent that the formation of 1 mol methane from acetate can yield only a fraction of a mol ATP.

It has already been mentioned that methanogenesis of methanol and of the methylamines can be subdivided into two processes: oxidation of one-fourth of the methyl groups to CO_2 and reduction of three-fourths of the methyl groups to CH_4 (Fig. 8.28). A methanol: coenzyme M and a trimethylamine: coenzyme M methyltransferase have been characterized. The methyl group is first transferred to protein-bound 5-hydroxybenzimidazolylcobamide which is the principal form of B_{12} in methanogens (not the dimethyl derivative as in the propionibacteria). Methyl group transfer proceeds further to coenzyme M. The carrier at which the methyl group is oxidized to CO_2 is unknown. Very likely the cytochromes present in those methanogens that grow on methanol and methylamines are involved in the first oxidation step ($X—CH_3$ to $X—CH_2OH$). Coupling of this oxidation ($E'_0 = -182$ mV) with reduction of factor F_{420} ($E'_0 = -373$ mV) requires reverse electron transfer that might proceed with the participation of cytochromes.

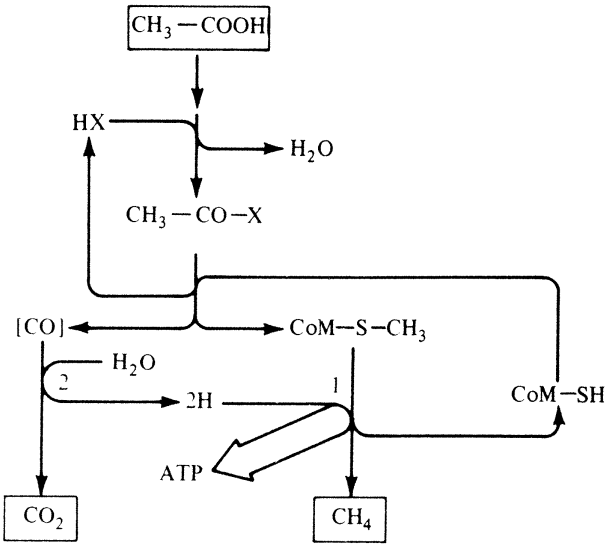


Figure 8.27. Tentative scheme for the formation of methane and carbon dioxide from acetate. 1, methyl-coenzyme M methylreductase; 2, CO dehydrogenase. Details of the other reactions are not yet known.

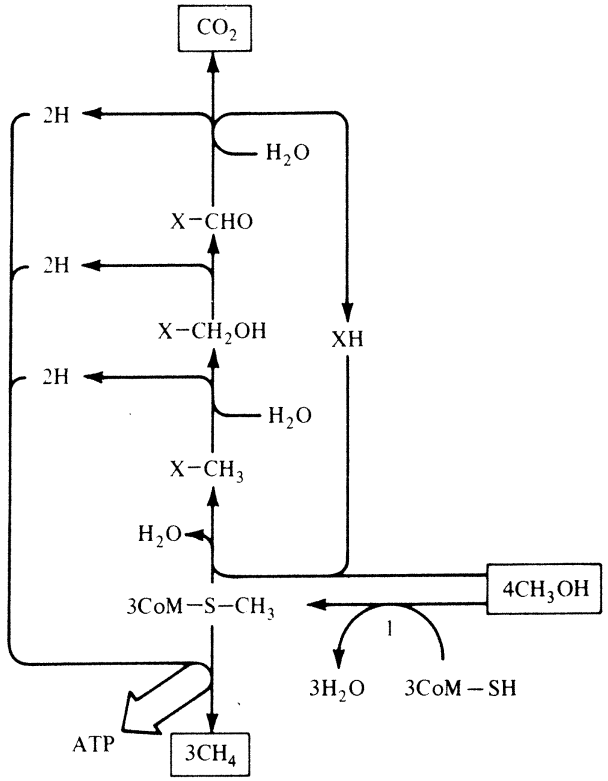


Figure 8.28. Tentative scheme for the formation of methane and carbon dioxide from methanol. 1, Methanol: coenzyme M methyltransferase (transfer via B_{12}; see text); X, unknown carrier.

D. Synthesis of cell carbon

M. thermoautotrophicum and a number of other species are able to grow in a mineral medium. Therefore, these organisms must be able to make all their cellular constituents from CO_2. Fuchs and co-workers have shown that the methanogens do not operate a cyclic CO_2 fixation pathway as do, for example, the phototrophs (see Chapter 9). The strategy is to synthesize first acetyl-CoA from $2CO_2$. For this purpose the organisms use a pathway that resembles the one present in acetogens (Fig. 8.23). Advantage is taken of the fact that carrier-bound methyl groups are intermediates of methane formation anyway. Not all of them are used to produce methane; some are carbonylated to give acetyl-CoA. The latter is reductively carboxylated to yield pyruvate. Ferredoxin or coenzyme F_{420} serve as H-donor. PEP synthetase and PEP carboxylase lead to oxaloacetate that is reduced to succinate; in *M. thermoautotrophium*, finally succinyl-CoA is reductively carboxylated, and α-oxoglutarate—the precursor of L-glutamate—is formed:

$$\text{succinyl-SCoA} + CO_2 + FdH_2 \longrightarrow \alpha\text{-oxoglutarate} + \text{CoASH} + Fd$$

Thus, in contrast to *Clostridium kluyveri* which also uses acetyl-CoA as starting material for biosyntheses, several methanogens lack citrate synthase, and employ the "dicarboxylic acid portion" of the citric acid cycle for glutamate synthesis rather than the "tricarboxylic acid portion". *M. barkeri*, however, has been shown to contain citrate synthase and to employ the "tricarboxylic acid portion" of the cycle.

VIII. Sulfide Fermentation (Desulfurication)

Most microorganisms use sulfate as the principal sulfur source and contain enzyme systems for the reduction of sulfate to sulfide. This process of assimilatory sulfate reduction has been discussed in Chapter 3 (see Fig. 3.3). In sulfide fermentation, sulfate is used as terminal electron acceptor, and the hydrogen sulfide formed is excreted. This process is therefore called **dissimilatory sulfate reduction**; it is carried out only by strictly anaerobic bacteria. On the basis of their oxidative abilities the sulfate-reducing bacteria (sulfidogenic bacteria) can be subdivided into two groups. Representative species of these groups are given in Table 8.11. The incomplete oxidizers, such as the *Desulfovibrio* species and most *Desulfotomaculum* species, have been known for a long time; they oxidize a number of organic acids and alcohols to acetate. The complete oxidizers were recently discovered by Pfennig and co-workers. *Desulfotomaculum acetoxidans* was the first; others, morphologically very different, followed.

Sulfate-reducing bacteria are loaded with electron and hydrogen carriers. Cytochrome c_3 discovered by Postgate, was the first cytochrome

Table 8.11. The two physiological groups of sulfate-reducing bacteria and some of their properties

species	substrates utilized[a]	growth with $H_2 + CO_2$	cytochromes
Group I			
Desulfovibrio desulfuricans	lactate, ethanol, malate	+[b]	*c*
Desulfovibrio vulgaris	lactate, ethanol, malate	+[b]	*c*
Desulfomonas pigra	lactate	−	*c*
Desulfotomaculum nigrificans	lactate, ethanol	+[b]	*b*
Desulfobulbus propionicus	propionate	−	*b*, *c*
Group II			
Desulfotomaculum acetoxidans	acetate	+	*b*
Desulfobacter postgatei	acetate	−	*b*, *c*
Desulfococcus multivorans	acetate, propionate, benzoate, fatty acids (C_1-C_{14})	−	*b*, *c*
Desulfonema limicola	formate, acetate, propionate, fatty acids (C_1-C_{12})	+	*b*, *c*
Desulfosarcina variabilis	acetate, propionate, benzoate, fatty acids (C_1-C_{14})	+	ND

ND, not determined.

[a] Substrates utilized in the presence of sulfate;

[b] Group 1 organisms require acetate for growth with $H_2 + CO_2$.

detected in strict anaerobes. All sulfate reducers contain cytochromes, which are of the *c*-type and/or of the *b*-type. In addition, menaquinone, several ferredoxins, flavodoxin, rubredoxin, and desulforedoxin (closely related to rubredoxin) are present. Another remarkable constituent of sulfate reducers is a siroheme protein, a protein containing two tetrapyrrol ring systems and iron sulfur centers. This protein exhibits sulfite reductase activity and may catalyze six-electron-transfer reactions; it is known as desulfoviridin (*Desulfovibrio*, *Desulfomonas*, *Desulfococcus*, *Desulfonema* species) or P582 (*Desulfotomaculum*, *Desulfonema* species). First the fermentation of lactate by the incomplete oxidizers will be discussed.

A. Fermentation of lactate and sulfate

This fermentation can be summarized as follows:

$$2CH_3—CHOH—COOH + 2H_2O \longrightarrow 2CH_3—COOH + 2CO_2 + 8H$$
$$SO_4^{2-} + 8H \longrightarrow S^{2-} + 4H_2O$$

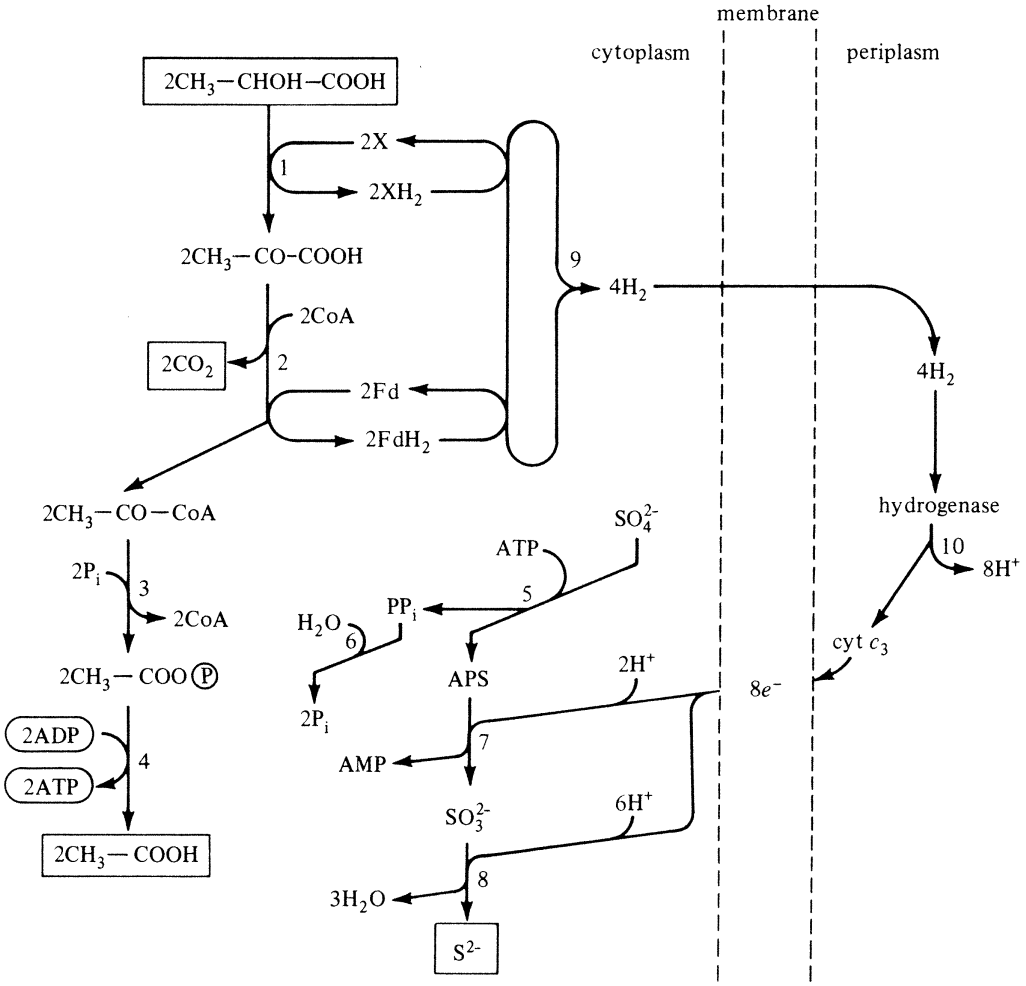
Lactate oxidation to acetate proceeds via pyruvate and acetyl-CoA. A flavoprotein probably functions as H-acceptor in the first oxidation step, and pyruvate-ferredoxin oxidoreductase is involved in acetyl-CoA formation. The final acetate production is coupled to ATP synthesis.

The 8H generated in lactate oxidation are used to reduce sulfate to sulfide. Prior to the first reduction step, sulfate is activated by conversion to adenosine-5′-phosphosulfate (APS) (Fig. 8.29), and the reduction products are sulfite and AMP. The further phosphorylated compound PAPS, which is an intermediate in assimilatory sulfate reduction (see Fig. 3.4), is not involved here. APS reductase contains FAD and iron-sulfur. The reaction is reversible; in vitro APS reduction is observed with reduced methyl viologen as H-donor and APS formation from sulfite and AMP with ferricyanide as H-acceptor.

The reduction of sulfite is catalyzed by the above-mentioned sulfite reductase. Until recently, the intermediary formation of trithionate and thiosulfate was assumed. It seems now that free intermediates do not occur during reduction of sulfite to sulfide.

Two aspects of dissimilatory reduction of sulfate to sulfide are interesting: the mode of H-transfer from lactate oxidation reactions to sulfate reduction reactions and the bioenergetics of sulfate reduction.

It has been demonstrated for *Desulfovibrio* species that one hydrogenase and its electron acceptor, cytochrome c_3, are localized in the periplasmic space. Other cytochromes, menaquinone, other redox carriers and lactate dehydrogenase are situated in the membrane whereas all the enzymes for pyruvate oxidation, for sulfate reduction, and a second hydrogenase are in the cytoplasm. This enzyme localization led Peck and co-workers to assume that a "hydrogen cycling" occurs in sulfate reducers. As indicated in Fig. 8.29, the reducing equivalents are released by the cytoplasmic



ATP balance

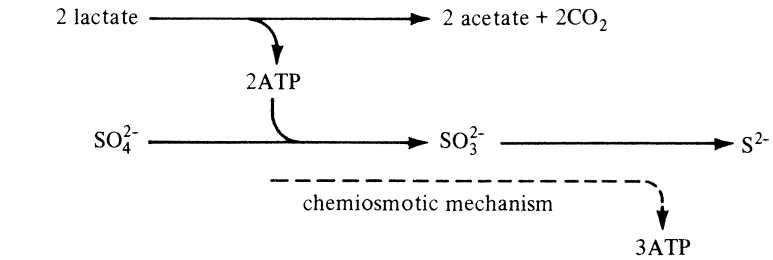


Figure 8.29. Pathway of dissimilatory sulfate reduction in *Desulfovibrio* species and "hydrogen cycling" hypothesis. 1, Lactate dehydrogenase, membrane-bound, H-acceptor not known; 2, pyruvate-ferredoxin oxidoreductase; 3, phosphotransacetylase; 4, acetate kinase; 5, ATP sulfurylase; 6, pyrophosphatase; 7, APS reductase; 8, sulfite reductase; 9, cytoplasmic hydrogenase; 10, periplasmic hydrogenase.

hydrogenase, picked up by the periplasmic hydrogenase, transferred to cytochrome c_3, and channelled via membrane-bound electron carriers to APS-reductase and sulfite reductase. It is obvious that hydrogen cycling allows the generation of a proton gradient at the cytoplasmic membrane.

The reduction of sulfate to sulfite via APS is connected to the hydrolysis of two high-energy phosphoester bonds. Since most sulfate-reducing bacteria are able to reduce sulfate with H_2 and since some group II species even grow with $H_2 + SO_4^{2-} + CO_2$, it is apparent that electron transport to SO_4^{2-} must yield at least 3ATP.

B. Fermentation of acetate and propionate by group II organisms

The conversion of ethanol to acetate and of L-malate to acetate by group I organisms proceeds along the usual pathways. Propionate is converted by *Desulfobulbus propionicus* to acetate via propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA. Formation of pyruvate from oxaloacetate is achieved by the action of methylmalonyl-CoA: pyruvate transcarboxylase.

Of course, it is most interesting that organisms such as *D. acetoxidans* can oxidize acetate completely to CO_2. It is clear now that the tricarboxylic acid cycle is employed for this purpose. The presence of all the enzymes has been demonstrated. Succinate and malate dehydrogenase are membrane-bound and transfer the reducing equivalents to menaquinone rather than to FAD or NAD^+.

C. Dissimilatory reduction of sulfur

Pfennig and collaborators isolated *Desulfuromonas acetoxidans* which grows with acetate and elemental sulfur; acetate is oxidized to CO_2 via the tricarboxylic acid cycle, and the reducing power generated is used to reduce sulfur to sulfide:

$$CH_3—COOH + 2H_2O \longrightarrow 2CO_2 + 8H \qquad \Delta G^{0\prime} = +106.7 \text{ kJ } (+25.5 \text{ kcal})$$

$$4S^\circ + 8H \longrightarrow 4H_2S \qquad \Delta G^{0\prime} = -130.5 \text{ kJ } (-31.2 \text{ kcal})$$

$$CH_3—COOH + 2H_2O + 4S^\circ \longrightarrow 2CO_2 + 4H_2S \qquad \Delta G^{0\prime} = -23.8 \text{ kJ } (-5.7 \text{ kcal})/\text{reaction}$$

Desulfuromonas is devoid of normal fermentative activities, but elemental sulfur can be replaced by fumarate which is reduced to succinate by a membrane-bound fumarate reductase.

Recently, the order Thermoproteales belonging to the archaebacteria was described by Zillig, Stetter, and co-workers. Its members, such as *Thermoproteus tenax* and *Pyrodictium occultum*, are extremely thermophilic organisms. Their growth is most interesting, occurring at the expense of

H_2S formation from H_2 plus elemental sulfur:

$$H_2 + S \longrightarrow HS^- + H^+ \quad \Delta G^{0\prime} = -28.0 \text{ kJ } (-6.7 \text{ kcal})$$

P. occultum is able to do so at a temperature of 105°C! *T. tenax* is a facultative chemolithotroph and grows also with organic substrates plus sulfur; *P. occultum*, however relies on molecular hydrogen and elemental sulfur as substrates.

IX. The Anaerobic Food Chain

The previous sections of this chapter have made us familiar with the various fermentations that are carried out by microorganisms. These fermentations lead to the formation of a number of products which, in natural habitats, accumulate only temporarily. The relationships between fermentative organisms are such that the products of one group of organisms can serve as substrates for another group of organisms. In other words, the various organisms are lined up with their catabolic activities and form a so-called anaerobic food chain. If sulfate is present in sufficient amounts the terminal process of this food chain is the sulfide fermentation; at limiting sulfate concentrations (fresh water environments) it is the methane fermentation.

A. Degradation of organic matter to CH_4, CO_2, and minerals

We have seen that the substrate spectrum of the methanogenic bacteria is rather narrow. Only $H_2 + CO_2$, formate, methanol, methylamines, and acetate can be utilized. Therefore, all the other fermentation products have to be converted to these compounds in the anaerobic food chain. This is not such a problem with lactate; it can be converted by the propionibacteria to propionate and acetate or by *Clostridium tyrobutyricum* to butyrate (lactate + acetate → butyrate + CO_2), but how are ethanol, propionate, and butyrate converted to acetate? The thermodynamics of reactions in which these compounds yield acetate and H_2 (and CO_2 in the case of propionate) is as follows:

$$\text{ethanol} + H_2O \longrightarrow \text{acetate} + H^+ + 2H_2$$
$$\Delta G^{0\prime} = +9.6 \text{ kJ/reaction } (+2.3 \text{ kcal/reaction})$$

$$\text{butyrate} + 2H_2O \longrightarrow 2 \text{ acetate} + H^+ + 2H_2$$
$$\Delta G^{0\prime} = +48.1 \text{ kJ/reaction } (+11.5 \text{ kcal/reaction})$$

$$\text{propionate} + 3H_2O \longrightarrow \text{acetate} + \text{bicarbonate} + H^+ + 3H_2$$
$$\Delta G^{0\prime} = +76.1 \text{ kJ/reaction } (+18.2 \text{ kcal/reaction})$$

The free energy changes are positive, and the reactions will not proceed from left to right except under conditions in which a product is kept at an

extremely low concentration. Due to the high affinity of the methanogenic bacteria towards H_2 the partial pressure of H_2 is kept as low as 10^{-4} atm in the presence of these organisms. This is low enough to make the formation of H_2 from NADH + H^+ and also from the above-mentioned substrates thermodynamically feasible (Fig. 8.30). Microbial associations in which a H_2-producing organism can grow only in the presence of a H_2-consuming organism are called **syntrophic associations**. The coupling of formation and use of H_2 is called **interspecies hydrogen transfer**, a term coined by Wolin, Bryant, and Wolfe.

A well-known syntrophic culture is "*Methanobacillus omelianskii.*" It consists of the S organism that oxidizes ethanol to acetate + H_2 and a methanogenic bacterium (Fig. 8.31a). Other syntrophic partners of H_2-consuming organisms are *Syntrophobacter wolinii* and *Syntrophomonas wolfei* which oxidize propionate (*S. wolinii*) or butyrate (*S. wolfei*) according to the above equations, but only in the presence of H_2-consuming organisms. They are obligate syntrophs.

Another type of relationship between H_2-producing and H_2-consuming organisms is found with various clostridia, *Ruminococcus albus*, etc. on one side and methanogens on the other (Fig. 8.31b). These organisms grow on carbohydrates and ferment glucose to ethanol/acetate + CO_2 + H_2 or butyrate/acetate + CO_2 + H_2. In the presence of a H_2 utilizer the fermentation is shifted towards an acetate fermentation:

$$\text{glucose} + 4H_2O \longrightarrow 2\ \text{acetate}^- + 2HCO_3^- + 4H_2 + 4H^+$$

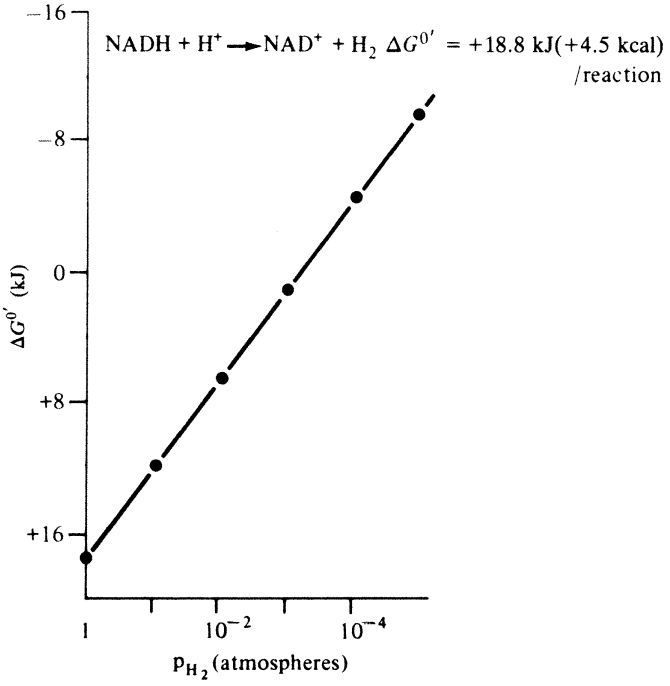


Figure 8.30. The free energy change for the oxidation of NADH to NAD^+ and H_2 as dependent on the partial pressure of hydrogen.

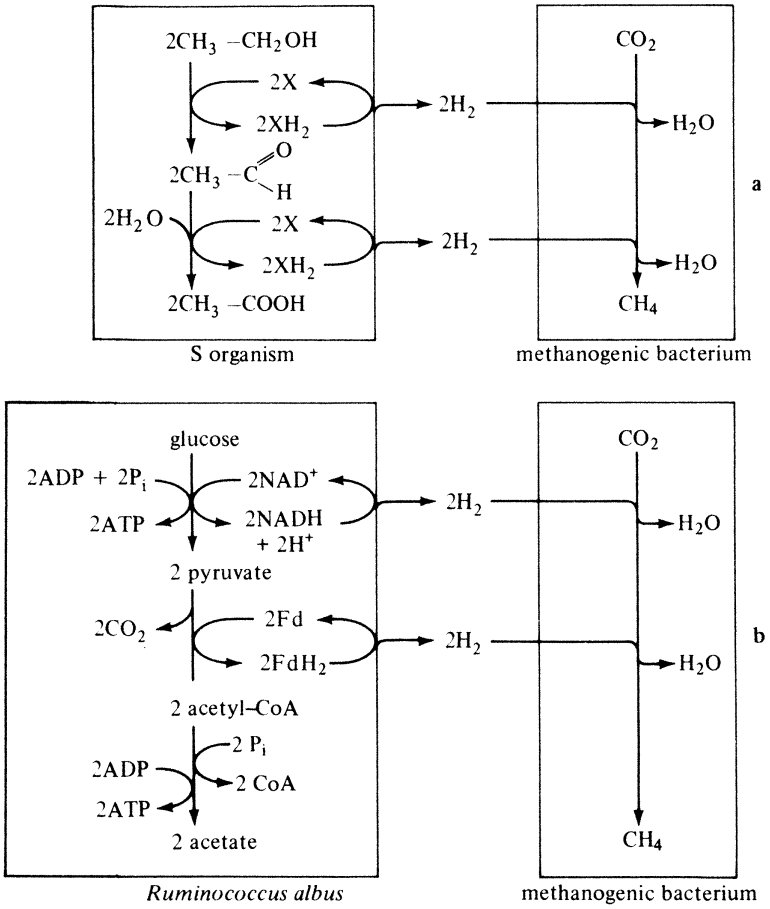


Figure 8.31. Examples of interspecies hydrogen transfer. At low p_{H_2} ethanol is oxidized by the S organism to acetate and H_2 (**a**) and glucose is oxidized by saccharolytic clostridia or *Ruminococcus albus* to acetate + CO_2 + H_2 (**b**). The p_{H_2} is kept low by the action of methanogenic bacteria.

Such shifts are possible if organisms are able to evolve H_2 and have branched fermentation pathways at their disposal (see Fig. 8.12).

The special fermentations under low partial pressures of H_2 make it understandable that fermentation products others than CH_4 + CO_2 do not accumulate in the anaerobic food chain. The importance of a low P_{H_2} was first recognized by Hungate. A scheme of the anaerobic food chain is given in Fig. 8.32.

B. Food chains in marine environments and in the rumen

It has already been mentioned that sulfate reducers predominate in marine environments as compared to methanogenic bacteria. Here the food chain is largely directed towards formation of CO_2 and H_2S. The reason for this is that the substrate spectrum of the sulfate-reducing bacteria is much wider, that they can utilize H_2 very effectively, and that their affinity

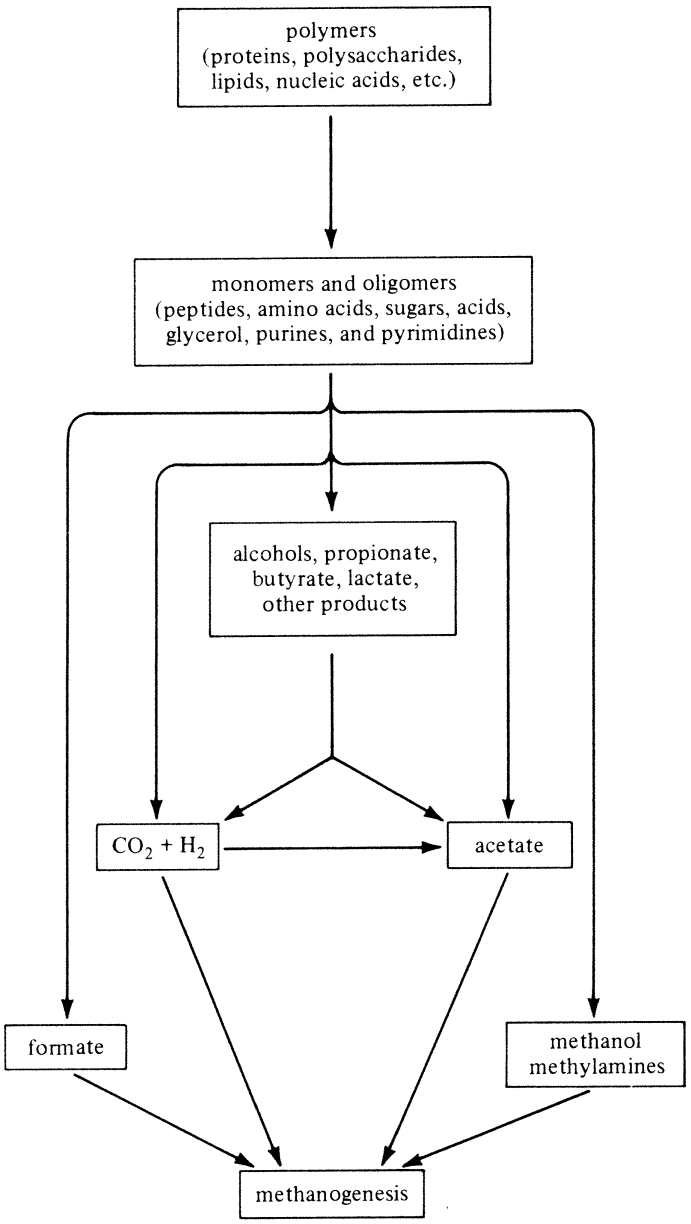


Figure 8.32. The anaerobic food chain.

towards acetate is much higher than the one of methanogens. It is apparent from Fig. 8.32 that acetate is the most important substrate for the terminal food chain reactions. In the presence of sulfate it is not split to $CH_4 + CO_2$ but oxidized to CO_2.

A special situation is met in the rumen. The ruminant is, of course, not interested in a complete anaerobic food chain. Thus, the partial pressure of H_2 is kept low in the rumen by methanogenic bacteria, thereby directing the fermentations towards the formation of organic acids (mainly acetic and propionic acids). These compounds are then taken up by the ruminant.

X. Fermentation of Nitrogenous Compounds

Sugars and organic acids are not the only substrates for anaerobes. Amino acids (formed from proteins by extracellular proteases) and purine and pyrimidine bases are fermented by a variety of microorganisms.

A. Single amino acids

A number of single amino acids can serve as energy and carbon source for anaerobes. **Alanine** is fermented by *Clostridium propionicum* via the acrylate pathway (see Fig. 8.18). *Peptostreptococcus micros* (*Diplococcus glycinophilus*) ferments **glycine** according to the following equation:

$$4H_2N{-}CH_2{-}COOH + 2H_2O \longrightarrow 4NH_3 + 2CO_2 + 3CH_3{-}COOH$$

Originally it was found that the CO_2 produced in this fermentation was derived from the carboxyl group of glycine and that both carbons of acetate originated partly from the methylene carbon of glycine and partly from CO_2. This was in agreement with a pathway in which glycine was hydroxymethylated to serine and then converted to acetate via pyruvate. Recently, Andreesen and co-workers showed that the pathway indicated was only used by the organism in selenium-deficient media and for biosynthetic purposes. In the presence of selenite, the organisms grow much better and the pathway used is different; it is depicted in Fig. 8.33. Part of the glycine is oxidized via methylenetetrahydrofolate to CO_2. The reducing equivalents generated are used to reduce 3 molecules of glycine to acetate + ammonia. The first reaction, the oxidation of glycine to CO_2 and methylenetetrahydrofolate, is very complex. Four proteins are involved: a decarboxylase, a hydrogen carrier protein, a lipoamide dehydrogenase, and a transferase. All subsequent steps are similar to the ones in acetate fermentation, but proceed in the reverse reaction (see Fig. 8.23). It should be noted that the formyltetrahydrofolate synthetase reaction yields ATP by substrate-level phosphorylation.

The most interesting reaction of this pathway is the glycine reductase reaction. As shown by Stadtman and co-workers (working with *Clostridium sticklandii*), the protein complex responsible for this reaction consists of three proteins; the so-called protein A contains selenocysteine. Thus it is clear that the operation of the pathway depicted in Fig. 8.33 depends on the presence of a selenium compound in the growth medium. The glycine reductase reaction is apparently coupled to ATP synthesis which proceeds most likely by substrate-level phosphorylation and not by a chemiosmotic mechanism. A scheme indicating the involvement of the proteins in the reaction is depicted in Fig. 8.34.

The fermentation of **threonine** (Fig. 8.35) is initiated by threonine

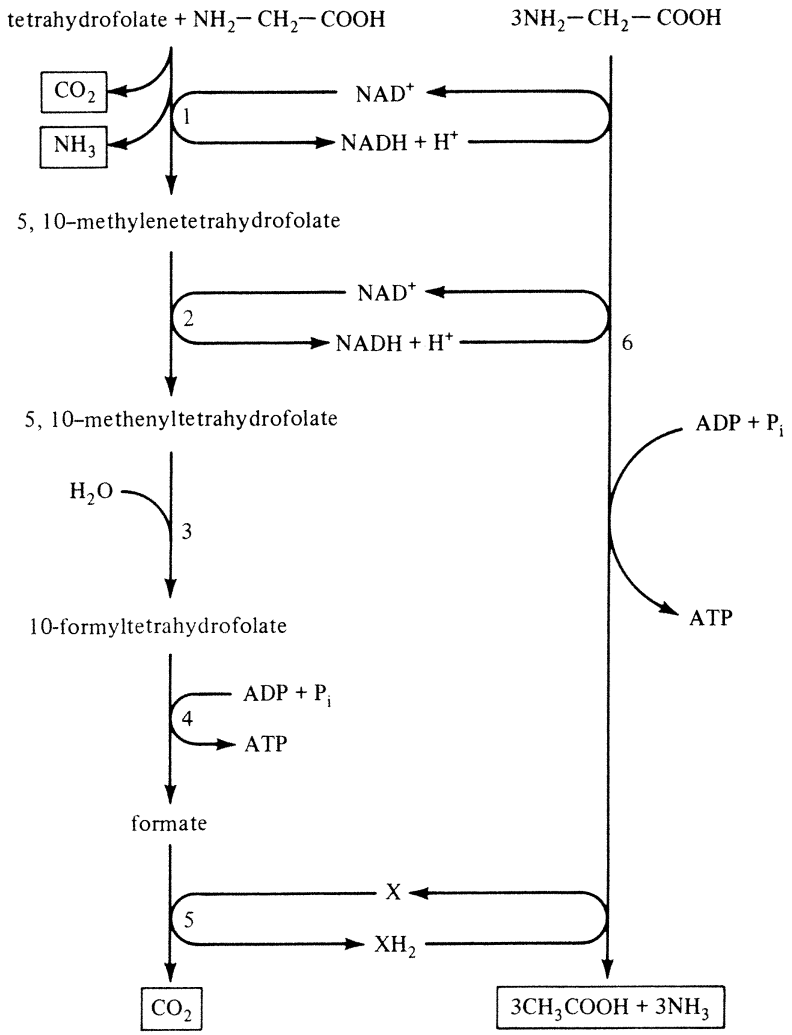


Figure 8.33. Fermentation of glycine by *Peptostreptococcus micros*. 1, Glycine decarboxylase complex; 2, methylenetetrahydrofolate dehydrogenase; 3, methenyltetrahydrofolate cyclohydrolase; 4, formyltetrahydrofolate synthetase; 5, formate dehydrogenase; 6, glycine reductase.

dehydratase (*C. propionicum*, *Peptostreptococcus prevotii*). The α-oxobutyrate formed is further converted to propionate in a reaction sequence involving an enzyme similar to pyruvate-ferredoxin oxidoreductase.

Aspartate is fermented by many facultative and some obligate anaerobic bacteria; it is deaminated to fumarate, which is partly reduced to succinate and partly oxidized to acetate. The pathways involved are similar to those of fumarate and malate fermentation. Some clostridial species, e.g., *C. novyi*, contain a decarboxylase that converts aspartate into alanine or β-alanine. Both are substrates for *C. propionicum*:

$$HOOC-CH_2-CH(NH_2)-COOH \longrightarrow CO_2 + CH_3-CH(NH_2)-COOH$$

$$HOOC-CH_2-CH(NH_2)-COOH \longrightarrow CO_2 + NH_2-CH_2-CH_2-COOH$$

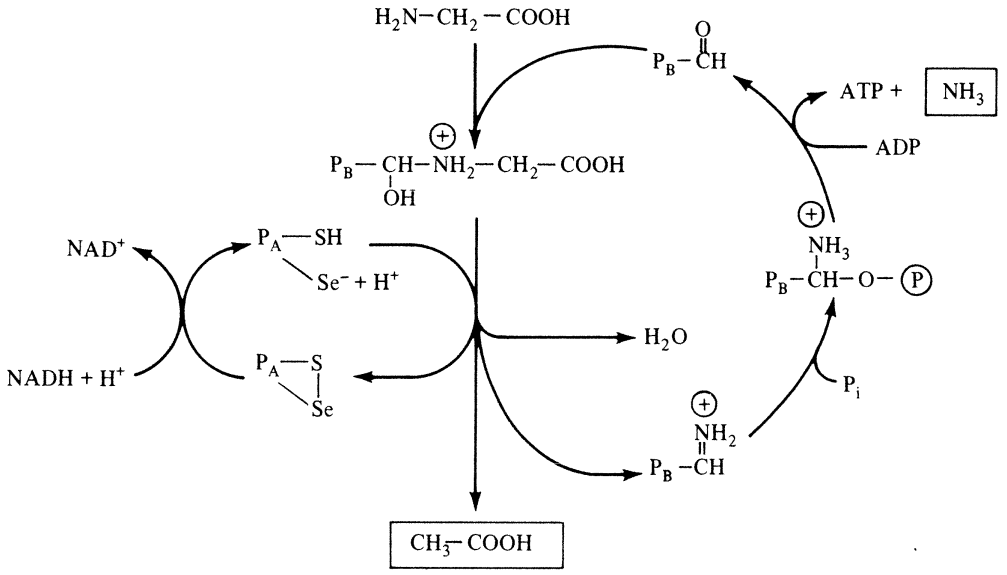


Figure 8.34. Tentative scheme of the glycine reductase mechanism. Only the possible function of two of the three proteins required is indicated. –CHO at P_B indicates a prosthetic group. [Redrawn from G. F. Barnard and M. Akhtar. *Eur. J. Biochem.* **99**, 593–603 (1979)].

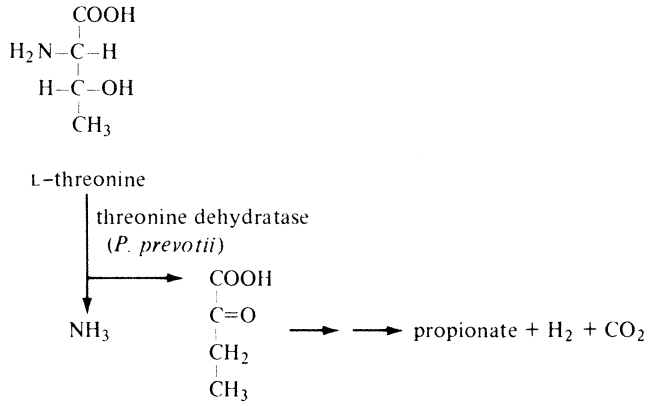


Figure 8.35. Initial reactions in the fermentation of threonine.

The fermentation of **glutamate** by obligate anaerobic bacteria has received considerable attention. This amino acid seems to be the preferred substrate of *Clostridium tetanomorphum*, which employs a rather unusual pathway for its breakdown. The elucidation of this pathway by Barker and collaborators led to the discovery of the first B_{12}-dependent enzyme, **glutamate mutase**. The rearrangement reaction catalyzed by this enzyme has already been discussed in connection with other B_{12}-dependent enzymes (Chapter 8, Section V). As shown in Fig. 8.36a, the product of the mutase reaction, β-methylaspartate, is deaminated to yield mesaconate. Addition of water leads to citramalate, which subsequently is cleaved to acetate and pyruvate. This reaction resembles the citrate lyase reaction, and the citramalate and citrate lyases are closely related to one another.

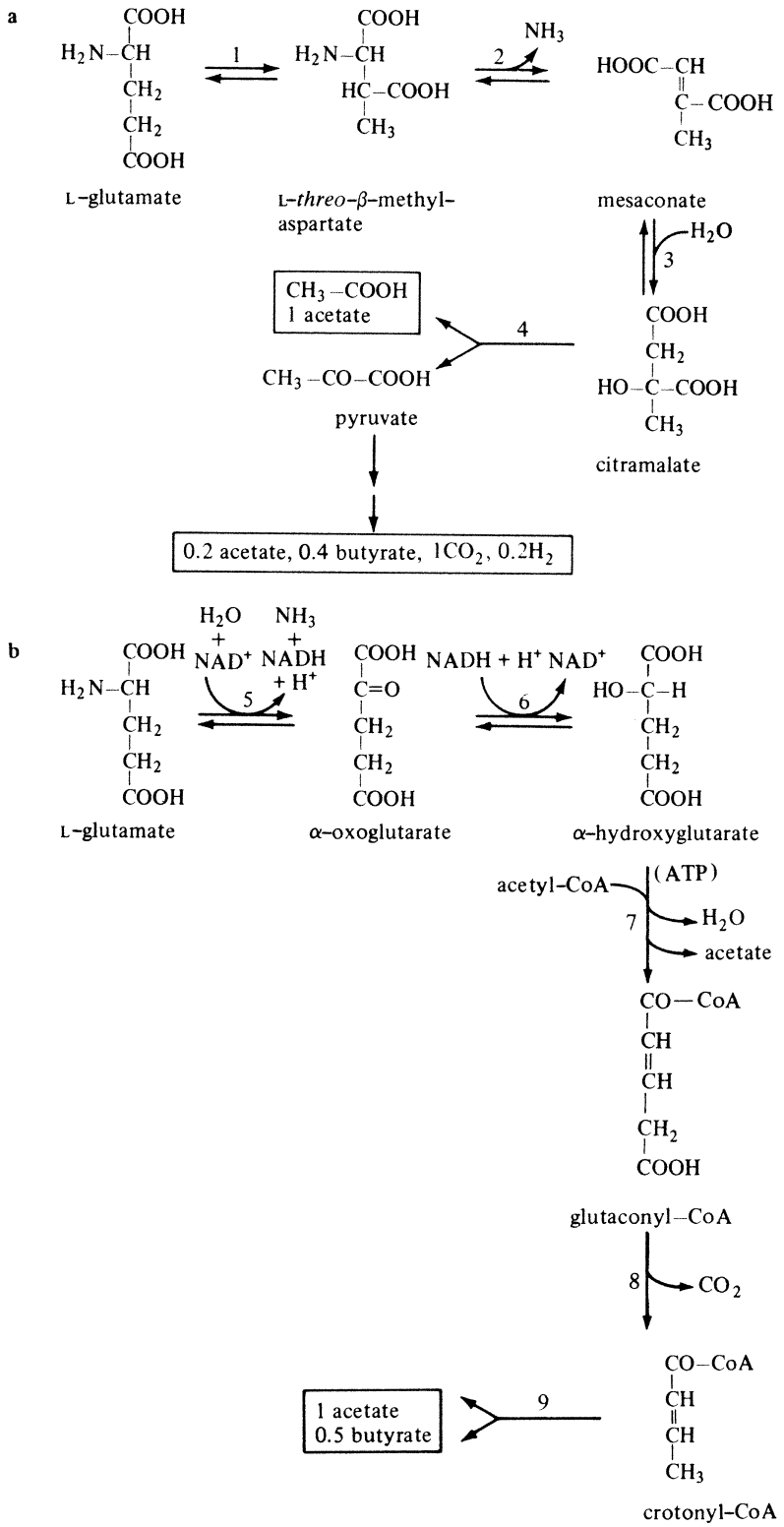


Figure 8.36. Pathways of glutamate fermentation by *Clostridium tetanomorphum* (**a**) and by *Peptostreptococcus asaccharolyticus* (**b**). 1, Glutamate mutase; 2, β-methylaspartase; 3, citramalate dehydratase; 4, citramalate lyase; 5, glutamate dehydrogenase; 6, α-hydroxyglutarate dehydrogenase; 7, a dehydratase and a CoA transferase are involved; 8, glutaconyl-CoA decarboxylase (Na^+-dependent); 9, dismutation of crotonyl-CoA.

Pyruvate is then further degraded to acetyl-CoA, CO_2, and reduced ferredoxin. Little hydrogen is evolved from the latter. Acetyl-CoA is only partly converted to acetate; the rest yields butyrate.

Acidaminococcus fermentans and *Peptostreptococcus asaccharolyticus* ferment glutamate to the same products as *C. tetanomorphum* but use another pathway. As is apparent from Fig. 8.36b, glutamate is first converted to α-hydroxyglutarate. Then glutaconyl-CoA is formed which is decarboxylated to crotonyl-CoA—a reaction that is biotin-dependent and coupled with Na^+ export (see Fig. 8.17). Finally crotonyl-CoA dismutates to yield acetate and butyrate: 1 crotonyl-CoA is oxidized to 2 molecules of acetate (via acetoacetyl-CoA) and 1 crotonyl-CoA is reduced to butyrate (via butyryl-CoA). The mechanism of α-hydroxyglutarate dehydration is not fully understood. The hydroxyl group may be removed from the molecule in a radical reaction.

It should also be mentioned that a glutamate decarboxylase yielding 4-aminobutyrate from glutamate is present in clostridia (e.g., *C. perfringens*), in enterobacteria (e.g., *E. coli*), and others. 4-Aminobutyrate is a substrate for certain clostridia.

Lysine is fermented by *Clostridium sticklandii* and by *C. subterminale* to acetate and butyrate. The interesting pathway employed is summarized in Fig. 8.37; it involves two shifts of amino groups. One of them, L-β-lysine mutase, requires coenzyme B_{12}. The C_6 carbon skeleton is cleaved in a very interesting reaction; the terminal $—CH_2—COOH$ group of 3-oxo-5-aminohexanoate is transferred to acetyl-CoA so that acetoacetate and 3-aminobutyryl-CoA are formed as products. The final products are butyrate, acetate, and ammonia.

Arginine is fermented to ornithine, CO_2, and NH_3 by clostridia, streptococci, halobacteria, eubacteria, and mycoplasmas. This fermentation is unusual in that ATP is formed from carbamoyl phosphate. Ornithine is degraded further by *C. sticklandii*. As in the fermentation of lysine, ornithine breakdown also involves a shift of an amino group. First, L-ornithine is converted to the D-enantiomer by a racemase. Then the amino group is shifted from C_5 to C_4 in a coenzyme B_{12} and pyridoxal phosphate depending reaction. 2-Amino-4-oxopentanoate is subsequently formed which is cleaved with CoA to yield acetyl-CoA and L-alanine. This is another variation of the arginine degradation pathways that were outlined in Chapter 6 (see Fig. 6.4).

B. Stickland reaction

Although a number of clostridial species grow with some single amino acids, many clostridia prefer to ferment mixtures of amino acids. They carry out coupled oxidation-reductions between pairs of amino acids. One amino acid, e.g., alanine, is oxidized, and a second one, e.g., glycine, is

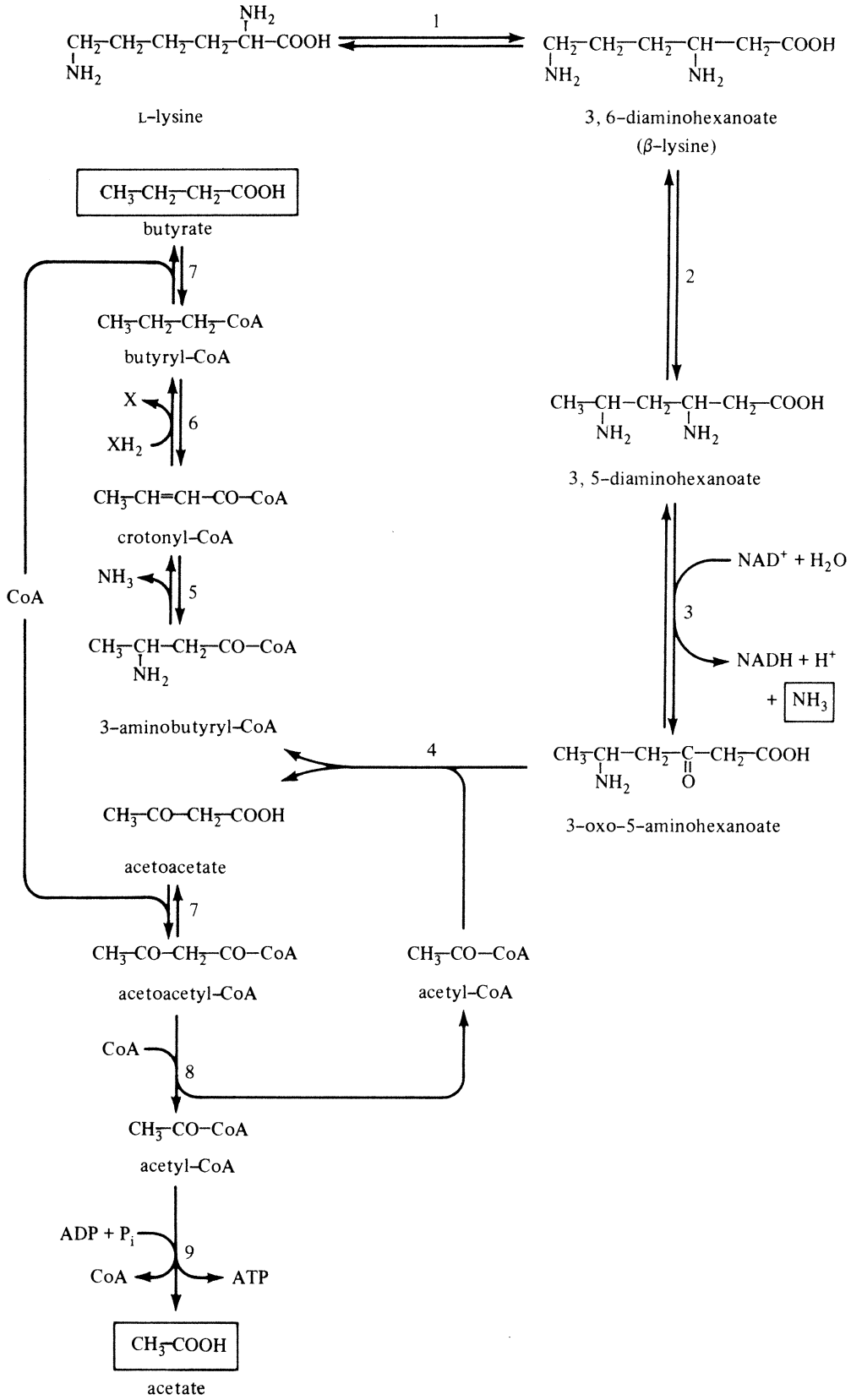


Figure 8.37. The clostridial pathway of L-lysine fermentation. 1, L-Lysine-2,3-aminomutase, pyridoxal-P and Fe^{2+}-dependent; 2, β-lysine mutase, B_{12}-dependent; 3, 3,5-diaminohexanoate dehydrogenase; 4, 3-oxo-5-aminohexanoate cleavage enzyme (acetyl-CoA requiring); 5, L-3-aminobutyryl-CoA deaminase; 6, butyryl-CoA dehydrogenase; 7, CoA transferase; 8, β-ketothiolase; 9, phosphotransacetylase + acetate kinase.

reduced:

$$CH_3-CH(NH_2)-COOH + 2H_2O \longrightarrow CH_3-COOH + CO_2 + NH_3 + 4H$$

$$2NH_2-CH_2-COOH + 4H \longrightarrow 2CH_3-COOH + 2NH_3$$

This type of fermentation was discovered by Stickland in 1934; it is carried out by practically all proteolytic clostridia, such as *C. sporogenes*, *C. sticklandii*, *C. histolyticum*, and *C. botulinum*. Some amino acids are preferably used as H-donors and others as H-acceptors. The most suitable donors and acceptors are given in Table 8.12. Depending on the microorganism, the aromatic amino acids and leucine may function either as H-donor or as H-acceptor.

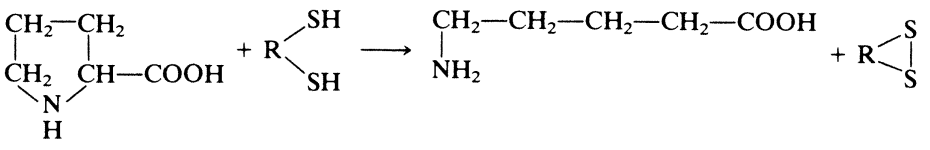
Amino acid oxidation proceeds via the corresponding α-oxoacid:

$$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH \xrightarrow[2H]{+H_2O} NH_3 + R-CO-COOH \xrightarrow[2H]{+H_2O} R-COOH + CO_2$$

The first step, the oxidative deamination, is accomplished either by an enzyme of the type of glutamate dehydrogenase or by transamination with α-oxoglutarate as NH_2-acceptor and subsequent regeneration of α-oxoglutarate by glutamate dehydrogenase. The oxidative decarboxylation is catalyzed by enzymes analogous to pyruvate-ferredoxin oxidoreductase. ATP is formed from the CoA-esters by the action of CoA-transferase, phosphotransacetylase, and acetate kinase.

Amino acid reduction is a rather complex reaction. The glycine reductase system has already been discussed in connection with the metabolism of *P. micros* (see Fig. 8.34). Reduction of betaine or sarcosine proceeds by analogous Se-requiring reductases.

The use of L-proline as oxidant is initiated by a racemase, and the D-enantiomer actually becomes reduced. In contrast to the glycine reductase, the D-proline reductase does not contain selenium, but it contains a pyruvate moiety linked to the protein by a peptide bond. This moiety is essential for catalytic activity. Purified D-proline reductase does not react with NADH as H-donor; a dithiol compound can be used as artificial H-donor. A flavoprotein and an iron-sulfur protein are involved in H-transfer to the enzyme in vivo.



D-proline 5-aminovalerate

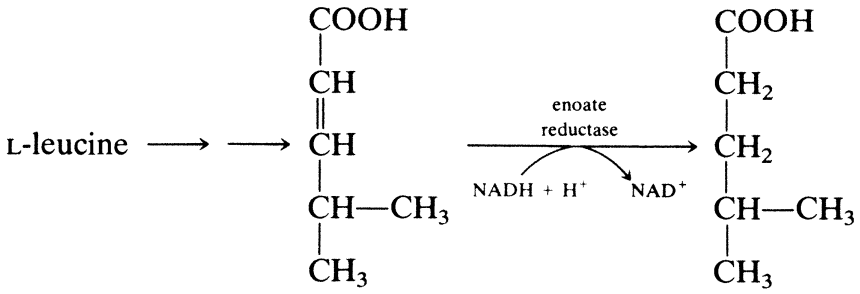
Organisms such as *C. sporogenes* or *C. botulinum* contain an enzyme—ornithine cyclase—that converts ornithine via Δ'-pyrroline-2-carboxylate

Table 8.12. Amino acids that function as H-donors and as H-acceptors in Stickland reactions.

H-donor		H-acceptor	
substrate	product	substrate	product
alanine	acetate + CO_2 + NH_3	glycine	acetate + NH_3
leucine	3-methylbutyrate + CO_2 + NH_3	proline	5-aminovalerate
		phenylalanine	phenylpropionate + NH_3
isoleucine	2-methylbutyrate + CO_2 + NH_3	tryptophan	indolpropionate + NH_3
valine	2-methylpropionate + CO_2 + NH_3	ornithine	5-aminovalerate + NH_3 (via proline)
phenylalanine	phenylacetate + CO_2 + NH_3	leucine	4-methylvalerate + NH_3
tryptophan	indolacetate + CO_2 + NH_3	betaine	acetate + trimethylamine
histidine	glutamate + CO_2 + NH_3	sarcosine	acetate + monomethylamine

to proline so that ornithine can also function as oxidant in Stickland reactions.

It is interesting that the aromatic amino acids and leucine can also function as oxidants. They apparently are converted directly or via the α-hydroxy acids to the α,β-unsaturated acids, which are then reduced by an enzyme present in many clostridia, enoate reductase. It is not very specific and acts on many unsaturated acids.



Since L-leucine can be oxidized and reduced it is not surprising that several proteolytic clostridia can carry out a Stickland reaction with leucine alone—part of it is oxidized to 3-methylbutyrate and another part is reduced to 4-methylvalerate.

C. Heterocyclic compounds

Purines and pyrimidines are readily fermented under anaerobic conditions. The long known species *Clostridium acidiurici* and *C. cylindrosporum* ferment guanine, hypoxanthine, urate, and xanthine; they are so specialized that they will not grow with any other substrate except the intermediate glycine.

Recently, *C. purinolyticum* was described; it grows on a much greater variety of purines including adenine, and it also grows on glycine. Growth is strictly selenium-dependent. Four products are formed by these species from purines: carbon dioxide, ammonia, acetate, and formate. Because of the high N content of the substrates and the two single carbons in the purine skeleton, NH_3 and CO_2 are the major fermentation products. The fermentation of purines is generally initiated by their conversion into xanthine (Fig. 8.38). In the case of guanine this is accomplished by guanine deaminase; in the case of adenine by adenine deaminase plus xanthine dehydrogenase. The latter is an interesting enzyme. It is a selenoflavoprotein containing iron-sulfur centers and molybdenum cofactor, and it catalyzes a variety of oxidation/reduction reactions at the purine ring and other N-heterocyclic compounds: the oxidation of hypoxanthine to xanthine, the reduction of urate to xanthine, the oxidation of purine to hypoxanthine, and the oxidation of, for instance, pterins and 4-hydroxypyrimidine.

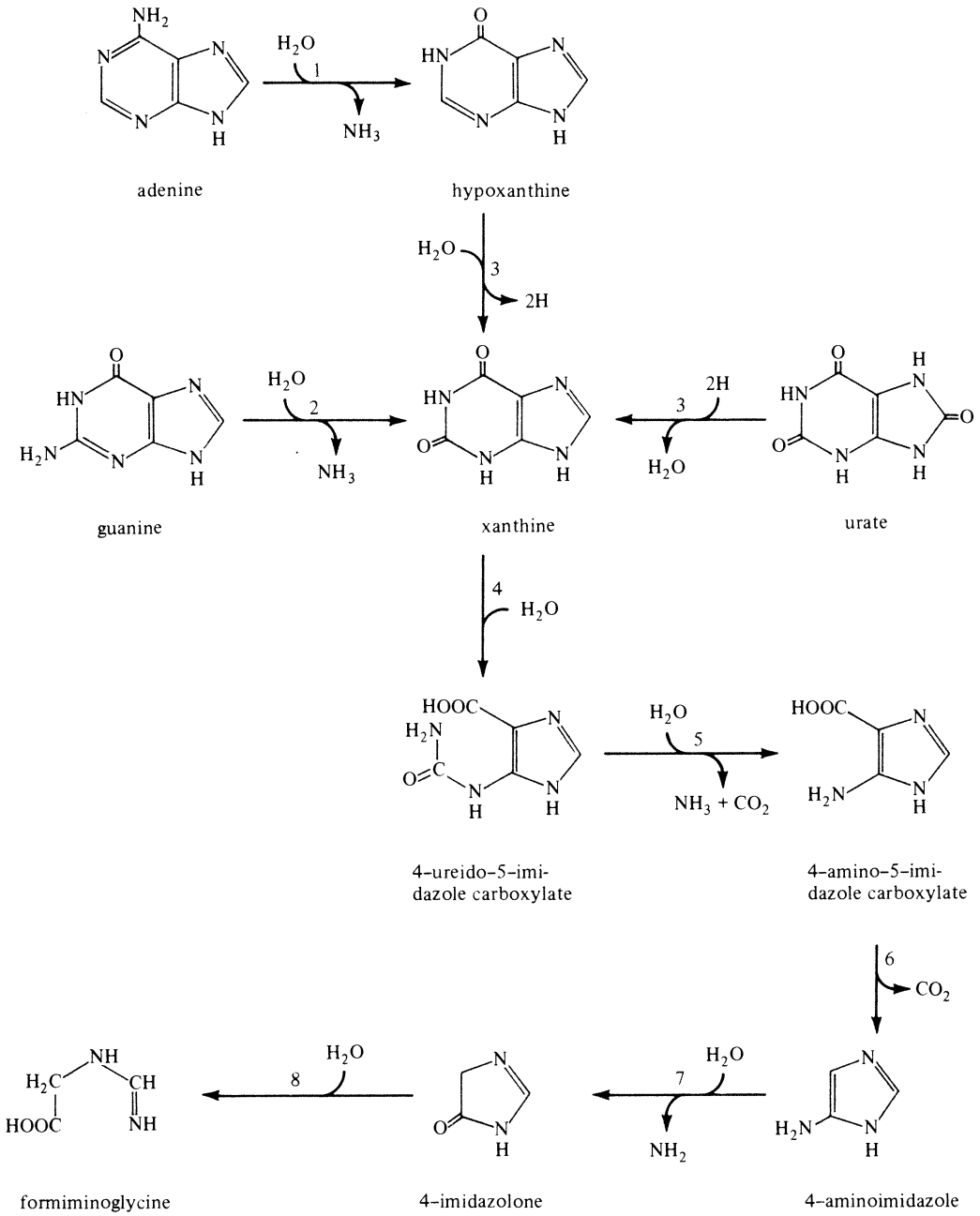


Figure 8.38. Conversion of adenine, guanine, and urate to formiminoglycine by purinolytic clostridia. 1, Adenine deaminase; 2, guanine deaminase; 3, xanthine dehydrogenase; 4, xanthine amidohydrolase; 5, 4-ureido-5-imidazole carboxylate amidohydrolase; 6, 4-amino-5-imidazole carboxylate decarboxylase; 7, 4-amino-imidazole deaminase; 8, 4-imidazolonase.

In the purine fermentation pathway, xanthine is then converted to formiminoglycine by a series of deaminations and decarboxylations. The fate of formiminoglycine is depicted in Fig. 8.39. The formimino group is converted to formate and further to CO_2. The reducing equivalents produced in the formate dehydrogenase reaction are used to drive the

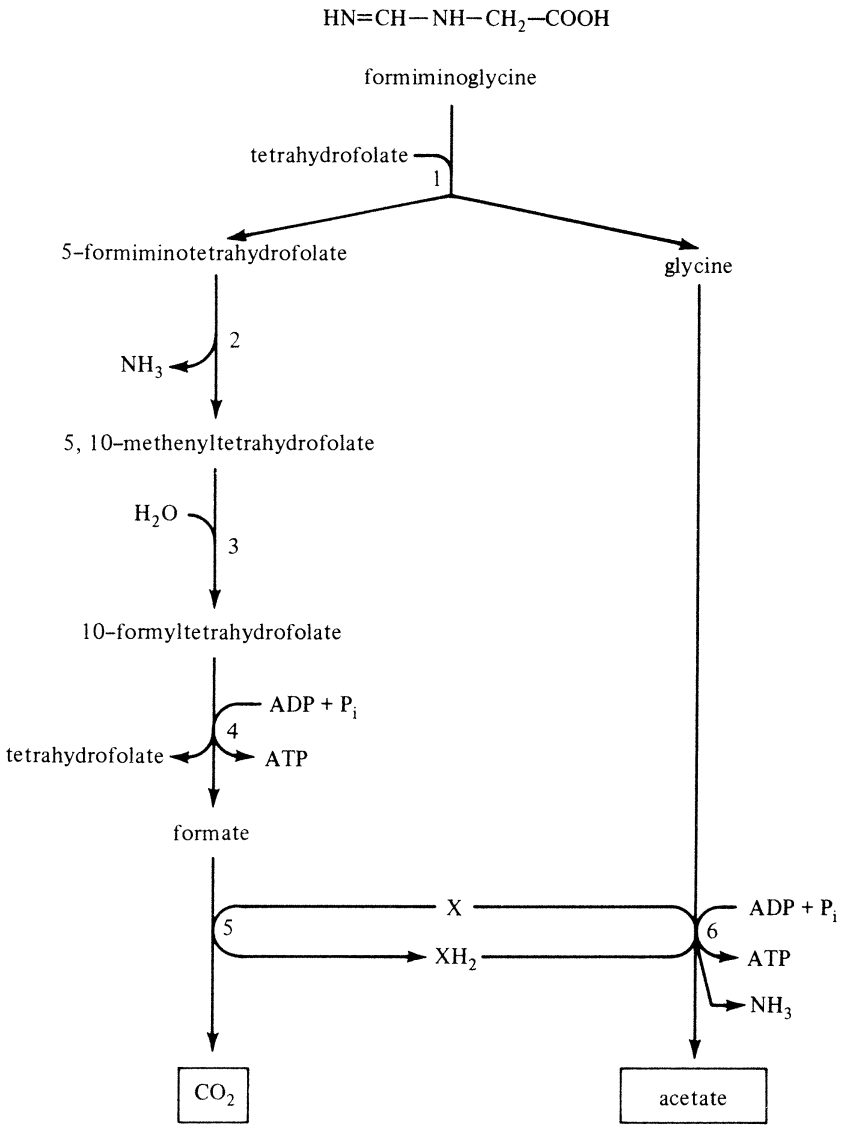


Figure 8.39. Conversion of formiminoglycine to acetate, CO_2, and ammonia. 1, Glycine formimino transferase; 2, formiminotetrahydrofolate cyclodeaminase; 3, methenyltetrahydrofolate cyclohydrolase; 4, formyltetrahydrofolate synthetase; 5, formate dehydrogenase; 6, glycine reductase.

glycine reductase reaction. If reducing equivalents are generated by oxidation of hypoxanthine to xanthine, formate does not need to be oxidized to CO_2 and is excreted. If urate serves as substrate, reducing equivalents have to be generated by an additional pathway, e.g., by oxidation of glycine to CO_2 as outlined in Fig. 8.33.

A number of bacteria are known that ferment pyrimidines. Uracil is degraded by *C. glycolicum* to β-alanine, CO_2, and NH_3 and orotic acid by *C. oroticum* to acetate, CO_2, and NH_3. In general pyrimidines are not fermented as readily as purines. The degradation of nicotinic acid by *Eubacterium barkeri* is initiated by a dehydrogenase that resembles xanthine dehydrogenase. Creatinine is converted by some clostridial

species to *N*-methylhydantoin; the further degradative pathway of this compound remains to be elucidated.

It can be concluded that the fate of a large number of nitrogenous compounds under anaerobic conditions is now known. Details have still to be worked out.

XI. Summary

1. Fermentations are anaerobic dark processes. ATP is formed by substrate-level and/or electron transport phosphorylation.

2. Most strictly anaerobic bacteria lack catalase and superoxide dismutase; strictly anaerobic bacteria require a low redox potential for growth.

3. Yeasts ferment glucose to ethanol and CO_2. The key enzyme of this fermentation is pyruvate decarboxylase. Two mol of ATP are formed per mol of glucose fermented; yeasts increase the rate of glucose breakdown when transferred from aerobic to anaerobic conditions (Pasteur effect).

Zymomonas species, *Sarcina ventriculi*, and *Erwinia amylovora* carry out alcohol fermentations. Ethanol produced in smaller amounts by lactic acid bacteria, enterobacteria, and clostridia is formed by reduction of acetyl-CoA.

4. Lactic acid bacteria employ the homofermentative, the heterofermentative, or the bifidum pathway for the fermentation of hexoses. The first pathway yields 2 lactate/glucose, the heterofermentative pathway yields lactate, ethanol, and CO_2, whereas acetate and lactate are formed in a ratio of 3:2 by the bifidum pathway. The key enzyme of the latter two pathways is phosphoketolase.

Lactic acid is produced in several forms—D(−), L(+), and DL—and lactic acid bacteria contain D-lactate dehydrogenase, L-lactate dehydrogenase, or a mixture of these enzymes. A few producers of the DL-form contain L-lactate dehydrogenase plus racemase.

Lactobacillus plantarum and some other species ferment malate to lactate and CO_2 (malo-lactate fermentation). *Streptococcus cremoris* and *Leuconostoc cremoris* produce diacetyl from citrate. The latter is cleaved into acetate and oxaloacetate by citrate lyase and the final step in diacetyl synthesis is the condensation of acetyl-CoA with hydroxyethylthiamine pyrophosphate.

From growth yield studies with lactic acid bacteria it was deduced that an average of 10.5 g of cells can be formed per 1 mol ATP produced, provided that all monomers required in biosynthesis are available to the cells.

5. The main fermentation product of many clostridia, eubacteria, fusobacteria, and butyrivibrios is butyrate. It is formed from sugars via pyruvate, acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA. The conversion of pyruvate to acetyl-CoA is catalyzed by pyruvate-ferredoxin ox-

idoreductase. In many fermentations H_2 is produced from reduced ferredoxin with hydrogenase. H_2 production from NADH allows the organisms to produce less butyrate, more acetate, and more ATP (advantage of branched pathways).

Some clostridia (e.g., *C. acetobutylicum*) form acetone and butanol at pH values below 4.5.

The hydrogen balance of fermentations can be determined on the basis of the O/R values of substrates and products or on the basis of the number of available hydrogens in substrates and products.

C. kluyveri ferments ethanol and acetate to butyrate, caproate, and molecular hydrogen. H_2 evolution is closely connected with ATP synthesis by this microorganism. Per mol H_2 evolved 0.5 mol of acetyl-CoA becomes available for ATP synthesis. Pyruvate is synthesized in *C. kluyveri* by reductive carboxylation of acetyl-CoA, and oxaloacetate by the pyruvate carboxylase reaction. Consequently, about 30% of the cellular material of *C. kluyveri* is derived from CO_2. *C. kluyveri* contains *re*-citrate synthase.

6. Microorganisms belonging to the genera *Escherichia*, *Salmonella*, and *Shigella* carry out a mixed acid fermentation and produce lactate, acetate, succinate, formate, CO_2, and H_2. Characteristic enzymes of this fermentation are pyruvate-formate lyase, which cleaves pyruvate into acetyl-CoA and formate, and formate-hydrogen lyase, which splits formate into $H_2 + CO_2$. Pyruvate-formate lyase is rapidly inactivated by oxygen.

7. Microorganisms belonging to the genera *Enterobacter*, *Serratia*, and *Erwinia* produce less acids than the above-mentioned enterobacteria but more CO_2, ethanol, and 2,3-butanediol. The first enzyme in 2,3-butanediol formation is α-acetolactate synthase. An oxaloacetate decarboxylase has been found in *Enterobacter* that couples decarboxylation with generation of a Na^+ gradient across the membrane.

8. *Clostridium propionicum* and *Megasphaera elsdenii* employ the acrylate pathway for the formation of propionate from lactate. Lactyl-CoA and acrylyl-CoA are intermediates of this pathway. Electron-transferring flavoprotein functions as H-carrier.

The propionibacteria and other propionate-forming microorganisms employ the succinate-propionate pathway in which succinyl-CoA and methylmalonyl-CoA function as intermediates. The interconversion of these two thioesters is catalyzed by methylmalonyl-CoA mutase, a coenzyme B_{12}-containing enzyme.

The reduction of fumarate to succinate by fumarate reductase is a process by which strict anaerobes gain ATP by electron transport phosphorylation. Fumarate reductase is membrane-bound and associated with menaquinone and in many organisms with a cytochrome of the *b* type.

9. *C. formicoaceticum* and *C. thermoaceticum* ferment 1 mol of hexose to almost 3 mol of acetate. Acetate is formed by the Embden–Meyerhof–Parnas pathway and by reduction of CO_2 to acetate. *Clostridium aceticum*

and *Acetobacterium woodii* ferment $H_2 + CO_2$ to acetate. CO is a precursor of the carboxyl group of acetate.

10. Methanogenic bacteria ferment $CO_2 + H_2$, formate, methanol, methylamines, and acetate. The reduction of CO_2 to CH_4 proceeds with methanofuran, tetrahydromethanopterin, and coenzyme M as carriers. ATP is produced in the last reduction step by a chemiosmotic mechanism. A nickel-tetrapyrrol is a constituent of the terminal reductase. A B_{12}-containing enzyme is involved in transfer of the methyl groups of methanol and methylamines to coenzyme M. Those methanogens that utilize methanol, methylamines, and acetate (e.g., *Methanosarcina barkeri*) contain cytochromes. Coenzyme F_{420} is an important hydrogen carrier in methanogenic bacteria.

11. In sulfide fermentation, the oxidation of organic compounds is coupled to the reduction of sulfate to sulfide. The substrate for reduction is adenosine-5′-phosphosulfate (APS), and reduction proceeds via sulfite to sulfide. Electron carriers such as ferredoxin, cytochrome c_3, rubredoxin, and menaquinone are involved in sulfate reduction and in ATP synthesis by electron transport phosphorylation. Group I sulfidogenic bacteria oxidize substrates to acetate, group II organisms all the way to CO_2.

12. The partial pressure of hydrogen in mud, in anaerobic digesters, and in the rumen is kept low by the action of methanogenic bacteria. This favors organisms that produce hydrogen (interspecies hydrogen transfer) and acetate and is a prerequisite for the functioning of the anaerobic food chain. In marine environments, sulfidogenic organisms predominate, and $H_2S + CO_2$ are produced from acetate, formate, and H_2.

13. Single amino acids are fermented by a number of anaerobic bacteria: alanine by *Clostridium propionicum*, glycine by *Peptostreptococcus micros*, glutamate by *C. tetanomorphum*, and lysine by *C. sticklandii*. The fermentation of glutamate and lysine involves coenzyme B_{12}-containing enzymes.

Pairs of amino acids are fermented by a number of proteolytic clostridia (Stickland reaction). The oxidation of one amino acid (e.g., alanine) is coupled to the reduction of another amino acid (e.g., glycine).

Microorganisms such as *C. acidiurici* and *C. purinolyticum* are specialized for the fermentation of purine bases. Urate is fermented by *C. acidiurici* to acetate, CO_2, and ammonia.