

Energy Conservation in Chemotrophic Anaerobic Bacteria

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INTRODUCTION

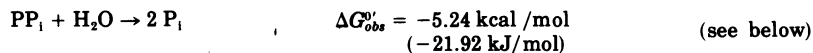
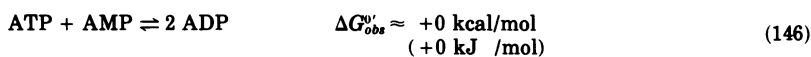
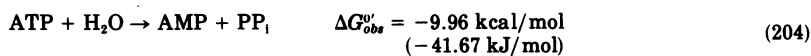
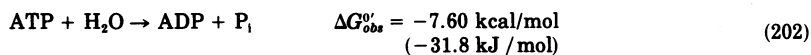
As representatives of the first living organisms or of bacteria which have adapted to extreme ecological conditions, the chemotrophic anaerobic bacteria exhibit characteristics which may be variously interpreted as being primitive or specialized (434). Consequently, a study of their biology frequently provides valuable insights into principles and

mechanisms of cellular processes. This is particularly true in the case of energy conservation.

The energy metabolism of chemotrophic anaerobes has been the subject of intensive investigations in the last few years. Evidence has accumulated that many strictly anaerobic bacteria can synthesize adenosine 5'-triphosphate (ATP) via electron transport phosphorylation

(ETP), a mechanism previously thought to occur only in chemotrophic aerobes and in phototrophic organisms (129). Many new observations have been made which are interesting from both mechanistic and thermodynamic points of view: a strictly anaerobic bacterium has been described which can oxidize acetate to CO₂ with elemental sulfur and obtain useful energy from this reaction as evidenced by growth (488); mixed cultures of anaerobic bacteria have been reported to metabolize long-chain fatty acids to acetate (109, 250); a sulfate-reducing bacterium was isolated that can oxidize methane to CO₂ (R. S. Hanson, personal communication); a microbial consortium of microbes was shown to anaerobically degrade benzoate to CO₂ and methane (168). The finding that an ATPase-dependent pH gradient (interior alkaline) (224) and a concomitant membrane potential difference (interior negative) (221, 222) are intrinsic properties of both anaerobic and aerobic procaryotic cells appears to have important evolutionary implications (528, 529). Of the regulatory effects described, the most intriguing one appears to be that, in many anaerobic bacteria, the amount of ATP formed per mole of energy substrate fermented can be regulated (262), thus permitting the organism to optimize the thermodynamic efficiency of energy transformation.

In view of these many new developments, it seems appropriate to bring up to date what is



known about energy transformation in this interesting group of organisms. In doing so we emphasize recent studies on ETP, on transport processes associated with energy metabolism, and on the regulation of thermodynamic efficiencies. A chapter on energy conservation via substrate level phosphorylation (SLP) has been included to give a complete picture.

The discussion includes ATP synthesis in facultative anaerobes growing under anaerobic conditions. Some of the known anaerobic bacteria appear to have originated from facultative anaerobic organisms, which in the anaerobic environment have lost the ability to use molecular oxygen as terminal electron acceptor of energy metabolism (129, 702). It is therefore frequently difficult or even impossible to decide

whether or not a reaction is typical for genuine anaerobic bacteria.

A recent review on the physiology of obligate anaerobiosis was done by Morris (434).

GENERAL FEATURES

Energy Transformation Via the ATP System

The metabolism of anaerobic bacteria as that of all living cells is an open system which is characterized by a continuous input and output of matter and energy. Each cell is endowed with a system that transforms the chemical and physical energy taken up into biologically useful energy and that utilizes the latter to perform work. The universal molecular carrier for biological energy is ATP, or more exactly the ATP system (Fig. 1). The energy taken up by the cells is used to drive the endergonic synthesis of ATP from adenosine 5'-diphosphate (ADP) and inorganic orthophosphate (P_i). The energy conserved in the "energy-rich" pyrophosphate bond of ATP (375) is then used to do work. ATP is either split to ADP and P_i or to adenosine 5'-monophosphate (AMP) and inorganic pyrophosphate (PP_i). An inorganic pyrophosphatase (EC 3.6.1.1.) (95, 286) and an adenylate kinase (EC 2.7.4.3.) (451) present in all living cells secure the connection of AMP and PP_i to the catabolism utilizing only ADP and P_i:

($\Delta G_{obs}^{0'}$ is the free energy change of the reactions at a free Mg²⁺ concentration of 10⁻³ M, an ionic strength of 0.25 and a pH of 7 [1 cal = 4.184 J]). The free energy change of hydrolysis of PP_i has been calculated from the free energy changes of ATP hydrolysis to ADP plus P_i and to AMP plus PP_i, respectively, and the $\Delta G^{0'}$ of the adenylate kinase reaction. The value obtained is by 1.2 kcal/mol [5.02 kJ/mol] more negative than the one determined recently by Flodgaard and Fleron (-4 kcal/mol) [172]. Note that ATP, ADP, AMP, and PP_i exist in solution as equilibrium mixtures of several polyanionic species, each of which can form complexes with divalent cations. Therefore, the observed free energy of hydrolysis [$\Delta G_{obs}^{0'}$] are functions of the acid dissociation constants of each species pres-

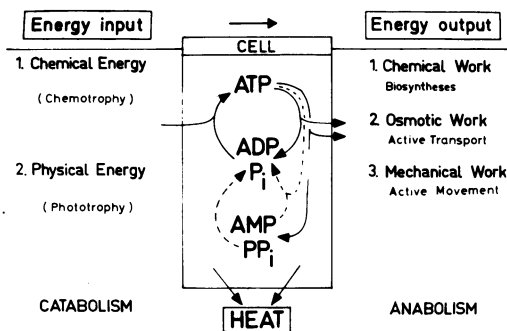


FIG. 1. Energy transformation via the ATP system. Abbreviations: P_i , inorganic phosphate; PP_i , inorganic pyrophosphate. ----- indicates connection of AMP and PP_i to the only ADP plus P_i utilizing catabolism via adenylate kinase and inorganic pyrophosphatase. Note that, in bacteria endowed with the mechanism of ETP, in mitochondria, and in chloroplasts, a proton-motive force (PMF) consisting of a pH gradient (ΔpH) and an electrical potential difference ($\Delta\psi$) may be equivalent to the ATP system with respect to some, but not all, transport processes rather than with respect to biosyntheses and active movement. Further details on the PMF as an intermediate in ATP formation during ETP and in transport processes are discussed in the section ATP Synthesis Via ETP and Transport of Energy Substrates and Products and the Proton-Motive Force.

ent and also of the stability constants with divalent cations. The observed standard free energies of hydrolysis for the reactions are therefore dependent upon the hydrogen ion and divalent cation concentrations.)

The standard free energy of hydrolysis of ATP to produce ADP and P_i (ΔG_{obs}°) is still a matter of considerable controversy (see Banks and Vernon [39]). Values of -6.79 kcal/mol (-28.4 kJ/mol) (543), of -7.6 kcal/mol (-31.80 kJ/mol) (202), and of -8.74 kcal/mol (-36.57 kJ/mol) (10, 489, 590) were recently obtained (for discussion see Guynn Veech [202]). The value used in this review is the one determined by Guynn and Veech [202], which is based on the ΔG° of the hydrolysis of acetyl coenzyme A (203), the energy-rich compound most frequently used by anaerobic bacteria to synthesize ATP via SLP (see section, Energy Conservation Via SLP).

It should be pointed out that the concept of ATP formation from ADP and P_i and its hydrolytic cleavage to ADP and P_i or AMP and PP_i is only a useful formalism to dissect complex processes into simple hydrolysis and condensation reactions, which in vivo do not occur (see Banks [38, 39, 402, 471]). Simple hydrolysis of energy-rich compounds must be prevented in any case

in the cell; otherwise the energy would be lost as heat without performing work. Since ATP is never directly hydrolyzed, the coupling between catabolism and anabolism is stoichiometric or chemical rather than energetic or physical (39). The hydrolysis potential of ATP and of other energy-rich compounds (see below) then is only a measure for a specific kind of chemical reactivity, i.e., a group transfer potential. The standard free energy of hydrolysis of ATP to product ADP plus P_i or AMP plus PP_i is a measure of its phosphoryl group transfer potential or adenylyl group transfer potential, respectively.

The amount of free energy required for the synthesis of 1 mol of ATP from ADP and P_i in a cell under reversible conditions is mainly dependent on the intracellular concentrations of ADP, ATP, P_i , and Mg^{2+} , and the pH. None of these parameters are known with certainty (for discussion of metabolic concentrations and the conservation of solvent capacity in living cells see Atkinson [30]). The ATP and ADP concentration in growing anaerobic bacteria has been determined to be approximately 2 mM and 1 mM, respectively (Table 1). The intracellular concentration of Mg^{2+} in anaerobic bacteria is not known. In *Bacillus cereus* (an aerobic bacterium), Mg^{2+} has been reported to be 6 mM and to be independent of the Mg^{2+} concentration in the growth medium (565). In *Escherichia coli*, the intracellular concentration of Mg^{2+} follows the concentration within the medium from 10^{-6} to 10^{-2} M (259). Reliable data for intracellular phosphate concentrations in anaerobic bacteria are not available. In *Strepto-*

TABLE 1. Steady-state levels of adenosine phosphates in chemotrophic anaerobic bacteria

Adenosine phosphate	<i>Clostridium kluveri</i> ^a (mM ^c)	<i>Methanobacterium</i> strain MOH ^b (mM ^c)
ATP ^d	2.2	1.68
ADP	0.83	1.76
AMP	0.3	0.29
ATP/ADP	<i>2.67</i> ^e	<i>0.95</i>

^a Growing cultures (from Decker and Pfitzer [130]).

^b Cell suspensions reducing CO_2 to CH_4 with H_2 as electron donor (from Robertson and Wolfe [537]).

^c Calculated from original data (moles per gram of dry cells), assuming 2.5 ml of cytoplasmic space per g of dry cells (see Riebeling et al. [529]).

^d The ATP concentration in *Selenomonas ruminantium* has been reported to be only 0.92 mM (249).

^e Italicized numbers are ratios.

coccus faecalis, the intracellular phosphate concentration has been reported to vary by a factor of 10, between 6 and 60 mM, dependent on the energy substrate fermented (223). The intracellular pH in anaerobic bacteria has been shown to be dependent on the pH of the growth medium (529) and to vary from organism to organism. The lowest pH observed is pH 6 (*Clostridium pasteurianum*) (529); the highest pH reported is 8.5 (*S. faecalis*) (224, 223). Taking these parameters into account, the free energy of hydrolysis of ATP becomes approximately -10.5 kcal/mol (-43.9 kJ/mol) at pH 7, -10.0 kcal/mol (-41.8 kJ/mol) at pH 6, and -12.0 kcal/mol (-50.2 kJ/mol) at pH 8.5 (for calculations see Alberty [10]; note that the difference between the free energy change from -7.6 under standard conditions to -10.5 kcal/mol under physiological conditions is mainly the result of the fact that two products are formed from one substrate at millimolar rather than molar concentrations).

Approximately 20 mmol of ATP is consumed during the synthesis of 1 g of cells (wet weight) in a growing culture of anaerobic bacteria (129). The intracellular concentration of ATP is about 2 mM (Table 1). Obviously the ATP system has a catalytic role only. During the doubling of the cell mass, ATP must be turned over approximately 10,000 times.

The ATP system must be in a kinetic equilibrium between ATP-producing and -consuming reactions. The processes with the smaller capacities become the rate-limiting factors for cell growth. As was outlined previously (129), anabolism appears to be rate limiting generally with aerobic organisms and catabolism appears to be rate limiting with anaerobes (see also 52):

$$v_{\text{ATP formation}} = v_{\text{ATP consumption}} = v_{\text{growth}}$$

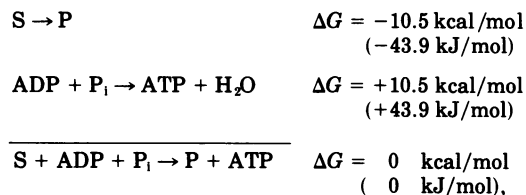
The catalytic role of the adenylate system in metabolic energy transformation requires a strict coordination of ATP consumption and ATP regeneration. The adenylate energy charge [(ATP + 0.5 ADP)/(ATP + ADP + AMP)] (28, 29, 31) (see Table 1) appears to be kept rather constant under different nutrient conditions (130) and absolute cellular contents (148; see however 176, 604).

Thermodynamic Efficiencies of Energy Transformation

It is clear that neither energy conservation during catabolism nor energy utilization during anabolism occurs with 100% efficiency. Part of the transformed energy is always lost as heat (Fig. 1).

As was pointed out above, dependent on the

intracellular pH, between -10 and -12 kcal (-41.8 and -50.2 kJ) is required for the synthesis of 1 mol of ATP from ADP and P_i in anaerobic bacteria. A catabolic process (formulated with the actual concentration of substrates and products) must therefore be associated with a free energy change of -10 to -12 kcal/mol so that it can be coupled with phosphorylation (for thermodynamic data of catabolic redox processes and the dependence of ΔG on pH and concentration see the Appendix). A value of -10 to -12 kcal/mol is sufficient, however, only if the ATP generating process is fully reversible, i.e., if the process is very near or at equilibrium:



where P = product and S = substrate. The thermodynamic efficiency (η) of such an idealized in vitro process is 100%:

$$\eta = \frac{n \times \Delta G (\text{ATP} \rightarrow \text{ADP} + P_i)}{\Delta G (S \rightarrow P)} \times 100\%$$

where n = moles of ATP formed in the reaction.

Krebs and collaborators recently demonstrated that the glyceraldehydephosphate dehydrogenase/phosphoglycerate kinase reaction (SLP) is very near equilibrium in the liver cells (333, 679). As in liver, glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3) are involved both in gluconeogenesis and glycolysis in many organisms. Evidence is accumulating that in mitochondria most of the reactions between reduced nicotinamide adenine dinucleotide (NADH) and cytochrome a_3 are virtually at equilibrium (464, 708, 709; cf. 603); ATP synthesis via ETP in the mitochondria is fully reversible at site 1 and site 2. Many enzymes involved in ATP synthesis via SLP in anaerobic bacteria catalyze completely reversible processes. However, dependent on the organism, either the forward or the backward reaction is only mediated by the enzymes in vivo, e.g., pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), formyltetrahydrofolate synthetase (EC 6.3.4.3), β -ketothiolase (EC 2.3.1.9), ornithine transcarbamoylase (EC 2.1.3.3) (see section, Energy Conservation Via Substrate Level Phosphorylation). These observations are taken to indicate that many of the reactions directly involved in energy conservation are very near equilibrium,

i.e., that the thermodynamic efficiency of ATP synthesis from ADP and P_i may approach 100% in the energy-conserving reaction proper. For a discussion of the role of equilibria in the regulation of metabolism see Krebs (331).

Living cells are open systems; life proceeds irreversibly. A system at equilibrium cannot perform work on its surroundings. At least one of the reactions of the system must be irreversible (for a discussion of non-equilibrium thermodynamics and its application to bioenergetics see Caplan [99]). The ATP-consuming processes (anabolism) generally proceed completely irreversibly; the thermodynamic efficiency of cell material synthesis is less than 10%. Therefore, theoretically all the reactions of energy metabolism (not only those directly coupled with phosphorylation) could operate near or at equilibrium without the organism losing the ability to do work. Such an energy metabolism could, however, not be regulated, as reactions at equilibrium cannot be. It is not only important for a living cell that ATP is synthesized, but also that the ATP is formed at the right time and at the right rate. This is probably the most decisive reason why at least one, but usually more than one, reaction of the overall energy metabolism of most organisms is found to proceed irreversibly under physiological conditions. Examples are the hexokinase reaction (EC 2.7.1.1), the phosphofructokinase reaction (EC 2.7.1.11), and the pyruvate kinase reaction (EC 2.7.1.40) in glycolysis and the oxidation of cytochrome α_3 with O_2 in the respiratory chain. As a consequence, the thermodynamic efficiency of ATP synthesis of the overall energy metabolism is always considerably lower than 100%.

The stoichiometric coupling of energy-supplying reactions and ATP formation leads to a "quantization" of the energy transfer, with the result that only packets of 10 to 12 kcal/mol can be utilized for energy conservation. Exergonic processes yielding substantially smaller amounts of free energy in fact reduce the efficiency of the overall process; this is also true of the part of the energy of strongly exergonic reactions that exceeds 10 to 12 kcal/mol (e.g., pyruvate kinase reactions [EC 2.7.1.40] and phosphoketolase reactions [EC 4.1.2.9] [129]).

A theoretical derivation of the upper efficiency limit for metabolic processes in living organisms has not yet been provided. Experience has shown, however, that values of more than 80% (pyruvate fermentation of *Proteus rettgeri* [341]) for overall processes are very unlikely. The majority of anaerobes work at efficiencies of 25 to 50%. With oxygen as an electron acceptor and a functional ETP, the ther-

modynamic efficiency is not greatly improved (e.g., glucose fermentation to lactic acid, $\eta = 44\%$; glucose respiration to CO_2 , $\eta = 59\%$), whereas the ATP gain (g , in moles of ATP formed per mole of substrate metabolized) increases drastically (glucose fermentation, $g = 2$; glucose respiration, $g = 38$).

The energy metabolism of most organisms is represented by a linear catabolic process with a constant ATP gain (Fig. 2). Examples are the aerobic respiration and the homolactic acid fermentation. The thermodynamic efficiency of these processes is invariable. The energy metabolism of many anaerobic bacteria, however, is branched (Fig. 2), each branch leading to a different ATP gain and thermodynamic efficiency of ATP synthesis. Product formation via the one branch would be characterized by a very high thermodynamic efficiency of ATP synthesis (too high to be able to represent the energy metabolism alone); product formation via the other would be characterized by a low efficiency (lower than required to maintain sufficient large metabolic fluxes) or even by an efficiency of zero. The relative rates of the two partial processes are adjusted so that the overall ATP gain and the thermodynamic efficiency of ATP synthesis are optimal. Examples for branched catabolic processes with variable ATP gains and thermodynamic efficiencies are the ethanol-acetate fermentation of *Clostridium kluveri* (131, 650), the glucose fermentation of *C. pasteurianum* (292, 296), the glucose fermentation of *Ruminococcus albus* (83, 262), and the glucose fermentation of *Lactobacillus casei* (143) (see section, Regulation of the Thermodynamic Efficiency of ATP Synthesis).

Energy-Providing Processes and the Synthesis of ATP

The energy required for the synthesis of ATP from ADP and P_i is generally, with a few excep-

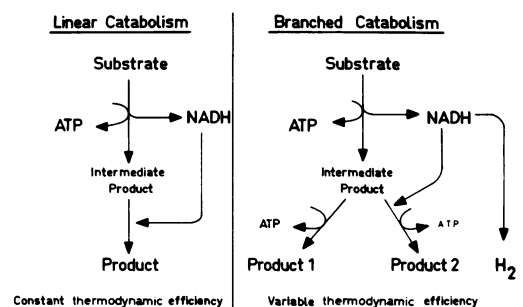


FIG. 2. Comparison of a linear catabolism having a constant thermodynamic efficiency of ATP synthesis with a branched catabolism having a variable efficiency.

tions discussed below, provided by redox processes. In both chemotrophic and phototrophic organisms the ATP-forming process can be viewed as an exergonic system with electron-accepting and -donating reactions or reaction chains which are coupled by electron carriers (Table 2). The donating part of the process comprises the flow of electrons from the donor substrates to the first electron carrier (with chemotrophs, usually nicotinamide adenine dinucleotide [NAD]); the accepting part is the flow from the first electron carriers to the final acceptor. Both partial reactions can be coupled with the phosphorylation of ADP. The mechanism of energy conservation differs in the electron-donating and electron-accepting partial processes. ATP is formed via SLP in the former and via ETP in the latter process.

Catabolic redox processes. The chemotrophic redox processes that occur in nature are characterized by the diversity of substrates that can be used for the production of energy. This is particularly true of the electron donors, but also to a considerable degree of the acceptors; both can be either organic or inorganic. Decker et al. (129) suggested that the energy metabolism of chemotrophic organisms formally be divided into H₂-forming and H₂-consuming partial reactions. This allows the assembly of the many types of metabolism by combination of a much smaller number of dehydrogenation and hydrogenation partial reactions. A detailed list of such partial reactions is given in the Tables 13 and 14 of the Appendix. H⁺ (pH 7) and H₂ were used as hypothetical electron acceptor and

donor, respectively, to allow calculations of the free energy changes associated with the partial reactions. The H⁺/H₂ couple rather than other reference systems (400, 401) was chosen, as the anaerobic degradation of organic material has been shown to proceed via a series of H₂-forming and H₂-consuming processes mediated by H₂-forming and H₂-utilizing anaerobic bacteria (for review see Pine [494]; for reviews on hydrogenases see 194 and 435). Thus the formulation of the partial reactions with H⁺ and H₂ as electron acceptor and donor, respectively, leads to reactions and free energy changes of direct biological significance for chemotrophic anaerobic bacteria.

Not every dehydrogenation reaction given in Tables 13 and 14 can be combined with every hydrogenation reaction. The overall redox process (formulated with the actual concentrations of the substrates and the products) must be sufficiently exergonic to allow the synthesis of 1 mol of ATP. (If the mechanism of ATP synthesis via electron transport phosphorylation should prove to require three [498] or four [71] protons per mole of ATP formed instead of two, then theoretically the overall redox process could be coupled with the formation of 2/3 or 1/2 mol of ATP only.) Thus not every dehydrogenation reaction can be coupled with the reduction of H⁺ to H₂ (H⁺ is the universal electron acceptor under anaerobic conditions). For example, the dehydrogenation of glucose with protons can proceed only to acetate plus CO₂, yielding 49.3 kcal/mol of glucose, but not beyond acetate, since the tricarboxylic acid cycle for the

TABLE 2. Energy metabolism as a system of electron-donating and electron-accepting partial processes^a

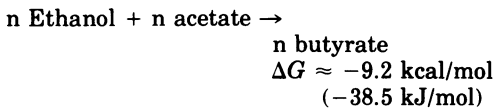
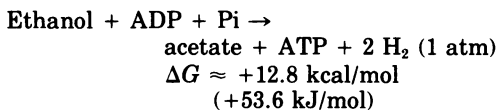
Example	Electron-donating partial process	Electron-accepting partial process
Lactic acid fermentation	glucose $\xrightarrow{\text{ATP (SLP)}}$ 2 pyruvate ⁻ + 2 H ⁺ + 4 H	4 H + 2 pyruvate ⁻ \rightarrow 2 lactate ⁻
Glucose respiration	glucose + 6 H ₂ O $\xrightarrow{\text{ATP (SLP)}}$ 6 CO ₂ + 24 H	24 H + 6 O ₂ $\xrightarrow{\text{ATP (ETP)}}$ 12 H ₂ O
Plant photosynthesis Noncyclic process	2 H ₂ O $\xrightarrow{h\nu}$ O ₂ + 4 H ⁺ + 4 e ⁻	4 e ⁻ + 4 H ⁺ $\xrightarrow{\text{ATP (ETP)}}$ 4 H
Cyclic process	Chl $\xrightarrow{h\nu}$ Chl ⁺ + e ⁻	e ⁻ + Chl ⁺ $\xrightarrow{\text{ATP (ETP)}}$ Chl

^a According to Decker et al. (129). Abbreviations: SLP, substrate level phosphorylation; ETP, electron transport phosphorylation.

dehydrogenation of acetate to CO_2 is endergonic, requiring 25 kcal/mol. Even up to the oxidation state of acetate, hydrogen formation can be observed as the sole electron-accepting reaction only if its concentration is kept low by hydrogen-consuming organisms (262). Thermodynamic considerations also practically rule out saturated carboxylic acids as energy substrates, since their dehydrogenation with H^+ as electron acceptor is thermodynamically so unfavorable that it can proceed only under very low H_2 pressures and without the synthesis of ATP.

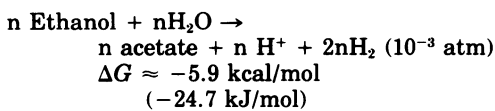
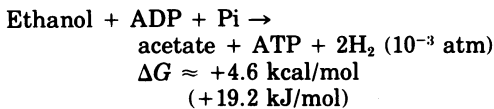
In *Clostridium kluyveri* and in the S-organism isolated from *Methanobacterium omelian-skii* the possibility appears to exist that the synthesis of ATP is linked to a dehydrogenation reaction which per se is endergonic. The overall energy metabolism is rendered exergonic by exergonic reactions running parallel to the endergonic one. The mechanism of energy coupling is not yet understood (see section on Ethanol-Acetate Fermentation of *C. kluyveri*).

C. kluyveri (650):



$n = 5 - 6$ (569a, 650)

S-Organism (84, 519, 520, 521):



$n = ?$

These exceptional cases have to be kept in mind when theoretical predictions on the combinability of dehydrogenation and hydrogenation reactions (Tables 13 and 14) are made.

Aerobic catabolic redox processes are always *intermolecular* between the substrate and the acceptor O_2 , which are coupled only via the electron carriers (Table 2, Respiration). *Anaer-*

obic energy metabolisms are, however, often *intramolecular*; electron-donating and -accepting steps are not only linked by the electron carrier but also by the electron acceptor, which must be formed as an intermediate product from the substrate (Table 2, Fig. 2). Intramolecular redox processes consequently lose one "degree of freedom", that is, the extent of possible dehydrogenations of the donor substrate is limited by the need for compensation of the hydrogen balance via an intermediate formed from the donor (intermediate product coupling) (129).

Carbohydrates, amino acids, carboxylic acids, alcohols, purines, and pyrimidines can be used as energy substrates under anaerobic conditions. In anaerobes the pathways of substrate conversion are often quite different from those found in aerobic organisms. Examples are the anaerobic catabolism of glutamate (43, 88, 154, 612, 633, 634, 688, 689), glycine (34, 33, 100, 311-314), lysine (35, 72, 108, 138, 276, 531, 623, 659, 735), ornithine (277), and purines (42, 554, 578). The difference of pathways is necessitated by the thermodynamic requirements imposed on anaerobic metabolism and by the need for compensation of the hydrogen balance via intermediate product coupling as discussed above.

Catabolic nonredox processes. There exist a few exceptions to the rule that biological energy is produced in redox processes. Several anaerobes have been shown to metabolize substrates by lysis rather than by dehydrogenation plus hydrogenation reactions. These processes are associated with SLP only. Examples are the arginine fermentation (50, 136) and the agmatine fermentation (136, 539) in *S. faecalis*, the xanthine fermentation in *Clostridium cylindrosporium* (42, 44), and the pyruvate fermentation to acetate and formate in *P. rettgeri* (341). Photophosphorylation in *Halobacterium halobium* (an aerobic organism) is the only example for the conversion of light energy without the participation of an electron transport chain (455, 456).

ATP synthesis via SLP. The dehydrogenation partial reactions and the nonredox processes can be associated with the synthesis of ATP via a mechanism referred to as SLP. Part of the energy released in these processes is initially conserved in "energy-rich" compounds (375). They are formed in dehydrogenase reactions or, in the case of nonredox processes, in lyase reactions (Table 3). The energy conserved is then transferred to the ATP system by kinase reactions. Thus, SLP requires the combination of a dehydrogenase with a kinase or of a "lyase" with a kinase, e.g.:

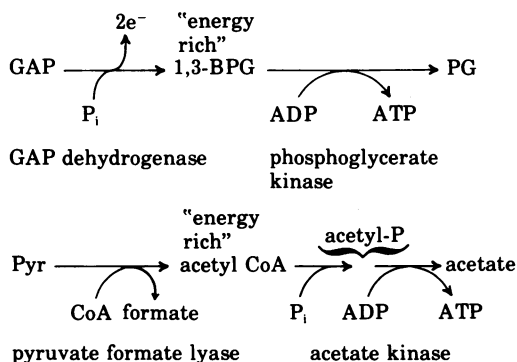
TABLE 3. Reactions coupled with SLP^a

Substrate	Products ^b	"Energy-rich" intermediate
$\boxed{\text{ADP}^{3-} + \text{P}_i^{2-} + \text{H}^+}$	$\boxed{\text{ATP}^{4-} + \text{H}_2\text{O}}$	
Dehydrogenation reactions		
1. Pyruvate ⁻ + 2H ₂ O	→ acetate ⁻ + HCO ₃ ⁻ + H ₂ + $\boxed{\text{H}^+}$ ^c	Acetyl CoA/acetyl P
2. Acetaldehyde + H ₂ O	→ acetate ⁻ + H ₂ + $\boxed{\text{H}^+}$	Acetyl CoA/acetyl P
3. GAP + H ₂ O	→ PG ⁻ + H ₂ + $\boxed{\text{H}^+}$	Bisphosphoglycerate
4. α-Ketoglutarate ²⁻ + 2H ₂ O	→ succinate ²⁻ + HCO ₃ ⁻ + H ₂ + $\boxed{\text{H}^+}$ ^c	Succinyl CoA
5. HSO ₃ ⁻ + H ₂ O	→ SO ₄ ²⁻ + H ₂ + $\boxed{\text{H}^+}$	Adenylyl sulfate
Lyase reactions		
6. Pyruvate ⁻ + H ₂ O	→ acetate ⁻ + HCOO ⁻ + $\boxed{\text{H}^+}$	Acetyl CoA/acetyl P
7. Acetoacetyl CoA + H ₂ O	→ acetyl CoA + acetate ⁻ + $\boxed{\text{H}^+}$	Acetyl CoA/acetyl P
8. Xylulose-5-P	→ acetate ⁻ + GAP + $\boxed{\text{H}^+}$	Acetyl P
9. Citrulline + H ₂ O	→ ornithine + NH ₂ COO ⁻ + $\boxed{\text{H}^+}$	Carbamyl phosphate
10. Formyl FH ₄ + H ₂ O	→ FH ₄ + HCOO ⁻ + $\boxed{\text{H}^+}$	N ¹⁰ -formyl FH ₄

^a Abbreviations: GAP, glyceraldehyde phosphate; PG, phosphoglycerate; FH₄, tetrahydrofolate.

^b For free energy changes of the reactions see section, Energy Conservation Via SLP.

^c Note that CO₂ rather than HCO₃⁻ has been shown to be the active species of "CO₂" formed by pyruvate:ferredoxin oxidoreductase (646a) and by pyruvate decarboxylase (332); the proton is formed only after equilibration with H₂O via the carbonic anhydrase reaction.



where GAP = glyceraldehydophosphate; 1,3-BPG = 1,3-bisphosphoglycerate; PG = phosphoglycerate; Pyr = pyruvate; and acetyl-P = acetyl phosphate.

"Energy-rich" compounds are characterized by their free energy of hydrolysis ("group transfer potential") (ΔG^0), which lies in the range of -5 to -15 kcal/mol (-20.9 to -62.8 kJ/mol) and by the fact that they exist in an enzymatic equilibrium with the ATP system. These "energy-rich" compounds are acid anhydrides or thioesters (Table 4), i.e., derivatives of carboxylic acids which represent the highest oxidation

level of the carbon atom in organic compounds. Acetals, thioacetals, ethers, and thioethers are not energy rich. Therefore "energy-rich" compounds can only be formed by the dehydrogenation of carbonyl groups or by lysis of carboxyl functions that are on the highest oxidation level already. Therefore, SLP can only be linked to the electron-donating part of catabolic redox processes or to the lytic part of catabolic non-redox processes.

The reactions known to be coupled with SLP are summarized in Table 3. In addition to the reactions listed, a few anaerobic bacteria can derive useful energy for growth from the oxidation of α-ketoacids other than pyruvate (e.g., α-ketobutyrate) in analogy to reaction 1 of Table 3 or from fructose-6-phosphate in analogy to reaction 8 of Table 3. The different reactions have in common that, per mole of ATP synthesized, 1 mol of protons is formed. This is the result of the fact that the energy-rich compounds being acid derivatives are generated from uncharged carbonyl functions by dehydrogenation or lysis (note that actually the proton is formed only after the ATP is hydrolyzed again). Thus the amount of ATP generated via SLP in anaerobically growing organisms can easily be deter-

TABLE 4. "Energy-rich" compounds involved in SLP

Type of compounds	Energy-rich compound	- ΔG_{obs}° of hydrolysis ^a		References
		kcal/mol	kJ/mol	
Acyl thioester	Acetyl CoA	8.5	35.7	203
	Propionyl CoA	8.5	35.6	
	Butyryl CoA	8.5	35.6	209a
	Succinyl CoA	8.4	35.1	
Phosphoacyl anhydride	Acetyl phosphate	10.7	44.8	620
	Bisphosphoglycerate	12.4	51.9	334, 584
	Carbamyl phosphate	9.4	39.3	517
Acyl anilide	N ¹⁰ -formyltetrahydrofolate	5.6	23.4	242
Phosphosulfuryl anhydride	Adenylyl sulfate ^b (APS)	21	88	9, 141, 534
Phosphoenol-ester	Phosphoenol ^c pyruvate	12.3	51.6	66

^a ΔG_{obs}° is the free energy change at a free Mg^{2+} concentration of 10^{-3} M, an ionic strength of 0.25 and a pH of 7; ΔG_{obs}° has been approximated from ΔG_{obs}° for ATP hydrolysis to form ADP and P_i ($\Delta G_{obs}^{\circ} = -7.6$ kcal/mol) and the free energy changes associated with the respective kinase reactions.

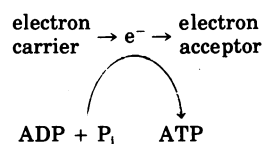
^b ATP synthesis via APS occurs only in a few chemotrophic aerobes and in a few phototrophic anaerobes (481).

^c Generally the formation of ATP from phosphoenolpyruvate via pyruvate kinase is considered as a site of SLP; however, the pyruvate kinase reaction does not lead to a de novo synthesis of ATP, rather, the phosphate needed for carbohydrate or glycerol activation and derived from ATP is transferred back to ADP; no orthophosphate is consumed in the pyruvate kinase reaction (305).

mined by simply measuring the amount of H^+ formed (650), provided that no ATP is synthesized via ETP. Only the bacteria that ferment glucose via the Entner-Doudoroff pathway (165) (e.g., *Zymomonas mobilis* = *Pseudomonas lindneri* [50] and *Zymomonas anaerobia* [405]) and the heterofermentive lactic acid bacteria growing on hexoses (e.g., *Leuconostoc mesenteroides* [258]) appear to make an exception to this rule (for biochemical pathways see Wood [719]). In these organisms 2 mol of H^+ are formed per mol of ATP synthesized since one of the dehydrogenation reactions in the fermentation (glucose-6-phosphate dehydrogenase reaction [EC 1.1.1.49]) associated with the formation of a proton is not coupled with the synthesis of ATP.

ATP synthesis via ETP. During the flow of electrons from the first electron carrier to the final electron acceptor, i.e., during the hydrogenation part of catabolic redox processes, ATP may be generated via a mechanism termed ETP (see Table 2). In this mechanism the electrochemical potential between redox partners of different redox potential (Table 5) is used to drive the phosphorylation of ADP. Assuming a two-electron transferring mechanism ($n = 2$), a potential difference ($\Delta E'$) of approximately 250 mV is required to allow the synthesis of 1 mol of ATP from ADP and P_i ($\Delta G' = -nF\Delta E' = 2 \times$

23.06×0.25 kcal/mol = -11.5 kcal/mol [-48 kJ/mol]; see footnote c, Table 5):



With aerobes the reduction of O_2 to H_2O in the respiratory chain is coupled very efficiently to ATP formation. With anaerobes the reduction of CO_2 to CH_4 , of NO_3^- to NO_2^- or N_2 , of SO_4^{2-} to H_2S , and of fumarate to succinate is evidently associated with phosphorylation whereas the reduction of H^+ to H_2 , of pyruvate to lactate, of crotonyl CoA to butyryl CoA, or of acetaldehyde to ethanol is not (see section, Energy Conservation Via ETP).

The mechanism of ETP is still a matter of considerable controversy. Three hypotheses have been advanced which are briefly discussed below (Table 6).

The *chemical hypothesis* assumes that an "energy-rich" intermediate "X~I" with a high hydrolysis potential be formed during the redox reactions of electron transport and that the energy conserved in X~I be transferred to the ATP system (600-602). This postulate is analogous to the well-known mechanism of SLP. The chemical hypothesis then does not assign any

TABLE 5. Redox potential of electron donors and electron acceptors involved in ETP

Redox compound	E_0' (mV)	References
$\text{SO}_4^{2-}/\text{HSO}_3^-$	-516	- ^b
$\text{CO}_2/\text{formate}^a$	-432	- ^b
H^+/H_2	-414	- ^c
$\text{S}_2\text{O}_3^{2-}/\text{HS}^- + \text{HSO}_3^-$	-402	- ^b
Flavodoxin ox/red (E'_{01})	-371 ^d	397
Ferredoxin ox/red (E'_{01})	-398 ^e	157
NAD/NADH	-320	91
Cytochrome c_3 ox/red	-290	728
$\text{CO}_2/\text{acetate}^-$	-290	- ^b
S^0/HS^-	-270	- ^b
CO_2/CH_4	-244	- ^b
FAD/FADH ₂	-220	- ^f
Acetaldehyde/ethanol	-197	- ^b
Pyruvate ⁻ /lactate ⁻	-190	- ^b
FMN/FMNH ₂	-190	- ^g
Dihydroxyacetone phosphate/glycerol-phosphate	-190	94
$\text{HSO}_3^-/\text{S}_2\text{O}_6^{2-}$	-173	- ^b
Oxaloacetate ²⁻ /malate ²⁻	-172	- ^b
Flavodoxin ox/red (E'_{02})	-115 ^d	397
$\text{HSO}_3^-/\text{HS}^-$	-116	- ^b
Menaquinone ox/red (MK)	-74	569, 685
APS/AMP + HSO_3^-	-60	594
Rubredoxin ox/red	-57	155
Acrylyl CoA/propionyl CoA	-15	232
Glycine/acetate ⁻ + NH_4^+	-10	- ^b
2-Demethylvitamin K ₂ ox/red	+25	569, 250a
$\text{S}_4\text{O}_6^{2-}/\text{S}_2\text{O}_3^{2-}$	+24	- ^b
Fumarate/succinate	+33	- ^b
Ubiquinone ox/red	+113	569
$\text{S}_2\text{O}_3^{2-}/\text{S}_2\text{O}_3^{2-} + \text{HSO}_3^-$	+225	- ^b
NO_2^-/NO	+350	- ^b
$\text{NO}_3^-/\text{NO}_2^-$	+433	- ^b
$\text{Fe}^{3+}/\text{Fe}^{2+}$	+772	- ^b
$\text{O}_2/\text{H}_2\text{O}$	+818	- ^b
$\text{NO}/\text{N}_2\text{O}$	+1175	- ^b
$\text{N}_2\text{O}/\text{N}_2$	+1355	- ^b

^a CO_2 rather than HCO_3^- has been shown to be the active species of " CO_2 " utilized or formed by formate dehydrogenases (336, 645, 646b).

^b Calculated from ΔG° for redox compound reduction with H_2 ($\Delta G^\circ = -n \cdot F \cdot \Delta E_0'$, $\Delta E_0' = E_0'$ (redox compound) - E_0' (H^+/H_2); n = number of electrons transferred in the reaction; F [Faraday constant] = 23060.9 cal/V equivalent) (110). The ΔG° values were calculated from the ΔGf° values given in Table 15 (CO_2 , CH_4 , H_2 , N_2 , NO , and N_2O in the gaseous state, all other compounds in aqueous solution).

^c At 25°C.

^d *Peptostreptococcus elsdenii*; for E_0' of clostridial flavodoxins see (394).

^e *Clostridium pasteurianum* ($E'_{02} = -367$ mV).

^f The redox potential of flavin enzymes may differ by as much as 200 mV from the values of the free coenzymes.

intrinsic function to the membranes. Membranes may be required, however, as organizers for the components of the electron transport

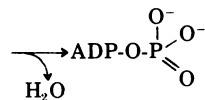
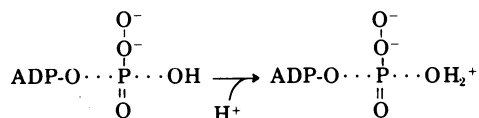
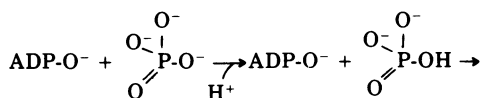
chain and/or as a hydrophobic solvent for the metastable X~I. This might explain why "energy-rich" intermediates of the X~I type could never be isolated (124, 329, 419, 587, 705).

The *conformational hypothesis* assumes that an energy-rich strained conformation of a protein component be induced by the redox reactions of electron transport and that the strained conformation would relax only in the presence of ADP and P_i with concomitant ATP formation (67, 68, 70). This view is based on the analogous mechanism of a muscular contraction operating, however, in the reverse direction, in that ATP splitting induces a conformational change (261, 441). Again in principle membranes should not be required; the generation and relaxation of conformational strain should be possible in homogeneous systems as with the actomyosin transitions during muscular contraction. The conformational hypothesis then does not assign an intrinsic function to the membranes either. They may be required, however, as an environment for the metastable strained conformation. Conformational changes have been observed with a number of components of the electron transport chain of mitochondria, but there is not yet evidence that they are the obligatory coupling link between electron transport and phosphorylation (37).

The *chemiosmotic hypothesis* assumes that a proton-motive force consisting of a pH gradient (ΔpH) and an electrical potential difference ($\Delta \psi$) be generated by the redox reactions of electron transport and that this proton-motive force drives the synthesis of ATP (196, 414, 415, 417). This view bears obvious analogies to ATP-driven transport processes in higher organisms, which operate, however, in the reverse direction, in that a Na^+/K^+ -ATPase builds up a sodium-motive force driving the sodium-linked transport of solutes (122, 123, 190, 191, 416). The chemiosmotic hypothesis, then, assigns an intrinsic function to the membranes. In principle not only intact membranes but even topologically closed membrane vesicles are required for the generation of a proton-motive force. The key question distinguishing the chemical and conformational hypotheses from the chemiosmotic hypothesis is whether the assembly of the electron transport chain in the membrane is merely for the purpose of obtaining the high efficiency of compact organization and of supplying optimal solvent conditions or whether the process of ETP is linked in a compulsory manner to the ability of the membrane to separate compartments (511).

The chemiosmotic hypothesis is based on three postulates. (i) The membrane, in which electron transport is coupled to phosphorylation, is impermeable to protons and hydroxyl

ions. (ii) The electron transport chain is oriented in the membrane such that the result of the redox process is the generation of a proton-motive force consisting of two components, a gradient of pH (inside alkaline) and of electrical potential (inside negative) (Fig. 3). (iii) The proton-motive force drives the synthesis of ATP mediated by reversed ATPases or ATP synthases (2, 485). A simple chemiosmotic molecular mechanism has been proposed (420). The condensation of $\text{ADP}^{3-} + \text{P}_i^{2+} + \text{H}^+$ to $\text{ATP}^{4-} + \text{H}_2\text{O}$ is thermodynamically unfavorable; it becomes possible by vectorial organization over the membrane. The ADP anions and phosphate anions have access to the reaction center only from the inside, the protons have access from the outside. First one phosphate O^- group is protonated (the others being shielded by the enzyme) to an OH group facilitating the attack of an ADP anion on phosphate, and then the phosphate OH group is further protonated to an OH_2^+ group, thus allowing the exit of water. This mechanism:



does not involve covalent intermediates and uses a proton-motive field across the reaction center of the enzyme to poise the process in the direction of ATP formation. With ATPases, a ratio of H^+ translocated per ATP hydrolyzed between 2 and 4 has been observed experimentally (see 71, 101, 290, 423, 437, 498, 653, 654; for reviews see 211, 233, 466, 660, 661). As formulated the mechanism has a H^+/ATP ratio of 2; with a more protonated form of phosphate or ADP entering, the active center ratios of 3 or 4 might be accounted for equally well. The proposal is primarily intended to define the general principle of a feasible type of molecular mechanism. The scientific issue has been discussed repeatedly with a great number of arguments and counterarguments on the conceptions and misconceptions involved (69, 420, 421, 706).

At present the chemiosmotic hypothesis may be favored over the other proposals due to avail-

able evidence, as follows. (i) ATP formation coupled to electron transport could only be convincingly demonstrated with topologically intact membrane vesicles (13, 365, 512, 516; cf. 116, 707, 734). (ii) The spatial arrangement, i.e., the sidedness of the components of the electron transport chain in the coupling membrane, appears to be consistent with the hypothesis (511). (iii) The generation of a proton-motive force could be measured in mitochondria (5, 424, 449, 465, 547, 599), in chloroplasts (236, 547-550, 575, 576, 694), in bacteria (14, 197, 198, 244, 524, 570, 571, 710), and in artificial planar phospholipid membranes containing cytochrome oxidase or bacteriorhodopsin, or H^+ -ATPase (151). (iv) ATP could be synthesized using an artificially imposed proton-motive force in chloroplasts (274, 589, 669, 733), in mitochondria (113, 189, 526, 545), in submitochondrial particles (654, 655), and in bacteria (385, 386, 710). (v) Uncouplers, which separate electron transport from phosphorylation, are all proton conductors, i.e., they can permeate the membrane both in the protonated and unprotonated form, leading to an equilibration of protons and thus to the dissipation of the proton-motive force (218, 219, 251). The mode of action of uncouplers can hardly be explained with the competing hypotheses (Table 6). (vi) Finally, the common chemical intermediate required by the chemical and conformational hypothesis could never be isolated (124, 329, 419, 587, 705).

Energy-Consuming Processes and the ATP Requirement for Bacterial Growth

Every cell has to fulfill specific functions, i.e., to perform work. This may be chemical work as with biosynthesis, osmotic work as with active transport, or mechanical work as with active movement (Fig. 1). The energy for these processes is provided by the ATP system.

The overall amount of ATP required during the synthesis of 1 g (dry weight) of bacterial cells is equal to $1/Y_{\text{ATP}}$. Y_{ATP} (grams of cells synthesized per mole of ATP produced) can be determined from growth yield studies (determinations of the amount of cells formed per mole of substrate fermented or product formed) if the ATP gain (moles of ATP formed per mole of substrate fermented) is known (50, 106). Measurements of Y_{ATP} in a great number of anaerobic bacteria resulted in values close to 10 g/mol (50), i.e., approximately 0.1 mol of ATP is generally required during the synthesis of 1 g of cells. Y_{ATP} values have been compiled by Decker et al. (129) and by Stouthamer (628). Note, however, that Y_{ATP} is not a constant

TABLE 6. Comparison of the three major hypotheses of ETP

Hypothesis	Type of energy-rich intermediate	Structural requirements	Mode of action of uncouplers
Chemical	X ~ I Compound with high hydrolysis potential formed during redox reactions of electron transport	<i>No intrinsic function for membranes!</i> In principle, membranes should not be required. Reactions should be possible in homogeneous soluble systems as with substrate level phosphorylation. Membranes may be required, however, as organizers for the components of electron transport phosphorylation and/or as a hydrophobic environment for a metastable X ~ I.	Cannot be explained satisfactorily
Conformational	~ Conformation High-energy conformation of protein components induced by redox reactions of electron transport	<i>No intrinsic function for membranes!</i> In principle, membranes should not be required. The generation of conformational strain and its relaxation should be possible in soluble, homogeneous systems as with the actomyosin transitions during muscular contraction. Membranes may be required, however, as an environment for a metastable strained ~ conformation.	Cannot be explained satisfactorily
Chemiosmotic	$\Delta pH + \Delta\psi$ Proton-motive force generated by redox reactions of electron transport	<i>Intrinsic function for membranes!</i> In principle, not only intact membranes but even topologically closed membrane vesicles are required for the generation of a proton-motive force.	Can be explained by proton conductor properties of uncouplers

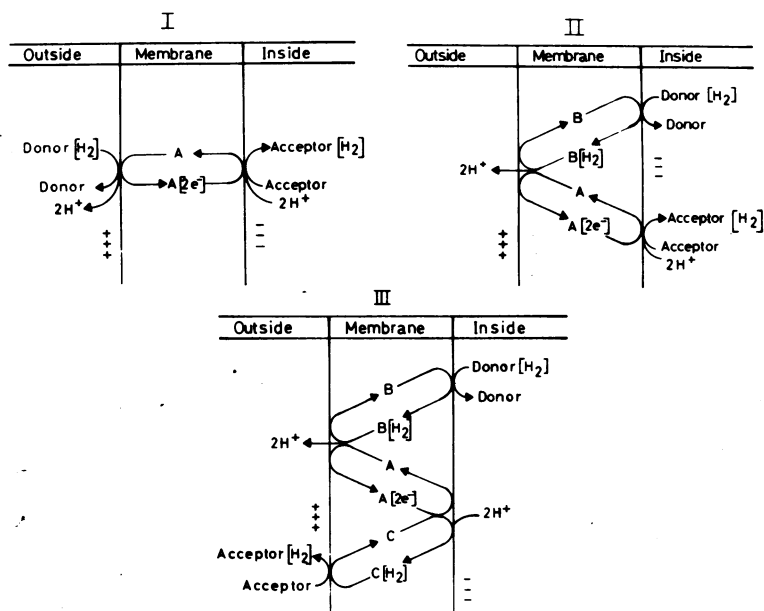


FIG. 3. Three examples of vectorial redox processes leading to the generation of a proton-motive force consisting of two components, a gradient of pH (inside alkaline) and of electrical potential (inside negative). (I) Donor outside, acceptor inside; (II) donor inside, acceptor inside; (III) donor inside, acceptor outside. Symbols: A, electron carrier (electrogenic electron transport); B and C, hydrogen carriers (electroneutral electron transport). (For more sophisticated versions (proton-motive Q cycle) see Mitchell [422].)

(630). Its value is dependent on the growth rate of the organism (495), on the substrates used for the synthesis of the cell material, and on the composition of the cell material (see below). A constant value is generally obtained only because the experimental conditions used to determine Y_{ATP} are frequently very similar: energy-limited growth, similar growth rates, comparable growth media, as well as similar contents of cell constituents. This is also the reason why determinations of ATP gains from growth yield studies using a Y_{ATP} value of 10 generally lead to the correct ATP gain. This method has been critically reviewed by Payne (472), Decker et al. (129), Forrest and Walker (177), Stouthamer and Bettenhausen (630), and Stouthamer (625, 628).

The amount of ATP required for biosynthesis only (to do chemical work) can be calculated if the composition of the cellular matter and the biosynthetic routes leading to the cellular components are known. In growing cultures of bacteria (active growth phase), the turnover of cell material may be disregarded since it consumes only a small fraction of the ATP produced. Most microorganisms seem to show a similar pattern of components (433). Furthermore, the ATP requirement for protein, ribonucleic acid, and deoxyribonucleic acid synthesis *de novo* is rather similar (129, 626); deviations of their relative proportion affect the amount of ATP required only slightly. Fat synthesis requires about 10% more ATP on a weight basis; since the fat content of anaerobes is small (5% of the dry weight of *C. kluyveri* [129]), little error is introduced by possible differences between species. The theoretical amount of ATP required for biosynthetic purposes is considerably different, however, if the cell material has to be synthesized from such different precursors as CO_2 and glucose (Table 7). Y_{ATP}^{max} values (the yield of cell mass per mole of ATP assuming that ATP is used for biosyntheses purposes only) of approximately 5 and 27, respectively, have been calculated (177, 626). A thorough treatise on the theoretical ATP requirement for the synthesis of microbial

cell material has been presented by Stouthamer (626).

From the experimentally determined Y_{ATP}^- value and the calculated Y_{ATP}^{max} value, the ATP requirement for the non-biosynthetic functions can be calculated. For example, Y_{ATP} for growing cultures of *C. kluyveri* has been determined to be approximately 9 g of cells per mol of ATP; Y_{ATP}^{max} for this organism growing on ethanol, acetate, and CO_2 , or crotonate plus CO_2 as sole carbon and energy source has been calculated to be 15.4 (129). Thus, approximately 50% of the ATP produced in the catabolism of this strictly anaerobic bacterium is used to do work other than chemical. This fraction of the ATP turnover has frequently been called "maintenance energy," a term whose use should be discouraged since it involves free energy expenditure not only for the compensation during the turnover of cellular matter but also for active movement (motility), active substrate, and ion transport. Pirt (495) has given a method to estimate this fraction of ATP consumption (maintenance energy) on the basis of specific growth rates and molar growth yields in continuous cultures (see Stouthamer and Bettenhausen [630]): $1/Y_{ATP} = m_s/\mu + 1/Y_{ATP}^{max}$, where m_s = maintenance coefficient (moles of ATP consumed per gram of dry cells per hour; μ = specific growth rate (hour⁻¹)). Y_{ATP}^{max} can be obtained in a double reciprocal plot of $1/Y_{ATP}$ versus $1/\mu$ from the intercept at the ordinate, the maintenance coefficient m_s can be obtained from the slope. The theoretically calculated and the experimentally determined values for Y_{ATP}^{max} are in good agreement (630).

The active movement of bacteria and the compensation for the insignificant turnover of cell material during active growth may be disregarded as consuming only a small fraction of the ATP produced. Thus most of the ATP consumed for non-biosynthetic purposes is probably required for transport functions, i.e., to perform osmotic work (see section, Transport of Energy Substrates and Products, and the Proton-Motive Force). The energy substrates are probably taken up actively, with the result that the transport requires a stoichiometrically related fraction of the free energy generated by their catabolism. Similarly, inorganic ions including protons may be co- or anti-transported together with the nutrient or may have to cross the cell membrane to preserve the ionic equilibrium during metabolic activity.

ENERGY CONSERVATION VIA SLP

Despite the large number of carbon substrates available to anaerobic organisms, there

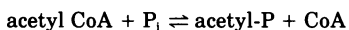
TABLE 7. ATP requirement for biosyntheses during bacterial growth

Carbon source	ATP requirement (mol of ATP/g of cells)	Y_{ATP}^{max} (g of cells/mol of ATP)	Reference
Glucose	0.037	27	626
Lactate ⁻	0.075	13.4	626
Malate ²⁻	0.065	15.4	626
Acetate ⁻ + CO_2	0.065	15.4	129, 642
Acetate ⁻	0.1	10	626
CO_2	0.2	5	177

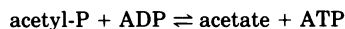
are only a few reactions conserving metabolic energy by SLP (Table 3). From the various different substrates (Fig. 4) only a few "energy-rich" intermediates are formed (acyl CoA, acetyl phosphate, 1,3-bisphosphoglycerate, carbamyl phosphate, formyltetrahydrofolate, and succinyl CoA). From these, acetyl CoA appears to be the most important. The inability of anaerobically growing bacteria to oxidize acetyl CoA via the citric acid cycle to CO₂ (see below) makes acetyl CoA the most frequently used source of high-energy phosphate in anaerobically growing bacteria.

Acetyl CoA as the Primary "Energy-Rich" Intermediate

Acetyl CoA is formed from a variety of different substrates in the energy metabolism of both anaerobic and aerobic organisms. In aerobically growing organisms, acetyl CoA is oxidized to CO₂ via the citric acid cycle. In anaerobically growing organisms, with a few exceptions acetyl CoA (488, 702a), cannot be oxidized to CO₂. The citric acid cycle can function only if succinate can be oxidized to fumarate (succinate/fumarate; E₀' = +33 mV). This is possible only with electron acceptors with a redox potential more positive than +33 mV such as O₂, nitrate, nitrite, Fe³⁺, trithionate, and tetrathionate (see Table 5). Part or all of the acetyl CoA is therefore available for the synthesis of ATP, which proceeds via the phosphotransacetylase (EC 2.3.1.8) (613) and the acetate kinase reactions (EC 2.7.2.1) (540, 541):

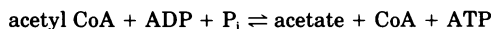


$$\Delta G'_{obs} = +2.2 \text{ kcal/mol} \quad (+9.0 \text{ kJ/mol})$$



$$\Delta G'_{obs} = -3.1 \text{ kcal/mol} \quad (-13 \text{ kJ/mol})$$

calculated from $\Delta G'_{obs}$ of hydrolysis of acetyl CoA and acetyl phosphate (Table 4) and $\Delta G'_{obs}$ (ATP → ADP + P_i) = -7.6 kcal/mol (-31.8 kJ/mol). Phosphotransacetylase of *E. coli* (632) and acetate kinase of *Veillonella alcalescens* (484) are regulatory enzymes indicating that in these organisms the respective reactions are not at equilibrium. Phosphotransacetylase (74, 80, 452, 538, 591, 699) and acetate kinase (79, 80, 484, 562, 656) are found in all anaerobic bacteria that form acetyl CoA in their energy metabolism and use the acetyl CoA to synthesize ATP. The two enzymes also occur in a few aerobic bacteria, e.g., *Acetobacter xylinum* (574) and *Azotobacter vinelandii* (74, 98, 205). They never have, however, been definitely detected in eucaryotic organisms (128, 540). In anaerobic protozoa, which form acetate from acetyl CoA, this step appears to be catalyzed by a novel acetate thiokinase, which regenerates CoA and conserves the energy of the thioester bond in phosphorylation of ADP or GDP (369a, 442).



$$\Delta G'_{obs} = -0.9 \text{ kcal/mol} \quad (-4.0 \text{ kJ/mol})$$

In the energy metabolism of anaerobically growing organisms, acetyl CoA may be formed from pyruvate via pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) or pyruvate formate lyase, from acetaldehyde via acetaldehyde dehydrogenase (EC 1.2.1.10) or from acetoacetyl CoA via thiolase (EC 2.3.1.9):

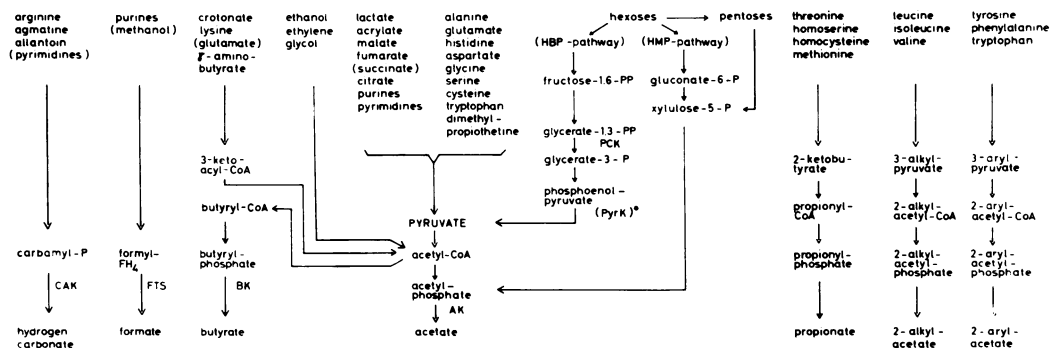
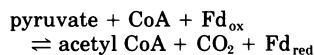
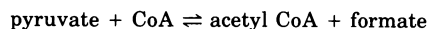


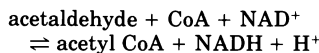
FIG. 4. Substrate level phosphorylations in the anaerobic catabolism of carbohydrates, amino acids, carboxylic acids, alcohols, purines and pyrimidines. Abbreviations: HBP, hexosebisphosphate pathway; HMP, hexose monophosphate pathway; CAK, carbamate kinase; FTS, formyltetrahydrofolate synthetase; AK, acetate kinase; PK, propionate kinase; BK, butyrate kinase; AKK, alkyl (aryl) acetate kinase; PGK, phosphoglycerate kinase; PyrK, pyruvate kinase. Asterisk after parentheses indicates involvement in ATP regeneration rather than in substrate level phosphorylation: the enzyme transfers the phosphate, expended for carbohydrate activation in the hexokinase and phosphofructokinase reaction and derived from ATP, back to ADP.



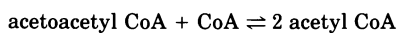
$$\Delta G^{0'} = -4.6 \text{ kcal/mol} \\ (-19.2 \text{ kJ/mol})$$



$$\Delta G^{0'} = -3.9 \text{ kcal/mol} \quad (317) \\ (-16.3 \text{ kJ/mol})$$

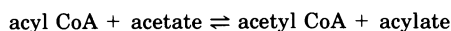


$$\Delta G^{0'} = -4.2 \text{ kcal/mol} \quad (622) \\ (-17.5 \text{ kJ/mol})$$



$$\Delta G^{0'} = -6.0 \text{ kcal/mol} \quad (128, 226) \\ (-25.1 \text{ kJ/mol})$$

($\Delta G^{0'}$ of the pyruvate:ferredoxin oxidoreductase reaction was calculated from ΔG_f^0 values with CO_2 in the gaseous state [Table 15], from E'_{01} ($\text{Fd}_{\text{ox}}/\text{Fd}_{\text{red}}$) (Table 5), and $\Delta G^{0'}$ for acetyl CoA hydrolysis [Table 4]). In addition, acetyl CoA is in equilibrium with other CoA esters via thiophorase (CoA transferase) (275) in many of the anaerobic bacteria:



($\Delta G^{0'}$ is dependent on the acids involved in CoA transfer [275])

Pyruvate:ferredoxin oxidoreductase catalyzes the reversible dehydrogenation of pyruvate to acetyl CoA and CO_2 with ferredoxin $E'_{01} = -398 \text{ mV}$ (157) as electron acceptor (646a, 670). The enzyme is found in many anaerobic bacteria (for reviews, see 85, 86) and in the hydrogen-forming protozoa (370, 371, 442). The enzyme has also been detected in facultative bacteria (681) and blue-green algae (64, 350). In the latter organism the pyruvate:ferredoxin oxidoreductase appears to have an anabolic function rather than a catabolic one, i.e., to provide the organisms with reduced ferredoxin which is required for the reduction of nitrogen or NAD phosphate (NADP). In *C. kluveri*, pyruvate:ferredoxin oxidoreductase physiologically mediates the formation of pyruvate from acetyl CoA and CO_2 rather than the reverse reaction (25, 648, 649).

Pyruvate formate lyase catalyzes the thiolitic cleavage of pyruvate to acetyl CoA and formate (317). This reaction is the main acetyl CoA generating process in anaerobically growing *Enterobacteriaceae* and in anaerobically dark-fermenting *Rhodospirillaceae* (291). The *Enterobacteriaceae* also contain the enzyme when growing aerobically. Under these conditions the lyase has been shown, however, to be completely inactive (316). Pyruvate formate lyase also occurs in *S. faecalis* (372) and in several clostridia (647, 648, 720). In clostridia the func-

tion of the enzyme has been shown to provide the cells with formate for the synthesis of one carbon unit rather than with acetyl CoA for the synthesis of ATP (647).

Aldehyde dehydrogenase (acylating) catalyzes the reversible dehydrogenation of acetaldehyde to acetyl CoA (93, 622). The enzyme occurs in many bacteria (127, 182, 299, 551, 622, 657). In most anaerobic and facultative bacteria the enzyme is involved in the formation of ethanol or butanol from acetyl CoA or butyryl CoA, respectively. In a few anaerobic bacteria, however, the enzyme has been shown to be involved in acetyl CoA formation, e.g., *C. kluveri* (93) and *Clostridium glycollicum* (182, 618). This reaction probably also occurs in *Desulfuromonas acetoxidans* growing on ethanol plus elemental sulfur (488) and in the S-organism (84, 519) isolated from *Methanobacillus omelianskii* (76, 521), and in a few sulfate-reducing bacteria growing on ethanol in association with hydrogen-utilizing methanogenic bacteria. In *C. kluveri* and *C. glycollicum*, the aldehyde dehydrogenase (s) has been shown to be operative with both NAD and NADP as electron acceptor with a preference for NAD (93, 182, 239, 240). In the S-organism the oxidation of acetaldehyde appears to be dependent on ferredoxin rather than on NAD, but a dependence on CoA or P_i could not be demonstrated (76, 520, 521). The oxidation of acetaldehyde to acetate must, however, in some way be coupled with phosphorylation, as the organism can grow on ethanol as sole energy source with the concomitant formation of stoichiometric amounts of acetate and H_2 , provided the H_2 is continuously removed to keep the H_2 pressure low and thus to make the reaction thermodynamically feasible. The finding that cell-free extracts of the S-organism contain phosphotransacetylase and acetate kinase (521) suggests that acetyl CoA may be an intermediate in acetate formation from ethanol despite the fact that a CoA dependence could not be demonstrated in cell-free extracts.

β -*Ketothiolases* catalyze the reversible formation of acetyl CoA from CoA esters of β -ketoacids (for a review, see Gehring and Lynen [185]). The equilibrium of the thiolase reaction strongly favors the thiolitic cleavage (128, 226). The enzyme occurs with varying specificity in aerobic and in anaerobic organisms. In aerobic organisms it is involved in fatty acid degradation, the synthesis of β -hydroxy- β -methylglutaryl CoA, and the synthesis and reutilization of poly- β -hydroxybutyric acid. In many anaerobic bacteria the main function of the enzyme is to catalyze the formation of acetoacetyl CoA from acetyl CoA (55). This reaction is the initial step in the synthesis of butyric acid in the

butyric acid fermentations. Acetyl CoA is thus used up rather than generated via β -ketothiolase. In a few anaerobic bacteria, however, the thiolase reaction has been shown to be involved in the formation of acetyl CoA, which is used to synthesize ATP via phosphotransacetylase and acetate kinase, e.g., *C. kluyveri* growing on crotonate (54, 650) and *Clostridium aminobutyricum* growing on α -aminobutyrate (215, 217, 216, 618).

There are indications that some anaerobic bacteria can oxidize butyric acid and other saturated fatty acids to acetate, presumably via butyryl CoA and acetoacetyl CoA (for literature, see 109, 250). Protons are assumed to be the electron acceptor. Under standard conditions the formation of acetate and H_2 from butyrate is an endergonic reaction ($\Delta G^\circ = +11.6$ kcal/mol [+48.4 kJ/mol]). If the hydrogen partial pressure is, however, one-thousandth of an atmosphere rather than one atmosphere as in anaerobic sludge digesters, then the reaction could proceed if the formation of acetate is not coupled with phosphorylation. Fatty acid degradation under anaerobic conditions has so far only been observed in mixed cultures of hydrogen-forming plus hydrogen-utilizing bacteria. No bacterium is known that can cause the reaction alone.

The dehydrogenation of butyryl CoA to crotonyl CoA ($E_0' = -15$ mV) (232) under anaerobic conditions with protons (H^+/H_2 ; $E_0' = -420$ mV) as electron acceptor is very difficult to envisage. An explanation could be that the degradation of fatty acids is catalyzed by a multienzyme complex without the formation of freely diffusible intermediates. Thus the dehydrogenation of acyl CoA with H^+ as electron acceptor could become thermodynamically feasible.

In *aerobically* growing organisms, acetyl CoA is formed mainly from pyruvate or from the CoA esters of β -ketoacids. The formation of acetyl CoA from pyruvate proceeds via oxidation with NAD and is catalyzed by the lipoate-dependent pyruvate dehydrogenase complex (522). This enzyme has not been found in any anaerobic organism. It is present in some facultative bacteria when growing under anaerobic conditions. Evidence is available, however, that under anaerobic conditions the pyruvate dehydrogenase complex is inactive (214, 237).

Thioesters Other Than Acetyl CoA as Primary "Energy-Rich" Intermediates

Acyl CoA esters other than acetyl CoA (propionyl CoA, butyryl CoA, *p*-hydroxyphenylacetyl CoA) are formed in anaerobic oxidations of amino acids such as threonine, leucine, and

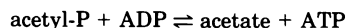
tyrosine (Fig. 4). It is probable that each of these energy-rich compounds can be used to synthesize ATP via the corresponding acyl phosphate. An enzyme catalyzing the formation of butyryl phosphate from butyryl CoA is present in *Clostridium butyricum* and several other clostridia (666, 671). A kinase has been purified from *C. butyricum* that utilizes propionyl phosphate and butyryl phosphate (665). Relatively little is known, however, about the kinases and phosphotransacetylases acting on the other acyl phosphates (44).

Acetyl Phosphate as the Primary "Energy-Rich" Intermediate

Acetyl phosphate is formed from acetyl CoA via phosphotransacetylase in most bacteria. In a few anaerobic bacteria, however, acetyl phosphate may be generated from other precursors. In *Lactobacillus delbrückii*, acetyl phosphate rather than acetyl CoA is formed during pyruvate dehydrogenation (374, 555). The reaction is catalyzed by a pyruvate dehydrogenase which uses flavin adenine dinucleotide as electron acceptor (210). In *Micrococcus lactilyticus* (= *Veillonella alcalescens*), an enzyme has been reported to exist that catalyzes the formation of formate and acetyl phosphate from pyruvate. CoA appears not to be required in the reaction (403, 404). In heterofermentative lactic acid bacteria, acetyl phosphate is generated from xylulose-5-phosphate via phosphoketolase (EC 4.1.2.9) (234) rather than from acetyl CoA. The acetyl phosphate formed is used to synthesize ATP if the bacteria grow on pentoses (235). If the bacteria grow on hexoses, however, 2 mol of NADH are formed in the reactions leading to the formation of xylulose-5-phosphate (258). In the absence of other electron acceptors, the acetyl phosphate is used to reoxidize the NADH, and ethanol is formed. Acetyl phosphate is therefore not available for the synthesis of ATP under these conditions.



$$\Delta G'' = -10.5 \text{ kcal/mol} \\ (-43.9 \text{ kJ/mol})$$



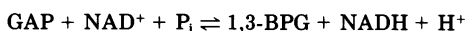
$$\Delta G''_{obs} = -3.1 \text{ kcal/mol} \\ (-13 \text{ kJ/mol})$$

(GAP = glyceraldehydophosphate; the $\Delta G''$ of the phosphoketolase reaction has been calculated from ΔGf° values [Table 15] assuming ΔGf° [xylulose] = ΔGf° [ribose] and $\Delta G''$ of hydrolysis of xylulose-5-phosphate and of glyceraldehydophosphate to be identical, and from $\Delta G''$ associated with acetyl phosphate hydrolysis [Table 4].)

In *Bifidobacteria* (142, 560, 561; see also 300) and in *Acetobacter xylinum* (574), phosphoketolase catalyzes both the cleavage of xylulose-5-phosphate to acetyl phosphate and glyceraldehydephosphate and the cleavage of fructose-6-phosphate to acetyl phosphate and erythrose-4-phosphate. Three moles of acetyl phosphate and 2 mol of glyceraldehyde-phosphate are formed from 2 mol of fructose-6-phosphate in these organisms (via phosphoketolase, transaldolase [EC 2.2.1.2] and transketolase [EC 2.2.1.1]). As no dehydrogenation reactions are involved in this process, all of the acetyl phosphate formed is available for the synthesis of ATP. Phosphoketolase is found only in procarotytic organisms.

1,3-BPG as "Energy-Rich" Intermediate

The oxidation of GAP to 3-phosphoglycerate (PG) is an important intermediary step in the energy metabolism of all anaerobic and aerobic organisms that use either carbohydrates or glycerol as energy source. The reaction proceeds via 1,3-bisphosphoglycerate (1,3-BPG) as energy-rich compound and is catalyzed by two enzymes, glyceraldehydephosphate dehydrogenase (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3). One mole of ATP is formed per mole of glyceraldehydephosphate oxidized:



$$\Delta G_{obs}^{\circ} = +2.4 \text{ kcal/mol} \\ (+10 \text{ kJ/mol})$$



$$\Delta G_{obs}^{\circ} = -4.8 \text{ kcal/mol} \quad (334, 584) \\ (-20.1 \text{ kJ/mol})$$

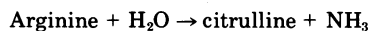
(ΔG_{obs}° of the glyceraldehydephosphate dehydrogenase reaction was calculated from $K = [\text{PG}][\text{ATP}][\text{NADH}][\text{H}^+]/[\text{GAP}][\text{NAD}][\text{P}_i][\text{ADP}] = 5.9 \cdot 10^{-6}$ [679] and ΔG_{obs}° of the phosphoglycerate kinase reaction).

The electron acceptor for the oxidation of GAP is NAD in almost every case examined. Even in *Clostridium thermoaceticum*, in which reduced NADP (NADPH) rather than NADH is required for catabolic reduction reactions, the GAP dehydrogenase (GAPDH) is specific for NAD (643) (for a possible exception see Senior and Dawes [588]). In aerobic organisms the NADH generated in the GAPDH reaction is reoxidized with O_2 via the respiratory chain. In anaerobic organisms O_2 is substituted by a variety of different electron acceptors such as pyruvate, acetyl CoA, and even protons. Thus in saccharolytic clostridia a considerable part of the NADH generated in the GAPDH reaction is used to form H_2 (296). NADH:ferredoxin oxidoreductase and ferredoxin hydrogenase (EC

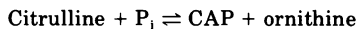
1.12.7.1) have been shown to catalyze this reaction (293–295, 651).

CAP As "Energy-Rich" Intermediate

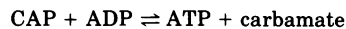
Ureido compounds (R-NH-CO-NH_2) are formed as intermediates in the energy metabolism of a few bacteria that can grow on arginine (*S. faecalis* [50, 136, 436]; *Mycoplasma hominis* [564]), agmatine (*S. faecalis* [136, 430, 539]), or allantoin (*Streptococcus allantoicus* [59, 672, 673]) as energy source. The ureido compounds are degraded by phosphorolytic cleavage to carbamyl phosphate (CAP) and the respective amines (amides). The carbamyl phosphate formed is used to synthesize ATP via carbamate kinase (EC 2.7.2.2). (Certain strains of *S. faecalis* can grow on media containing some glucose and high concentrations of arginine (136). The fermentation products are ornithine, CO_2 , and 2 mol of ammonia (arginine dihydrolyase pathway). Arginine is first hydrolyzed to citrulline (487) via arginine deimidase (EC 3.5.3.6) which then undergoes a phosphoroclastic cleavage to ornithine and CAP (114, 283) via ornithine carbamoyltransferase (EC 2.1.3.3). Bauchop and Elsdon (50) have shown that ATP formed in this way is used efficiently to increase the growth of *S. faecalis* when other essential nutrients are present in excess.



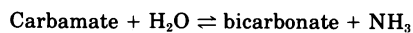
$$\Delta G^{\circ} = -9 \text{ kcal/mol} \\ (-37.7 \text{ kJ/mol})$$



$$\Delta G^{\circ} = +6.8 \text{ kcal/mol} \quad (114) \\ (+28.5 \text{ kJ/mol})$$



$$\Delta G_{obs}^{\circ} = -1.8 \text{ kcal/mol} \quad (284, 517) \\ (-7.5 \text{ kJ/mol})$$



$$\Delta G^{\circ} = -0.8 \text{ kcal/mol} \quad (517) \\ (-3.3 \text{ kJ/mol})$$

(ΔG° of arginine hydrolysis was calculated from the equilibrium constant of the following reactions: argininosuccinate lyase reaction [EC 4.3.2.1][$K = 1.14 \times 10^{-2}$ M]; argininosuccinate synthetase reaction [EC 6.3.4.5][$K = 8.9$]; aspartate ammonia lyase reaction [EC 4.3.1.1][$K = 2.3 \times 10^{-2}$ M]; $\text{ATP} + \text{H}_2\text{O} = \text{AMP} + \text{P}_i$, [$\Delta G_{obs}^{\circ} = -9.96$ kcal/mol] [data from Barman, reference 45; for thermodynamic data on allantoin degradation see Bojanowski et al., reference 59].)

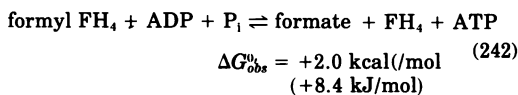
The generation of CAP from ornithine is thermodynamically rather unfavorable. The formation of citrulline from arginine, however, is an irreversible reaction. Thus the overall

process becomes exergonic enough to allow the synthesis of 1 mol of ATP from ADP and P_i .

There is some evidence that creatine and creatinine can be utilized as another source of carbamyl phosphate by anaerobic bacteria (635). It is also possible, but it has not yet been demonstrated, that CAP may be formed during the fermentation of some pyrimidines (42, 241).

Formyltetrahydrofolate as "Energy-Rich" Intermediate

Formyltetrahydrofolate synthetase (EC 6.3.4.1) catalyzes the reversible formation of formate and tetrahydrofolate (FH_4) from formyl- FH_4 with the concomitant phosphorylation of ADP to form ATP (242, 243, 510, 273, 287, 288, 509):

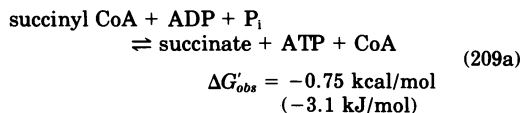
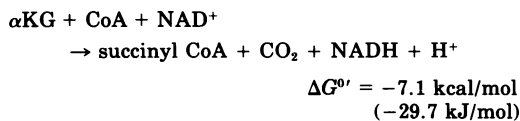


The ATP formation via this reaction is an extremely specialized process. Only one purine-fermenting clostridium, *C. cylindrosporium*, is presently believed to use this mechanism as a major path of ATP generation (42, 44). Even closely related purine-fermenting clostridia, e.g., *C. acidi-urici*, degrade the purines so that acetyl phosphate rather than formyltetrahydrofolate is the source of high-energy phosphate (44). There are some indications, however, that formyl FH_4 may be used to synthesize ATP also in the fermentation of methanol by *Methanosarcina barkeri* (619, 726).

Formyltetrahydrofolate synthetase is found not only in *C. cylindrosporium* and *M. barkeri*, but also in many anaerobic and aerobic bacteria, yeast plants, and animals. In these organisms the function of formyltetrahydrofolate synthetase is, however, to synthesize formyl FH_4 from formate and tetrahydrofolate rather than the reverse reaction (for literature see Thauer et al. [645]).

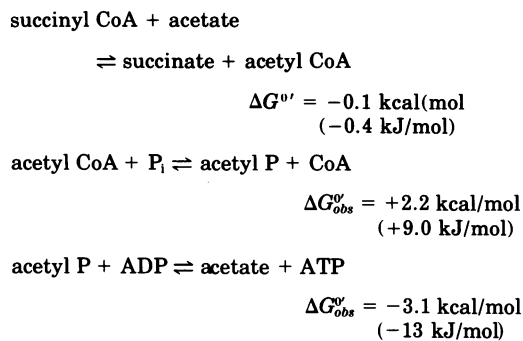
Succinyl CoA as "Energy-Rich" Intermediate

In the citric acid cycle succinyl CoA is formed from α -ketoglutarate (α KG) via α -ketoglutarate dehydrogenase (522) and succinate is formed from succinyl CoA via succinate thiokinase (succinyl-CoA synthetase [EC 6.2.1.4 and EC 6.2.1.5]). The latter reaction is coupled with the phosphorylation of GDP or ADP (for review, see Bridger [75]):



($\Delta G^{0'}$ of the ketoglutarate dehydrogenase reaction was calculated from $\Delta G^{0'}$ values with CO_2 in the gaseous state [Table 13], $\Delta G_{obs}^{0'}$ for hydrolysis of succinyl CoA [Table 4], and $\Delta G_{obs}^{0'}$ for NAD reduction with H_2 [Table 5]).

The formation of succinate from α KG is generally not believed to occur in anaerobically growing organisms. An exception appears to be found in *P. rettgeri* (341). This organism can anaerobically grow on fumarate as sole energy source; 7 mol of fumarate is disproportionated to 6 mol of succinate and 4 mol of CO_2 . One mole of the 6 mol of succinate formed is derived from α KG via α KG dehydrogenation rather than via fumarate reduction. From growth yield studies evidence is available that 1 mol of ATP is formed in this reaction. Thus succinate is probably formed from α KG via α KG dehydrogenase (NAD dependent?) and succinate thiokinase. It cannot be excluded, however, that succinate formation is coupled with phosphorylation via the following reactions:



Both acetokinase and phosphotransacetylase have been found in *P. rettgeri*. Whether the organism contains a thiophorase that can catalyze a CoA transfer from succinyl CoA to acetate (11) is not known.

Desulfuromonas acetoxidans catalyzes the oxidation of acetate to CO_2 with elemental sulfur as the electron acceptor (488). As succinate cannot be oxidized to fumarate (succinate/fumarate; $E_o' = +33 \text{ mV}$) with elemental sulfur (S^0/S^- ; $E_o' = -270 \text{ mV}$) (see Table 5), the participation of the citric acid cycle in the formation of CO_2 from acetate appears rather unlikely.

Succinate thiokinase is found in many anaerobically growing organisms. The function of the enzyme is, however, to catalyze the formation of succinyl CoA from succinate

rather than the reverse reaction. The succinyl CoA is required for biosynthetic purposes, i.e., the synthesis of tetrapyrroles and of cystathionine.

ENERGY CONSERVATION VIA ETP

In chemotrophic anaerobes, ATP synthesis is frequently considered to be associated only with electron-donating, formally hydrogen-forming reactions and thus to proceed only via SLP. However, in recent years, direct and indirect evidence has accumulated showing that, in many both facultatively and obligately anaerobic bacteria, ATP generation can also be coupled to the electron accepting, formally hydrogen-consuming reactions of energy metabolism. The most convincing demonstration is the finding that many strictly anaerobic bacteria can carry out mixed fermentations with H₂ as electron donor and either fumarate (382, 715), sulfate (608), nitrate (263), or CO₂ (740) as electron acceptor and obtain useful energy (ATP) from the intermolecular redox process as evidenced by growth. Since no mechanisms are known for SLP coupled to H₂ oxidation, ATP is presumably formed via ETP (44) (see sections, ATP Synthesis Via SLP and ATP Synthesis Via ETP).

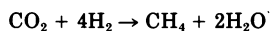
It is important to note that the demonstration of ATP synthesis coupled to a "hydrogenation" reaction in one organism does not necessarily mean that the reaction is always linked with phosphorylation in all anaerobic organisms. In some anaerobic bacteria the hydrogenation reaction may serve only as a sink for electrons, the function of which is to drive SLP.

Hydrogenation Reactions Evidently Coupled with Phosphorylation

The reactions evidently coupled with phosphorylation will be discussed in the order of increasing free energy changes associated with the transfer of an electron equivalent from H₂ to the respective electron acceptor (Table 8).

All of the methanogenic and the sulfate-reducing bacteria and many of the fumarate-reducing bacteria are strict anaerobes. The percentage of facultative organisms is higher in the group of nitrate-reducing bacteria than in the group of fumarate-reducing bacteria and is almost 100% in the group of denitrifiers. From the electron acceptors listed in Table 8 the methane bacteria can only reduce CO₂. The sulfate-reducing bacteria, however, frequently can also use fumarate, the fumarate-reducing bacteria can also use either sulfate or nitrate, and the nitrate-reducing bacteria can also use either fumarate or nitrite.

CO₂ reduction to methane.



$$\Delta G^{\circ} = -31.3 \text{ kcal/mol} \\ (-131 \text{ kJ/mol})$$

(CO₂, H₂, and CH₄ in the gaseous state)

All methane bacteria known to date can grow on H₂ and CO₂ as sole energy source. With the exception of *Methanobacterium* strain MOH (81, 84), *Methanobacterium thermoautotrophicum* (740), *Methanosarcina barkeri* (619), *Methanobacterium arbophilicum* (739), and strain F5 isolated by Prins et al. (506), they can additionally use electrons generated from the oxidation of formate for growth and methane formation. *M. barkeri* can also grow on methanol (58), and *M. thermoautotrophicum* can metabolize acetate when grown on CO₂ plus H₂ (742). Free intermediates appear not to be formed during methane formation from CO₂ and H₂ (41, 44, 714). A consistent view of the reactions involved is not available. For a summary of what is known about the nutritional requirements of methanogenic bacteria, see the reviews by Pine (494) and Bryant et al. (82). The biochemistry of methane formation has been reviewed by Stadtman (619), Wolfe (713), and McBride and Wolfe (399). The most recent reviews on methane formation are by Taylor (637) and Zeikus (736).

Enzymes and electron carriers involved in CO₂ reduction to methane. CO₂ reduction to CH₄ is mediated by an electron transport system involving dehydrogenases, electron carriers, and probably four reductases (Fig. 5). The components of the system are found in the

TABLE 8. Reductive processes coupled with phosphorylation

Reaction ^a	-ΔG ^{0'} ^b	
	kcal/electron equivalent from H ₂	kJ/electron equivalent from H ₂
CO ₂ reduction to methane	3.9	16.4
Sulfate reduction to sulfide	4.5	18.8
Fumarate reduction to succinate	10.3	43.1
Nitrate reduction to nitrite	19.5	81.6
Nitrite reduction to N ₂	31.7	132.6
O ₂ reduction to H ₂ O (for comparison)	28.3	118.4

^a CO₂, CH₄, H₂, N₂, NO, and N₂O in the gaseous state, all other substances in aqueous solution.

^b ΔG^{0'} for the different reactions was calculated from ΔG⁰ values given in Table 15.

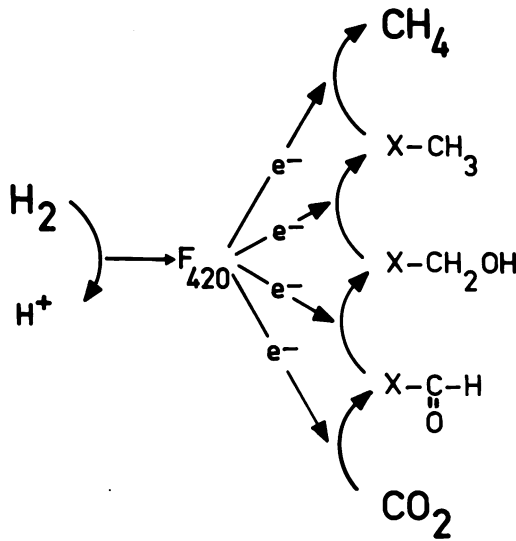


FIG. 5. Scheme of the electron transport system involved in CO₂ reduction to CH₄. Symbols: F₄₂₀, low-molecular-weight fluorescent electron carrier (105), which is reduced by H₂ via hydrogenase (667, 668); X, unknown cofactor (recent evidence indicates that X-CH₃ is CH₃-S-CoM [714]); - e⁻ →, electron transport chain, components of which are unknown.

30,000 × g supernatant of cell suspensions (1 g of cells plus 1 ml of buffer) subjected to sonic oscillation (536). The formation of methane from CO₂ in cell-free extracts is dependent on ATP (536, 717, 718).

(i) *Dehydrogenases*. Cell-free extracts of *Methanobacterium* strain MOH have been reported to catalyze the reduction of CO₂ to methane with H₂ as electron donor (536, 718). In cell-free extracts of *Methanobacterium formicicum*, formate can be used instead of H₂ (81). Cell-free extracts of *Methanobacterium ruminantium* contain a hydrogenase and a formate dehydrogenase. The two enzymes catalyze the reduction of F₄₂₀ (see below) with H₂ and formate, respectively. Ferredoxin and pyridine nucleotides are inactive as electron acceptors (667, 668).

(ii) *Reductases*. The reduction of CO₂ to CH₄ proceeds via a series of reactions of which only the reduction of methyl coenzyme M to methane and free coenzyme M (CoM) has unambiguously been identified (640, 641, 398, 714). CoM is a cofactor specific for methanogenic bacteria. The chemical structure has been elucidated only recently to be HS-CH₂-CH₂-SO₃⁻ (640). All methane-forming bacteria looked at have been shown to contain this interesting compound which has not been found in any other organism (398). CoM is a growth factor for some of the methanogens, whereas others are capa-

ble of synthesizing enough to even excrete it into the medium (638). Methyl CoM is the thioether of CoM. The reduction of methyl CoM with H₂ is catalyzed by a soluble enzyme system. The reduction is dependent on the presence of Mg²⁺ and of ATP which, however, does not appear to be required in stoichiometric amounts (398, 640, 641, 714).

The reactions leading to the formation of methyl CoM from CO₂ and CoM are not known. Free formate, formaldehyde, and methanol do not appear to be intermediates (Fig. 5). CO₂, rather than formate, and methanol are reduced to methane in cell-free extracts of *Methanobacterium* strain MOH (536) (see, however, Fina et al. [171]). Tetrahydrofolate- and corrinoid-activated one-carbon units are considered as intermediates; their role in methane formation is, however, being questioned (637, 714).

Methyltetrahydrofolate and serine have been shown to be reduced to methane in cell-free extracts of methane bacteria (58, 82, 726). The rate of methane formation from methyl-FH₄ in cell-free extracts of *Methanobacterium* strain MOH is much lower than the rate of methane formation from CO₂, which is not in favor of methyl FH₄ as an intermediate. *M. barkeri* and *M. thermoautotrophicum* have been reported to exhibit formyl tetrahydrofolate synthetase activity (58, 169, 639). It has been suggested that the enzyme may, however, be involved in CO₂ assimilation (e.g., formation of the positions 2 and 8 of the purines) rather than in methane formation from CO₂ (see 637).

Methyl-vitamin B₁₂ compounds have been shown to be reduced to methane in cell-free extracts of methane-forming bacteria (56, 57, 717, 721, 722, 725). The methyl group is transferred to CoM via methyltransferase to form a thioether, which is then reduced to give methane (398, 640, 641). The methyl derivative of B₁₂ may, however, form methyl CoM only when added in large amounts. A corrinoid protein that catalyzes the formation of methane from methyl B₁₂ and methyltetrahydrofolate was partially purified from *Methanobacillus omelianskii* (721). However, there is no indication as yet, that the protein is also present in the pure methanogenic organism (*Methanobacterium* strain MOH) derived from *M. omelianskii* (84). It is possible that biochemical differences exist when methanogenic bacteria grow under high and low partial pressure of H₂ as does *Methanobacterium* strain MOH when growing in pure culture on CO₂ plus H₂ as compared to growth on ethanol plus CO₂ in association with the S-organism. Chlorinated methanes have been shown to inhibit methane formation both in vivo (49, 537) and in vitro (723, 724), which

has been taken to indicate that a corrinoid is involved in CO₂ reduction to methane. Alkyl halides are known to alkylate reduced vitamin B₁₂ compounds. The finding that the demethylation of methyl CoM is inhibited by chloroform (398) and that the enzyme preparation mediating this reaction does not appear to contain a corrinoid (714) shows that the inhibitor experiments cannot be interpreted unambiguously.

(iii) *Electron carriers*. Besides CoM, methane-forming bacteria contain F₄₂₀, a compound not found in any other organism (105). F₄₂₀ is a low-molecular-anionic compound of molecular weight of approximately 630. It is fluorescent in the oxidized form and not fluorescent in the reduced form. The structure has not yet been elucidated. Evidence is, however, available that it is not a pteridine (R. S. Wolfe, personal communication). The redox potential of F₄₂₀ has not been determined. It is probably near -300 mV or more negative as indicated by the finding that hydrogenase from methanogenic bacteria catalyzes a rapid reduction of protons with reduced F₄₂₀ (667, 668). F₄₂₀ has the interesting property to be protein bound when in the reduced form and to be dissociated from the proteins when in the oxidized form.

Cells from *M. thermoautotrophicum* could

not be shown to contain menaquinone nor appreciable amounts of *b*- or *c*-type cytochromes (A. Kröger, personal communication). This finding is of special importance, as it shows that neither a quinone nor a cytochrome is required for ETP in the methanogenic bacteria. All other organisms equipped with the mechanism of ETP have been shown to contain both cytochromes and either a naphthoquinone or a ubiquinone. Whether methane bacteria contain flavodoxins and rubredoxins is not known. Ferredoxin has been identified in cell-free extracts of *M. sarcina* (56). Cell-free extracts of *M. ruminantium*, however, appear not to contain this iron sulfur protein (667).

(iv) *Topography*. Figure 6 is a high-power micrograph of a thin section of *M. thermoautotrophicum* showing numerous intracytoplasmic membranes. They have been shown to consist of closely apposed unit membranes that are formed from invagination of the plasmic membrane (741). The membraneous inclusions are found in *Methanobacterium* and *Methanosprillum* species (346, 737, 738). The amount of intracytoplasmic membranes appears to be correlated with the growth rate of the bacteria, e.g., the shorter the doubling time, the more membranes are formed, suggesting that in *Methano-*

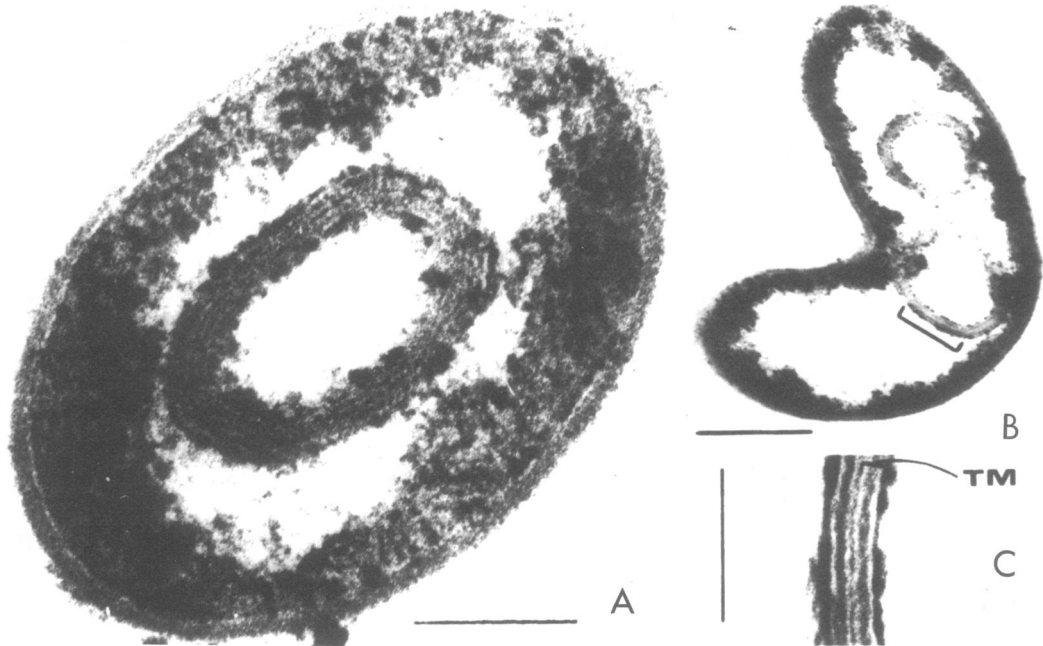


FIG. 6. (A) Cross-section of *M. thermoautotrophicum* illustrating closely stacked membranes in concentric circles. Bar indicates 0.13 μ m. (B) Section through *M. thermoautotrophicum* showing closely stacked triplet membranes (bracket). Bar indicates 0.25 μ m. (C) High magnification of bracketed area in part B demonstrating a pair of triplet membranes. Bracket delineates one triplet membrane (from Zeikus and Wolfe [741] with permission).

bacterium and *Methanospirillum* species the membranous system may be involved in energy conservation (737).

Thin sections of *Methanosarcina* and of *Methanococcus* species show internal membranes of the vesicular, mesosomal type. The membranous cytoplasmic bodies are often associated with cell division (737, 743).

Phosphorylation coupled to CO₂ reduction to methane. CO₂ reduction to methane is coupled with phosphorylation as evidenced by growth of *M. thermoautotrophicum* on CO₂ and H₂ as sole carbon and energy source (740). The finding that *M. thermoautotrophicum* can grow on mineral medium in the complete absence of organic compounds excludes the possibility that ATP synthesis proceeds via SLP. This is important, as the molar growth yields of methane bacteria growing on CO₂ and H₂ are low—only 1 to 3 g of cells (dry weight) are formed per mol of CH₄ produced from CO₂ and H₂ (537, 619, 637, 639; for a discussion see Quayle [508]). The Y_{ATP}^{max} for bacteria growing on CO₂ as sole carbon source has been calculated to be 5 (Table 7). As the experimentally found Y_{ATP} is always considerably lower than the calculated one, the growth yield data can be taken to indicate that probably 1 ATP is formed per mol of methane produced (see section, Energy-consuming processes and the ATP requirement for bacterial growth).

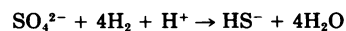
Under standard conditions the free energy change of CO₂ reduction to methane with H₂ is -31.3 kcal/mol (-131 kJ/mol) of methane formed. The methane bacteria usually grow, however, at H₂ concentrations of 1 μM, corresponding to a concentration of H₂ in the gas phase of approximately 10⁻³ atm. For example, the concentration of H₂ in the rumen is no higher than 1 μM (125, 256, 257). Taking this into account, the free energy change of CH₄ formation decreases from -31.3 kcal/mol (-131 kJ/mol) under standard conditions to -15 kcal/mol (-62.8 kJ/mol) under physiological conditions. This indicates that no more than 1 ATP will be formed during CO₂ reduction to methane.

The reduction of CO₂ to H₂ by cell suspensions of methane bacteria is inhibited by low concentrations of viologen dyes (716) and by uncouplers of oxidative phosphorylation, e.g., carbonylcyanide-*m*-chlorophenyl hydrazine (CCCP), pentachlorophenol, and dinitrophenol (537). Concomitantly, the ATP pool decreases and the concentration of AMP increases. Sulfate reduction to H₂S with H₂ by cell suspensions of sulfate-reducing bacteria is also inhibited by low concentrations of viologen dyes and by inhibitors such as dinitrophenol (477).

The effect of the uncouplers on both electron transport and phosphorylation is surprising. In the case of the sulfate-reducing bacteria the effect on both electron transport and phosphorylation is explicable by the requirement of ATP for the activation of sulfate via ATP sulfurylase (EC 2.7.7.4). In the absence of ATP, sulfate cannot be reduced, thus uncouplers of phosphorylation must also inhibit electron flow. In the case of the methane bacteria, a similar explanation appears likely. The reduction of CO₂ to the oxidation state of formaldehyde is an endergonic reaction (+5.4 kcal/mol; +22.6 kJ/mol) and can probably proceed, especially at low H₂ partial pressures (see above), only if CO₂ or formate is activated prior to their reduction. The reduction of formaldehyde to CH₄ is exergonic enough to allow the synthesis of more than 1 ATP (CH₂O + 2H₂ = CH₄ + H₂O; $\Delta G^\circ = -37.6$ kcal/mol (-157.4 kJ/mol). Note, however, that Robertson and Wolfe (536) have shown that less than 1 mol of ATP is required for the synthesis of 1 mol of CH₄ from CO₂ and H₂ in cell-free extracts of *Methanobacterium* strain MOH. Since under their experimental conditions the reduction of CO₂ to methane was not coupled with phosphorylation, this finding was interpreted to indicate that ATP is not required for the reduction of CO₂ to the redox level of formaldehyde (536, 713).

Viologen dyes are usually not considered uncouplers of phosphorylation. They have been shown, however, to uncouple electron transport from phosphorylation associated with fumarate reduction with H₂ (47, 48). The viologen dyes, which are reduced by hydrogenase from both sulfate-reducing bacteria and from methane bacteria, probably bypass the coupling site since they are also good artificial electron donors for most of the reductases involved. It is interesting in this respect that viologen dyes only slowly enter the cells. Thus the site of reduction and of reoxidation of the viologen dyes is probably on the outer side of the plasmic membrane. The hydrogenase of sulfate-reducing bacteria has been localized in the periplasmic space (53). Whether this is also the case for the hydrogenase of methane bacteria remains to be shown.

Sulfate reduction to sulfide.



$$\Delta G^\circ = -36.4 \text{ kcal/mol} \\ (-152.2 \text{ kJ/mol})$$

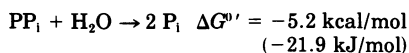
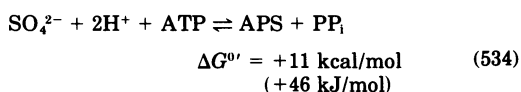
Several anaerobic bacteria (the genera *Desulfovibrio* and *Desulfotomaculum* [97, 502]) can utilize SO₄²⁻ as ultimate electron acceptor of energy metabolism. Sulfate can be replaced by sulfite, thiosulfate, tetrathionate (499), or

elemental sulfur (55a). Lactate, pyruvate, ethanol, and formate appear to be the preferred electron donors (501). Sulfate-reducing bacteria have been shown to grow on H₂ and sulfate as sole energy source (608). A consistent view of the reactions involved in dissimilatory sulfate reduction is lacking. Recent reviews on this subject are by Barton et al. (48), LeGall and Postgate (356), Peck (480), and Siegel (594).

Enzymes and electron carriers involved in dissimilatory sulfate reduction. The reduction of sulfate, sulfite, thiosulfate, and tetrathionate is mediated by an electron transport system composed of dehydrogenases, electron carriers, and a series of reductases. Some of the enzymes and electron carriers involved are found exclusively in the membrane fraction, others are solubilized on cell rupture.

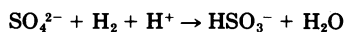
(i) *Dehydrogenases.* Lactate dehydrogenase (47), pyruvate:ferredoxin oxidoreductase (7), ethanol dehydrogenase (608), formate dehydrogenase, and hydrogenase are the dehydrogenases involved in dissimilatory sulfate reduction. The formate dehydrogenase (530, 727) and hydrogenase (228, 330, 357, 729) have been partially purified. The hydrogenase is an iron sulfur protein and is specific for cytochrome *c*₃ as electron acceptor. Hydrogenase activity is found in both the particulate and the soluble cell fraction. The formate dehydrogenase uses either cytochrome *c*₃ or *c*₅₅₃. The electron acceptors for lactate dehydrogenase and ethanol dehydrogenase are not known. They are not NAD or NADP. Both enzymes are tightly membrane bound. An NADH dehydrogenase capable of linking the reduction of inorganic sulfur compounds to the oxidation of NADH has not been reported. The presence of such a dehydrogenase appears not to be required under normal growth conditions, i.e., growth on lactate plus sulfate.

(ii) *Reductases.* There is general agreement that adenylylsulfate (APS) and hydrogen sulfite (HSO₃⁻) (p*K*₂ = 6.8) are free intermediates in dissimilatory sulfate reduction to sulfide (p*K*₁ = 7). They are formed via ATP sulfurylase (EC 2.7.7.4) and APS reductase (EC 1.8.99.2), respectively (264, 476, 478). Both enzymes are solubilized on cell rupture. The physiological electron donor for APS reductase is not known. Reduced viologen dyes are efficient artificial electron donors (for review, see 481).



$$\Delta G^{\circ} = -16.4 \text{ kcal/mol} \quad (156, 594)$$

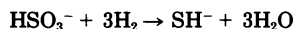
$$(-68.6 \text{ kJ/mol})$$



$$\Delta G^{\circ} = +4.7 \text{ kcal/mol}$$

$$(+19.7 \text{ kJ/mol})$$

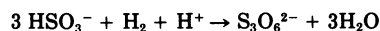
(The ΔG° of SO₄²⁻ reduction to HSO₃⁻ + H₂O was calculated from ΔG_f° values given in Table 15. ΔG° is +4.8 kcal/mol [20.1 kJ/mol] if calculated from ΔG° of the ATP sulfurylase reaction, the pyrophosphatase reaction, the APS reductase reaction, and the hydrolytic cleavage of ATP to AMP and PP_i.) The question whether additional free intermediates occur between sulfite and sulfide is still a matter of considerable controversy. At present two mechanisms of bisulfite reduction to sulfide are discussed. The literature was recently reviewed by Chambers and Trudinger (102) and by Siegel (594). The first mechanism of bisulfite reduction [mechanism (i)] assumes that bisulfite is reduced to hydrogen sulfide via one enzyme, bisulfite reductase (EC 1.8.99.1) (identical with desulfovridin in *D. gigas* [351], with desulfurubridin in *D. desulfuricans* strain Norway [352], and with CO binding pigment P 582 in *Desulfotomaculum* species [8]) without any free intermediates.



$$\Delta G^{\circ} = -41.0 \text{ kcal/mol}$$

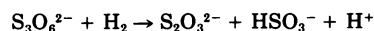
$$(-171.7 \text{ kJ/mol})$$

The second mechanism of bisulfite reduction [mechanism (ii)] assumes that sulfite is reduced to sulfide via three enzymes—bisulfite reductase (EC 1.8.99.1), trithionate reductase, and thiosulfate reductase—with trithionate and thiosulfate as free intermediates (Fig. 7). Respective activities are found in the particulate and soluble fraction of cell-free extracts of sulfate-reducing bacteria.



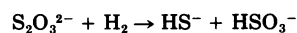
$$\Delta G^{\circ} = -11.1 \text{ kcal/mol}$$

$$(-46.3 \text{ kJ/mol})$$



$$\Delta G^{\circ} = -29.4 \text{ kcal/mol}$$

$$(-123 \text{ kJ/mol})$$



$$\Delta G^{\circ} = -0.5 \text{ kcal/mol}$$

$$(-2.1 \text{ kJ/mol})$$

In mechanism (i) (six-electron reduction), sulfide is the product of sulfite reduction via bisulfite reductase; in mechanism (ii) (recycling sulfite pool), trithionate is the product. The problem is that, dependent on the experimental

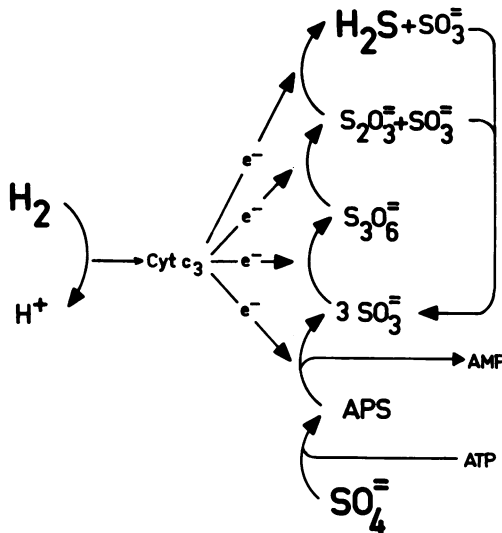


FIG. 7. Scheme of the electron transport system involved in sulfate reduction to sulfide in sulfate-reducing bacteria. Abbreviations: *cyt c₃*, cytochrome *c₃*; *APS*, adenylyl sulfite; $-e^-$, electron transport chain, components are unknown. The assumption was made that sulfite is reduced via trithionate and thiosulfate (recycling sulfite pool mechanism) to sulfide rather than directly.

conditions, either trithionate (pH 6, low concentrations of reduced methyl viologen, high concentrations of sulfite) or sulfide (pH 7, high concentrations of reduced methyl viologen, low concentrations of sulfite) is found as major or sole product(s) of sulfite reduction by purified bisulfite reductase from sulfate-reducing bacteria (152, 285, 322). Since the physiological electron donor of the dissimilatory bisulfite reductases and the intracellular pH and concentration of sulfite are not known, it is impossible to decide which of the products are formed *in vivo*. The situation is further complicated by the possible presence of an assimilatory type of sulfite reductase catalyzing a six-electron reduction of sulfite to sulfide (353). The finding that siroheme (sirochlorine) (440) is the prosthetic group of both assimilatory and dissimilatory sulfite reductases (438, 439) suggests a mechanistic relationship between the two groups of enzymes (594).

(iii) *Electron carriers.* The soluble cell fraction contains cytochrome *c₃* ($E_0' \approx -300$ mV) (500, 728), *c₅₅₃* ($E_0' \approx -100$ mV) (356) ferredoxin ($E_0' \approx -300$ mV) (6, 358), flavodoxin ($E_{01}' \approx -400$ mV) (153, 355), and rubredoxin ($E_0' \approx -60$ mV) (155, 354, 445). Cytochrome *c₃* is the coenzyme of hydrogenase (729); cytochrome *c₅₅₃* or *c₃* is the coenzyme of formate dehydrogenase (530, 727); ferredoxin is the coenzyme of pyruvate:ferredoxin oxidoreductase

(7). None of the electron carriers appears to be the direct electron donor for *APS* reductase or bisulfite reductase. Ferredoxin and flavodoxin have been shown to be involved in sulfate reduction to sulfide with H_2 (6, 48, 358, 355); the site of action of these electron carriers is, however, not yet clear. For a critical discussion of the role of soluble electron carriers in sulfate-reducing bacteria, see the review of LeGall and Postgate (356).

The particulate fraction contains *c*-type cytochromes (48), *b*-type cytochromes (231), and menaquinone-6 (387, 693). Whether the membrane-bound electron carriers are involved in dissimilatory sulfate reduction has not yet been determined. Menaquinone-6 ($E_0' = -74$ mV [569, 685]) is probably functionally associated with the membrane-bound fumarate reductase which is always present in high activities even in cells not grown on fumarate or malate (48). On the basis of thermodynamic considerations, Wagner et al. (685) proposed that menaquinone-6 could be involved in *APS* reduction ($E_0' = -60$ mV) and in trithionate reduction ($E_0' = +225$ mV) rather than in thiosulfate reduction (-402 mV) or sulfite reduction to trithionate ($E_0' = -173$ mV). The validity of the proposal is dependent on whether or not trithionate and thiosulfate are physiological intermediates in dissimilatory sulfate reduction (for E_0' values see Table 5).

(iv) *Topology.* Hydrogenase and cytochrome *c₃* were recently reported to be located exclusively in the periplasmic space of lactate-plus-sulfate-grown *D. gigas* (53, 359). *APS* reductase and bisulfite reductase in this organism are found exclusively in the cytoplasm (53). They are probably peripheral membrane proteins located on the cytoplasmic side of the membrane. If cytochrome *c₃* and the periplasmic hydrogenase are physiologically involved in dissimilatory sulfate reduction, then the reduction of sulfate with H_2 is a transmembrane redox process [see Fig. 3, (I)]. Hydrogenase and cytochrome *c₃* could, however, physiologically be involved in hydrogen formation rather than hydrogen uptake. Sulfate-reducing bacteria can, in the absence of sulfate, grow on ethanol or lactate, with the concomitant formation of acetate (CO_2) and H_2 , provided that the partial pressure of H_2 is kept low (for example, by growing the bacteria together with hydrogen-utilizing organisms [Bryant, personal communication]). One mole of *ATP* is synthesized per mol of acetate formed via acetyl CoA and acetyl phosphate under these conditions.

Sorokin (608) noted that acetate and CO_2 formed during the oxidation of ethanol and for-

mate had to be excreted from the cells prior to their assimilation. He postulated that the process of oxidation and of biosynthesis in *Desulfovibrio* are spatially separated. For a detailed discussion of these observations, see LeGall and Postgate (356).

The location of cytochrome c_3 (409) in the periplasmic space (53, 359) is of special interest. Cytochrome c_2 from *Rhodospseudomonas spheroides* and *Rhodospseudomonas capsulata* (505), cytochrome c from *Micrococcus denitrificans* (573), and cytochrome c -552 from *E. coli* (178) are all located in the periplasmic space of the respective bacteria. Racker et al. (513) showed that, in mitochondria, cytochrome c is outside the inner membrane. The location of the c cytochromes mentioned above on only one side of the membrane may be taken to indicate that the function of these cytochromes may be very similar.

Phosphorylation coupled to dissimilatory sulfate reduction: (i) in vivo evidence. Cell suspensions of sulfate-reducing bacteria catalyze the reduction of sulfate to sulfide with H_2 . Sulfate reduction is inhibited by 2,4-dinitrophenol (DNP) and by methyl viologen (see subsection, CO_2 reduction to methane). The reduction of sulfite and of thiosulfate, which are not dependent on ATP, is not inhibited by either of these compounds (477). These findings can only be interpreted to indicate that dissimilatory sulfate reduction is coupled with phosphorylation. At least two equivalents of ATP must be formed; these are required for the activation of sulfate. This inference is made on the premise that the pyrophosphate produced in the ATP sulfurylase reaction is completely hydrolyzed by pyrophosphatase present in sulfate-reducing bacteria (690), but the possibility exists that a portion or all of the pyrophosphate formed in the APS reductase reaction could be utilized (48, 118, 166, 523, 525). Since the bacteria have been reported to grow on minimal media with H_2 and SO_4 as sole energy source (608), additional ATP must also be formed. Considerations of the energetics of sulfate-reducing bacteria growing on lactate plus sulfate or ethanol plus sulfate lead to the same conclusion (44, 48, 129).

Growth yields of sulfate-reducing bacteria on lactate plus sulfate, on pyruvate plus sulfate, and on pyruvate plus sulfite have been reported to be almost identical (586, 682). Furthermore, the oxidation of H_2 appears to contribute little to the energy metabolism of sulfate-reducing bacteria growing on lactate plus sulfate (307). These findings can best be explained if it is assumed that all of the ATP required for sulfate activation to APS is formed during APS reduc-

tion to sulfite and that the reduction of 1 mol of sulfite to sulfide is coupled with the synthesis of only 1 mol of ATP ($P/2e^- = 1/3$).

(ii) In vitro evidence. The first demonstration of phosphorylation was provided by Peck (479). Cell-free preparations of *D. gigas* grown on lactate plus sulfate were shown to catalyze the reduction of sulfite to sulfide with H_2 as electron donor, with the concomitant esterification of phosphate. A soluble protein fraction and membrane particles were required for both electron transfer and phosphorylation. Phosphorylation was uncoupled by DNP, pentachlorophenol, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (369), and gramicidin (48, 479). Oligomycin and antimycin A were without effect. The observed P/H_2 ratios were 0.05 to 0.2 (479) (see above).

The membrane fraction of *D. gigas* has been reported to contain a membrane-bound ATPase which is activated by DNP (200). The latter finding indicates that the membrane-bound ATPase might be involved in phosphorylation coupled to the reduction of sulfate to sulfide.

(iii) Thermodynamic and mechanistic considerations. The relatively high redox potential of the $APS/HSO_3^- - AMP$ couple ($E_0' = -60$ mV) (594) allows the oxidation by APS of various metabolic hydrogen donors (lactate, pyruvate, formate, H_2). The free energy yields of the reactions are sufficient to drive the synthesis of 1 ATP from ADP and P_i . The finding that the sulfate-reducing bacteria couple the two-electron reduction of fumarate (succinate/fumarate; $E_0' = +33$ mV) with phosphorylation (47) indicates that ETP via APS reduction is also mechanistically possible.

A six-electron reduction of sulfite to sulfide ($E_0' = -116$ mV), e.g., with lactate (lactate/pyruvate; $E_0' = -190$ mV), could theoretically be linked with phosphorylation as, in a six-electron transferring process, a ΔE of 70 mV is sufficient to make the synthesis of ATP thermodynamically feasible. The overall mechanism of ATP synthesis would, however, have to be different from that of ETP coupled to a two-electron redox process (e.g., fumarate reduction and APS reduction), with approximately 220 mV potential differences. Different mechanisms of ETP in one bacterium are not considered to be very likely. Two electrogenic proton-extruding systems with different mechanisms, one with $2H^+/2e^-$ and the other with $2H^+/6e^-$, would be required. A reduction of sulfite to sulfide via trithionate and thiosulfate as free intermediates (three successive two-electron reactions) would avoid this problem. Of the three reactions involved, the two-electron reduction of trithionate to sulfite plus thiosulfate

($E_0' = +228$ mV) is exergonic enough to allow the synthesis of ATP on the $2H^+/2e^-$ basis. A phosphorylation coupled to the reduction of thiosulfate to sulfide plus sulfite ($E_0' = -402$ mV) and of sulfite to trithionate ($E_0' = -173$ mV) can theoretically be excluded. A phosphorylation coupled only to the reduction of trithionate would explain why only low $P/2e^-$ values are found for the reduction of sulfite to sulfide (see subsection, Tetrathionate reduction to thiosulfate).

It is interesting to note that the catabolic reduction of nitrite to N_2 via nitric oxide and nitrous oxide rather than the six-electron reduction of nitrite to ammonia has been shown to be coupled with phosphorylation (see subsection, Nitrite reduction to N_2).

Fumarate reduction to succinate.



$$\Delta G^{0'} = -20.6 \text{ kcal/mol} \\ (-86.0 \text{ kJ/mol})$$

Many bacteria use fumarate as electron acceptor in catabolic redox processes (Table 9). The most prominent representative is *Vibrio succinogenes* which can grow on H_2 and fumarate as sole energy source (715). Others are the strictly anaerobic bacteria *D. gigas* (413) and *Clostridium formicoaceticum* (193), and the facultative anaerobes *E. coli* (207, 238, 382) and *P. rettgeri* (341). The reduction of fumarate to succinate is also an important reaction in the energy metabolism of a few eucaryotes (protozoa [443] and helminths [147, 407, 408, 563]). *Ascaris lumbricoides*, *Fasciola hepatica*, and *Hymenolopis diminuta* are parasitic helminths which have adapted to anaerobic conditions by using fumarate instead of molecular oxygen as terminal electron acceptor of the mitochondrial respiratory chain (for literature, see 147, 407, 408, 563). Note that in many anaerobic bacteria the reduction of fumarate to succinate has an anabolic rather than a catabolic function, i.e., to provide the organism with succinate for the synthesis of tetrapyrroles.

Fumarate is readily available as an electron acceptor. It can easily be formed from widely abundant carbon compounds such as malate, aspartate, pyruvate plus bicarbonate, and glucose plus 2 bicarbonate. The relatively high re-

dox potential of the fumarate/succinate couple ($E_0' = +33$ mV) allows the oxidation by fumarate of various metabolic hydrogen donors (e.g., NADH, lactate, formate). The free energy yield of these reactions is sufficient for the synthesis of 1 ATP from ADP and inorganic phosphate (201). A recent review on dissimilatory fumarate reduction is by Kröger (336).

Enzymes and electron carriers involved in dissimilatory fumarate reduction. Dissimilatory fumarate reduction proceeds via an electron transport system involving specific dehydrogenases, electron carriers, and fumarate reductase (Fig. 8). The components of the system are all membrane bound. They are found in the particulate cell fraction of bacteria and in the mitochondrial fraction of eucaryotic helminths capable of dissimilatory fumarate reduction. Fumarate reductase of *V. alcalescens* (= *M. lactilyticus*) appears to be an exception in this respect. Most of the enzyme activity is found in the soluble cell fraction of this strict anaerobe (692).

(i) *Dehydrogenases.* H_2 , NADH, formate, lactate, and glycerol phosphate have been shown to be the electron donors involved in dissimilatory fumarate reduction (Table 9). Dependent on the organism and on the growth conditions, the membrane fraction contains a hydrogenase, an NADH dehydrogenase, a formate dehydrogenase, an NAD-independent lactate dehydrogenase, or an NAD-independent glycerolphosphate dehydrogenase. Some of the dehydrogenases can be readily solubilized; others are tightly membrane bound. Hydrogenase from sulfate-reducing bacteria (357), NADH dehydrogenase from *Propionibacterium shermanii* (581), formate dehydrogenase from *E. coli* (164), glycerol phosphate dehydrogenase from *Propionibacterium arabinosum* (607), and *E. coli* (410), and the lactate dehydrogenase of *Propionibacterium pentosaceum* (429) have been partially purified. The hydrogenase of *Desulfovibrio* is an iron-sulfur protein (357) (for a review on hydrogenases see Mortenson and Chen [435]). The NADH dehydrogenase contains riboflavine 5'-phosphate (FMN) and iron-sulfur clusters (581). The formate dehydrogenase of *E. coli* is a molybdoprotein containing selenium as a functional component (162, 164, 363, 593). The

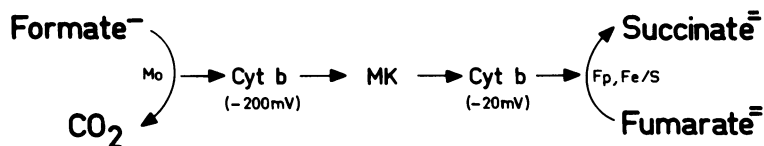


FIG. 8. Scheme of the electron flow from formate to fumarate in *Vibrio succinogenes* (according to Kröger [335]). Abbreviations: MK, menaquinone; Mo, molybdo-protein; cyt b, cytochrome b; Fp, Fe/S = FAD-iron-sulfur protein.

TABLE 9. List of some organisms that can use fumarate as electron acceptor of energy metabolism: electron carriers and electron donors involved in dissimilatory fumarate reduction, and evidence that fumarate reduction is coupled with phosphorylation^a

Organism	Electron carriers found in the cells and assumed to be involved in fumarate reduction		Electron donors involved in fumarate reduction	Inhibition of electron transport by:	ATP synthesis coupled to fumarate reduction as indicated by:
	Quinone	Cytochrome			
Anaerobic bacteria					
<i>Anaerovibrio lipolytica</i>	?	<i>b</i> -type (145)	NADH, glycerol-phosphate		Growth yields on fructose and on glycerol (248, 247)
<i>Actinomyces naeslundii</i>					Growth yields on glucose (87)
<i>Bacteroides fragilis</i>	MK (188)	<i>b</i> -type (383)	NADH (289)		Growth yields on glucose (248)
<i>Bacteroides melaninogenicus</i>	MK (188)	<i>b</i> -type (96, 698)	NADH (533)	NQNO (533)	No evidence
<i>Clostridium formicoaceticum</i>	MK (193)	<i>b</i> -type (193)	Formate		Growth on fumarate plus formate (193)
<i>Cytophaga succinicans</i> (17)					
<i>Desulfovibrio gigas</i> (199, 230, 367, 413)	MK (387, 693)	<i>b</i> -type (229)	H ₂ , lactate	HQNO (48)	Phosphorylation coupled to the oxidation of H ₂ with fumarate in cell-free extracts: P/2e ⁻ = 0.3 to 0.4 (47, 48)
<i>Propionibacteria</i> (347)	MK (606) MK-9(H ₂) (580)	<i>b</i> -type (583, 605, 606)	NADH (581), lactate (245), glycerol-phosphate (607)	NQNO (583) HQNO (144)	Growth yields on glucose, fructose, glycerol, and lactate of <i>P. freudenreichii</i> , and <i>P. pentosaceum</i> (50, 144).
<i>Ruminococcus flavefaciens</i> (252)			NADH (253)		No evidence
<i>Selenomonas ruminantium</i> (247)		<i>b</i> -type (145)	NADH (289)		No evidence (249)
<i>Desulfuromonas acetoxidans</i> (488)		<i>b</i> -type (488)			Growth yields on pyruvate plus malate (488)
<i>Streptococcus faecalis</i> (137)	DMK (51, 188)	No cytochromes (see however, 532)	NADH (167)	NQNO at high concentrations (167)	Phosphorylation coupled to the reduction of fumarate with NADH in cell-free extracts: P/2e ⁻ = 0.2 (167)
<i>Succinomonas amylolytica</i> (255)					
<i>Veillonella alcalescens</i> (= <i>Micrococcus lactilyticus</i> [328, 692])	MK (188)	<i>b</i> -type (145)	Lactate (145)		Growth yields on lactate (145; see also 328)

TABLE 9—continued

Organism	Electron carriers found in the cells and assumed to be involved in fumarate reduction		Electron donors involved in fumarate reduction	Inhibition of electron transport by:	ATP synthesis coupled to fumarate reduction as indicated by:
	Quinone	Cytochrome			
<i>Vibrio succinogenes</i>	MK (335, 338)	<i>b</i> -type (271, 272, 335, 339)	H ₂ , formate (27, 271, 272, 715)	HQNO (335, 272)	Growth on H ₂ plus fumarate (715); growth yields on formate plus fumarate (Kroger, unpublished data); phosphorylation coupled to the reduction of fumarate with formate in spheroplasts: P/2e ⁻ = 1 (335; for review, see 336).
Facultative bacteria					
<i>Bacillus megaterium</i>	MK (337)	<i>b</i> -type (337)	NADH, glycerol-phosphate (337)	HQNO (337)	
<i>Escherichia coli</i> (246, 507)	MK (447, 597)	<i>b</i> -type (597, 598)	NADH, lactate, glycerol-phosphate	HQNO (447, 597)	Phosphorylation coupled to the reduction of fumarate with glycerol-phosphate: P/2e ⁻ = 0.1 (412); can grow on fumarate plus H ₂ (382; growth yields [238]; see also 208, 327)
<i>Haemophilus parainfluenzae</i> (697)	DMK (364, 696)	(250a, 250c)		HQNO (596)	(250b)
<i>Proteus rettigeri</i>	MK (340)	<i>b</i> -type (340)	NADH, formate (340)	HQNO (340)	Growth yields on fumarate, citrate, and glucose plus CO ₂ (341)
Anaerobic eucaryotes					
<i>Ascaris lumbricoides</i> (315, 585)	Rhodiquinone (558)	<i>b</i> -type	NADH		Phosphorylation coupled to the reduction of fumarate in cell-free extracts (563)
<i>Hymenolepis diminuta</i> (563)			NADH		
<i>Fasciola hepatica</i> (408)			NADH		
Aerobic system under non-physiological conditions					
Beef heart mitochondria (206)	UQ		NADH		Phosphorylation coupled to the reduction of fumarate with NADH in digitonin particles: P/2e ⁻ = 0.7 to 0.9 (206)

^a See also Kröger (336). Abbreviations: MK, vitamin K₂ (menaquinone-6); DMK, 2-demethyl-vitamin K₂; MK-9(H₄), (II, III)-tetrahydromenaquinone-9; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; UQ, ubiquinone.

glycerolphosphate dehydrogenases appear to be flavoproteins (308). The different dehydrogenases have in common that they catalyze the reduction of membrane-bound electron carriers. They can be assayed by using artificial electron acceptors such as viologen dyes (hydrogenase and formate dehydrogenase), ferricyanide (NADH dehydrogenase), and phenazine methosulfate or 2,6-dichlorophenol indophenol (lactate dehydrogenase and glycerol phosphate dehydrogenase).

(ii) *Fumarate reductase*. The enzyme has been shown to be an iron-sulfur flavoprotein with a covalently bound flavine adenine dinucleotide (FAD) as the prosthetic group (335, 338). The FAD is linked at the position 8α to histidine. Thus the dissimilatory fumarate reductase is similar to the assimilatory fumarate reductase of yeast (658) and to the catabolic succinate dehydrogenase of mitochondria. The enzyme can be tested with reduced viologen dyes or reduced free flavins as electron donor. Activities between 40 nmol/min per mg of particulate protein (*S. faecalis* [167]) and 2,000 nmol/min per mg of membrane protein (*V. succinogenes* [335]) are found in cells grown under conditions of fumarate respiration. The fumarate-reducing system is not constitutive in most of the bacteria. Yet in *D. gigas* high activities of fumarate reductase are also found in lactate-plus-sulfate-grown cells (47). The growth medium contained yeast extract, however, indicating that fumarate reductase might have been induced by a component of the yeast extract, e.g., aspartate or malate. The finding that fumarate reductase activity is increased considerably when the cells are grown on a fumarate medium (231) is consistent with this view.

(iii) *Electron carriers*. The membrane fraction of all bacteria capable of using fumarate as electron acceptor of energy metabolism contains a naphthoquinone, usually menaquinone (Table 9). Between 1 μ mol (*S. faecalis* [51]) and 15 μ mol (*V. succinogenes* [335]) are found per g of particle-bound proteins. The mitochondria of helminths capable of dissimilatory fumarate reduction contain rhodoquinone (426, 554) rather than ubiquinone. Menaquinone appears to be a necessary redox mediator for dissimilatory fumarate reduction in bacteria as evidenced by (i) the presence of the quinone, (ii) the reduction of menaquinone by specific electron donors and its reoxidation by fumarate, and (iii) extraction reactivation experiments (note that in most of the bacteria listed in Table 9 the presence of menaquinone is the only indication that it might be involved in dissimilatory fumarate reduction). The specificity for menaquinone can be explained by the different redox potentials of

the lipid-soluble electron carriers (250a, 340). Menaquinone ($E_0' = -74$ mV) (569, 685) has a redox potential more negative than the succinate/fumarate couple ($E_0' = +33$ mV); ubiquinone ($E_0' = +113$ mV) (569) has a more positive one. The reduction of fumarate with ubiquinone is therefore thermodynamically unfavorable. In agreement with this interpretation is the finding that ubiquinone rather than menaquinone is involved in succinate oxidation to fumarate. The redox potential of rhodoquinone has not been reported. It should be more negative than that of ubiquinone if the above explanation for the involvement of menaquinone rather than ubiquinone in dissimilatory fumarate reduction is correct. The finding that menaquinone is specifically involved in nitrate reduction ($\text{NO}_3^-/\text{NO}_2^-$; $E_0' = +433$ mV) in gram-positive and ubiquinone in gram-negative bacteria indicates that the specificity for menaquinone in fumarate reduction might have other than thermodynamic reasons. For a discussion of the role of menaquinone and fumarate reductase in pyrimidine biosynthesis, see the review by Cox and Gibson (119).

b-Type cytochromes are usually present in the membrane fraction containing the fumarate reductase activity (Table 9). Between 0.1 μ mol (*C. formicoaceticum*) [193]) and 2 μ mol (*V. succinogenes* [335]) are found per g of membrane protein (for a list of cytochrome *b* contents of different bacteria see DeVries et al. [145]). A consistent view of the functional organization of the cytochromes with respect to fumarate reduction is lacking. In *P. rettgeri* the reduction of menaquinone by formate rather than by NADH appears to proceed via cytochrome *b* (340). In *Bacillus megaterium* evidence is available that the reduction of fumarate by reduced menaquinone involves a *b*-type cytochrome (337). In *P. shermanii*, the *b*-type cytochrome is assumed to be situated in the oxygen-linked chain of the electron transport system rather than in the chain linked to fumarate reduction (583). In *V. succinogenes*, two *b*-type cytochromes appear to be involved in fumarate reduction with formate, one (cytochrome *b*; $E_m = -200$ mV) being directly linked to formate dehydrogenase and one (cytochrome *b*; $E_m = -20$ mV) being linked to the fumarate reductase (335, 336, 338, 339). In *E. coli*, the reduction of fumarate with glycerolphosphate and NADH has recently been shown to occur in a cytochromeless mutant (597, 598), indicating that a cytochrome must not obligatorily be involved in fumarate reduction. This is also shown by the finding that in *S. faecalis*, which does not contain cytochromes (see, however, Ritchey and Seeley [532]), fumarate is reduced

with NADH, a reaction which might be coupled with phosphorylation (167). It is important to note that the rate of fumarate reduction in *S. faecalis* and in the cytochromeless mutant of *E. coli* is much lower (40 nmol/min per mg of protein with NADH) than usually found in bacteria containing cytochromes (300 nmol/min per mg of protein) and that the rate of growth of the cytochromeless mutant of *E. coli* on glycerol-fumarate medium is lower by a factor of three than the wild type (597). Assuming the fumarate reductase limits growth, this finding indicates that *b*-type cytochromes are essential for high fumarate-reducing activities.

HQNO and 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide (NQNO) (369) have been shown to inhibit fumarate reduction in most of the bacteria listed in Table 9, which has been interpreted to indicate that a *b*-type cytochrome is involved in fumarate reduction. It is of interest that HQNO inhibits NADH-fumarate oxidoreductase in the cytochromeless mutant of *E. coli* (597), since this clearly demonstrates that cytochrome *b* is not necessarily the site of action of this compound. This agrees with the previous suggestion that the site of action of HQNO is close to the site of quinone in the respiratory chain (229, 337; see also Cox et al. [120]).

(iv) *Topography*. A tentative scheme of the topography of fumarate reduction with formate in *V. succinogenes* was recently published by Kröger (335). A modified version of the scheme (336) assumes that the formate dehydrogenase is localized on the outer aspect of the cytoplasmic membrane and the fumarate reductase is located on the inner side. The main experimental evidence for the suggested topology is that impermeant electron donors fail to be oxidized by fumarate in the presence of intact cells, whereas impermeant electron acceptors are rapidly reduced by formate under these conditions. Thus dissimilatory fumarate reduction with formate as electron donor appears to be a transmembrane redox process in *V. succinogenes* as does the dissimilatory reduction of sulfate with H₂ or formate in sulfate-reducing bacteria. For tentative schemes of the topography of fumarate reduction in *E. coli*, see Hadcock and Jones (207).

According to Kröger's scheme (336), two protons are formed on the outer side and two protons are consumed on the inner side of the cytoplasmic membrane of *V. succinogenes* during the reduction of 1 mol of fumarate with formate (see Fig. 3, scheme D). The rate of acidification of the medium containing intact cells was shown to be in the same order of magnitude as that of fumarate reduction. Ratios of H⁺/fumarate up to 1.6 were found (335). Proton

ejection was abolished by uncoupling agents and dependent on the presence of potassium ions in the medium but it was not stimulated by valinomycin (335). Konings and Kaback (327) recently reported that the NADH and the formate-dependent fumarate reduction in *E. coli* are associated with the generation of a transmembrane pH gradient. Thus the formation of a pH gradient coupled to the reduction of fumarate appears to be a general phenomenon.

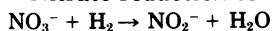
Phosphorylation coupled to dissimilatory fumarate reduction: (i) in vivo evidence. *V. succinogenes* (715) and *E. coli* (382) can grow on H₂ and fumarate as sole energy sources. Succinate appears to be the only end product formed (382, 715). Fumarate reduction must be coupled with phosphorylation in these organisms as evidenced by growth. An indirect demonstration of phosphorylation was recently provided by Kröger et al. (341). They showed that *P. rettgeri* can grow on fumarate as sole energy and carbon source (341), and that 7 mol of fumarate is disproportionated to 6 mol of succinate and 4 mol of bicarbonate in the energy metabolism of this facultative anaerobe. A 5.5-g amount of cells (dry weight) was obtained per mol of succinate formed. This growth yield can only be explained if it is assumed that the reduction of fumarate is coupled with phosphorylation. Growth yield studies with *V. succinogenes* (Kröger, personal communication) on fumarate plus formate and with *Desulfuromonas acetoxidans* (488) on acetate plus fumarate indicate that approximately 4 g (dry weight) of cells is formed per mol of fumarate reduced. The Y_{ATP}^{max} of cells growing on fumarate as sole carbon source is 15.4 (Table 7). Since the cells grow only slowly, the Y_{ATP} value is expected to be smaller than 10 (see section, Energy-consuming processes and the ATP requirement for bacterial growth). Whether the Y_{ATP} value is as small as 4 (and thus 1 mol of ATP formed per mol of fumarate is reduced) cannot be decided at present. (For growth yield on fumarate and succinate see Bongers [60].)

(ii) *In vitro evidence*. In spheroplasts of *V. succinogenes*, the phosphorylation of ADP and ATP was shown to be dependent on the reduction of fumarate to succinate (335). The rate of phosphorylation was approximately equal to that of fumarate reduction. Phosphorylation was inhibited by CCCP. Barton et al. (47, 48) demonstrated phosphorylation coupled to fumarate reduction in cell-free extracts of *D. gigas*. The particulate fraction was shown to catalyze the reduction of fumarate with H₂ and lactate, with the concomitant esterification of orthophosphate. The system exhibited a P/H₂ ratio of 0.3 to 0.4 and was uncoupled by gramicidin,

pentachlorophenol, dinitrophenol, and methyl viologen. Both fumarate reduction and ATP synthesis were inhibited by HQNO. (For an explanation of the uncoupling effect of methyl viologen see subsection, CO₂ reduction to methane.)

The oxidation of NADH by fumarate in digitonin fragments of beef heart mitochondria has been shown to be coupled with phosphorylation ($P/2 e^- = 0.7$ to 0.8) (206). Vertebrates physiologically cannot use fumarate as terminal electron acceptor. The finding does show, however, that ATP synthesis can be coupled to the reduction of fumarate under non-physiological conditions (high concentrations of both NADH and fumarate) and suggests that the fumarate-reducing system in the parasitic helminths (Table 9) may have evolved from Complex I plus Complex II of the respiratory chain of aerobic eucaryotes.

Nitrate reduction to nitrite.



$$\Delta G^{\circ\prime} = -39 \text{ kcal/mol} \\ (-163.2 \text{ kJ/mol})$$

Nitrate respiration is a widespread property of facultative anaerobic bacteria, and even in strictly anaerobic bacteria such as *Veillonella alicalescens* (263), *Selenomonas ruminantium* (145), and *Clostridium perfringens* (265) nitrate may serve as terminal electron acceptor of energy metabolism (Table 10; for a detailed list see the review by Payne [473]). In most of the organisms, nitrate is reduced no further than nitrite. In a limited number of bacteria, however, nitrite is reduced either to N₂ or NH₃. Nitrate reduction to nitrite will be discussed in this section; the reduction of nitrite to N₂ will be discussed in the following section. The reduction of nitrite to NH₃ will not be dealt with, as this six-electron redox process appears not to be coupled with phosphorylation (209). Recent reviews on nitrate respiration are by Kröger (336), by Haddock and Jones (207), and by Stouthamer (627).

Enzymes and electron carriers involved in dissimilatory nitrate reduction. Dissimilatory nitrate reduction to nitrite is catalyzed by a membrane-associated electron transport system consisting of dehydrogenases, electron carriers, and nitrate reductase (Pichinoty type A) (Fig. 9) (for reviews, see Payne [473], Pichinoty [491], and Stouthamer [627]). Respiratory nitrate reductases of *Spirillum itersoni* (183) and of *C. perfringens* (107) are found in the soluble cell fraction. In the case of *S. itersoni*, this may indicate unusual fragility of the spirillar membrane rather than non-membrane localization. In the case of *C. perfringens*, it may be a reflec-

tion of the finding that nitrate reduction is not coupled with phosphorylation in this organism (227, 265).

(i) *Dehydrogenases.* NADH, succinate, lactate, formate, glycerolphosphate, and hydrogen have been shown to reduce nitrate to nitrite via membrane-bound dehydrogenases linked via electron carriers (a quinone and a cytochrome) to dissimilatory nitrate reductase (Table 10). In enteric bacteria grown on carbohydrates, NADH and formate appear to be the preferred reductants. When *E. coli* is grown, however, on glycerol, succinate, or lactate plus nitrate, the membrane fraction contains an active succinate dehydrogenase, glycerolphosphate dehydrogenase, and lactate dehydrogenase, respectively, which mediate electron transport from succinate, glycerolphosphate, or lactate to nitrate (180, 323, 411). The formate dehydrogenase of *E. coli* is the dehydrogenase best investigated. It is a molybdoprotein containing selenium (162, 164, 363, 593) with a molecular weight of 590,000 (164). The electron acceptor appears to be a cytochrome *b* (cyt *b*_{FDH}, Fig. 9) (164, 267). The nitrate-inducible-type formate dehydrogenase is distinct from the enzyme associated with the formate hydrogen-lyase system of enteric bacteria (132, 133, 553, 552).

(ii) *Nitrate reductase A (EC 1.7.99.4).* The particle-bound nitrate reductase has a respiratory function in every bacterium that produces it. It is capable of reducing chlorate and bromate as well as nitrate and is very sensitive to the inhibition by azide. According to Pichinoty (490), this enzyme is designated nitrate reductase A. This is to discriminate it from the assimilatory nitrate reductase, which is found in the soluble cell fraction, and is designated enzyme B. Enzyme B is not capable of reducing chlorate (it is, in fact, inhibited by it), and is much less sensitive to the inhibition by azide (for reviews, see Payne [473], Pichinoty [491], and Stouthamer [627]). Nitrate reductase A has been purified from *E. coli* (164, 174, 380, 381, 592, 636), *Micrococcus denitrificans* (173), *Aerobacter aerogenes* (675, 676, 678), *Pseudomonas aeruginosa* (170), and *Neurospora crassa* (448) (for a detailed list see the review by Stouthamer [627]). They are similar in that they all contain molybdenum and iron, that they are inhibited by azide and inactivated by cyanide, and are not affected by CO (627). The enzyme from *E. coli* is a molybdo-iron-sulfur protein with a molecular weight near 230,000 (monomeric form) (73, 163, 207). It reacts with artificial electron donors such as reduced viologen dyes and FMNH₂ and can be purified as a complex with cytochrome *b*-556 (112), which is

its probable physiological reductant (cyt b_{NR} , Fig. 9).

(iii) *Electron carriers*. Ubiquinone has been demonstrated to be involved in nitrate respira-

tion in gram-negative bacteria (Table 10). Extraction of ubiquinone from the membrane fraction of *E. coli* (163, 266, 267) and of *A. aerogenes* (318, 319) results in loss of capacity for nitrate

TABLE 10. List of some organisms that can use nitrate as electron acceptor of energy metabolism: electron carriers and electron donors involved in dissimilatory nitrate reduction to nitrite, and evidence that nitrate reduction to nitrite is coupled with phosphorylation^a

Organism	Electron carriers found in the cells and assumed to be involved in nitrate reduction		Electron donors involved in nitrate reduction	Inhibition of electron transport by:	ATP synthesis coupled to nitrate reduction as indicated by:
	Quinone	Cytochrome			
Anaerobic bacteria					
<i>Clostridium perfringens</i> ($NO_3^- \rightarrow N_2$)		Ferredoxin (107)	Pyruvate		Nitrate reduction to nitrite is <i>not</i> coupled with phosphorylation (227, 265)
<i>Propionibacterium pentosaceum</i> ($NO_3^- \rightarrow N_2$)	MK (606, 580)	<i>b</i> -type	NADH, lactate, glycerol-phosphate	HQNO (184)	Growth yields on nitrate plus lactate, pyruvate, or glycerol (184)
<i>Selenomonas ruminantium</i> ($NO_3^- \rightarrow NH_3$)		<i>b</i> -type (145)			No evidence
<i>Staphylococcus aureus</i> ($NO_3^- \rightarrow NH_3$)	MK (557)	<i>b</i> -type (89, 103)	Lactate (89)	HQNO (89)	No evidence
<i>Veillonella alcalescens</i> (= <i>Micrococcus lactilyticus</i>) ($NO_3^- \rightarrow NH_3$)	MK (188)	<i>b</i> -type (145)	NADH, lactate, glycerol-phosphate (145)	HQNO	Growth can occur in a medium with H_2 and nitrate as sole energy sources (263); the reduction of nitrate in vesicles is coupled with the transport of amino acids (328); growth yield studies (145)
<i>Vibrio succinogenes</i> ($NO_3^- \rightarrow NH_3$)	MK (335, 338)	<i>b</i> -type (271, 272, 335, 339)	H_2 , formate (450)		Growth on formate plus nitrate as sole energy source (450)
Facultative bacteria					
<i>Aerobacter aerogenes</i> (= <i>Klebsiella aerogenes</i>) ($NO_3^- \rightarrow NH_3$) (175, 675-678)	UQ (318, 319)	<i>b</i> -type (320, 674)	NADH		Growth yields on nitrate (209)
<i>Bacillus steaerothermophilus</i> ($NO_3^- \rightarrow NH_3$) (579)	MK (150)	<i>b</i> -type (309)	NADH, succinate (310)		No evidence
<i>Citrobacter freundii</i> ($NO_3^- \rightarrow NH_3$)					Growth yields on nitrate (301)

TABLE 10—Continued

Organism	Electron carriers found in the cells and assumed to be involved in nitrate reduction		Electron donors involved in nitrate reduction	Inhibition of electron transport by:	ATP synthesis coupled to nitrate reduction as indicated by:
	Quinone	Cytochrome			
<i>Escherichia coli</i> ($\text{NO}_3^- \rightarrow \text{NH}_3$) (65, 112, 174, 180, 181, 208, 267, 306, 380, 381, 592)	UQ (266)	<i>b</i> -type (163, 181, 267, 552)	NADH, lactate, glycerol-phosphate, formate	HQNO (163)	Phosphorylation coupled to the reduction of nitrate with glutamate and citrate in cell-free extracts (459); $\text{P}/2e^- = 0.65$ and 1.1, respectively; the reduction of nitrate in vesicles is coupled with the transport of amino acids (327)
<i>Hyphomicrobium</i> species ($\text{NO}_3^- \rightarrow \text{N}_2$)					Growth on methanol plus nitrate as sole energy sources (611)
<i>Haemophilus parainfluenzae</i> (NO_3^- to NH_3)					
<i>Micrococcus denitrificans</i> ($\text{NO}_3^- \rightarrow \text{N}_2$) (173, 343, 344)	UQ (572)	<i>b</i> -type (278, 556, 572)	NADH, succinate (278, 444)		Growth can occur on H_2 and nitrate as sole energy sources (see 278); phosphorylation coupled to the reduction of nitrate with NADH: $\text{P}/2e^- = 0.9$ (278, 444)
<i>Pseudomonas aeruginosa</i> ($\text{NO}_3^- \rightarrow \text{N}_2$)		<i>b</i> -type, <i>c</i> -type (170)	NADH (170)		Phosphorylation coupled to the reduction of nitrate with NADH in cell-free extracts (444, 732)
<i>Pseudomonas denitrificans</i> ($\text{NO}_3^- \rightarrow \text{N}_2$) (515, 609)					Growth yields on nitrate plus glutamate (324, 325, 444); phosphorylation coupled to the reduction of nitrate with NADH: $\text{P}/2e^- = 0.25$ (457); Growth yields on nitrate (629)
<i>Proteus mirabilis</i> ($\text{NO}_3^- \rightarrow \text{NH}_3$) (132–135)	UQ	<i>b</i> -type	NADH, formate		
<i>Spirillum iterosonii</i> ($\text{NO}_3^- \rightarrow \text{NH}_3$)		<i>b</i> -type			No evidence
<i>Thiobacillus denitrificans</i> ($\text{NO}_3^- \rightarrow \text{N}_2$) (4, 15, 16)		<i>c</i> -type (15, 16)	Sulfide, sulfite (4)		Growth can occur on H_2 and nitrate as sole energy source in strains not containing APS reductase (see 15, 481, 594)

^a Abbreviations: MK, vitamin K₂ (menaquinone-6); UQ-ubiquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

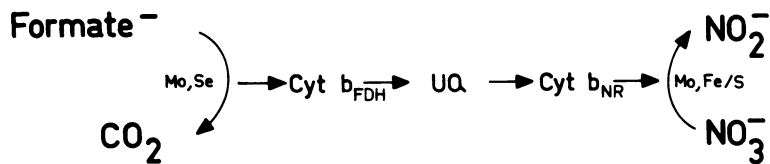


FIG. 9. Scheme of the electron flow from formate to nitrate in *Escherichia coli* (according to Enoch and Lester [163, 164]). Abbreviations: UQ, ubiquinone; Mo, Se, molybdo-seleno-protein; Mo, Fe/S, molybdo-iron-sulfur-protein; Cyt b_{FDH} = cytochrome b of formate dehydrogenase; Cyt b_{NR} = cytochrome b of nitrate reductase.

reduction with physiological electron donors. Addition of ubiquinone restores most of the activity, whereas addition of menaquinone does not. This specificity cannot be satisfactorily explained. In gram-positive bacteria, where ubiquinones are generally absent, dissimilatory nitrate reduction is dependent on naphthoquinones as demonstrated by extraction reactivation experiments (150). In addition, two menaquinone-deficient and one aromatic-deficient mutant of *Staphylococcus aureus* (557) were shown to be unable to reduce nitrate. Reinitiation of menaquinone synthesis in the aromatic-deficient mutant by growing it with shikimic acid restored its nitrate respiratory activity.

All bacteria investigated containing a particle-bound, active nitrate reductase contained at least one b -type cytochrome (Table 10). The b -type cytochrome is reduced by the different physiological electron donors, probably via the quinone, and is reoxidized by nitrate (163) (see also Boxer and Clegg [65]). HQNO and NQNO inhibit nitrate reduction with physiological electron donors (Table 10) (for a discussion of the site of inhibition see subsection, Fumarate reduction to succinate). Mutants of *E. coli* lacking cytochromes synthesize nitrate reductase and incorporate it into the membrane but can neither grow with nitrate as electron acceptor nor reduce nitrate with any of the physiological electron donors, indicating that cytochrome b is an essential component of the nitrate-reducing electron transport system (627).

A cytochrome c (c -552) (179) is produced in high quantity in *E. coli* and *A. aerogenes* growing on nitrate-containing media; its reduced form can be reoxidized by nitrate (195). The physiological significance of this finding is, however, questioned. Cytochrome c -552 is assumed to be associated with nitrite reduction rather than nitrate reduction (115, 117, 149).

(iv) *Topography*. Nitrate appears to be reduced at the outer side of the cytoplasmic membrane rather than at the inner side (for tentative schemes, see Haddock and Jones [207]). This is concluded from the observation that the rate of nitrate entry at 30°C into cells is about 0.1% of that required to support the observed rate of nitrate reduction (180). The localization

of the nitrate-reducing site of nitrate reductase on the outer side was also demonstrated by inhibition studies with azide and distribution studies of nitrate between the intracellular and extracellular space (180). FMNH₂, a nonphysiological reductant in the present context, reacts with nitrate reductase at the inner aspect of the cytoplasmic membrane: in a heme-less mutant of *E. coli* where membrane-bound nitrate reductase was active with artificial electron donors, FMNH₂ failed to be oxidized by nitrate in the presence of intact protoplasts whereas the reaction was rapidly catalyzed by membrane fragments of the mutant (306). FMNH₂ is not assumed to penetrate the plasmic membrane. These findings taken together show that nitrate reductase spans the membrane and can catalyze a vectorial reduction of NO₃⁻ on the outer side of the plasmic membrane with reducing equivalents from the inner (181).

A transmembrane orientation of the cytochrome b -556 nitrate reductase complex in *E. coli* with cytochrome b and nitrate reductase on the opposite faces of the cytoplasmic membrane has also been shown by labeling experiments with ¹²⁵J (lactoperoxidase/H₂O₂ method) (65). The subunits of nitrate reductase become labeled in inside-out vesicles but not in protoplasts. Cytochrome b is significantly labeled only in protoplasts and is not labeled to a corresponding degree in inside-out vesicles (181).

The reduction of nitrate to nitrite has been shown to be associated with proton translocation in *E. coli* (61, 208, 327). Observed stoichiometries (H⁺/NO₃⁻) for spheroplasts of *E. coli* grown anaerobically in the presence of nitrate were approximately 4 for malate oxidation and approximately 2 for succinate, D-lactate, and glycerol. The H⁺/NO₃⁻ ratio for formate oxidation was found to be greater than 2 (180).

A possible arrangement for the cytochrome b nitrate reductase complex in the cytoplasmic membrane of *E. coli* explaining the above finding was recently published by Garland et al. (181).

Suspensions of *E. coli* with high nitrite reductase activity release cytochrome c -552 when incubated with lysozyme and ethylenediamine-

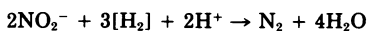
tetraacetate (178). Although the spheroplasts so obtained retained full NADH-nitrite oxidoreductase activity (assimilatory nitrite reductase), the glucose-supported rate of nitrite reduction (dissimilatory nitrite reductase) was considerably reduced. Full activity was restored when spheroplasts and cell washing were combined (691). This finding indicates that dissimilatory nitrite reductase in *E. coli* is accessible from the outside and suggests that nitrite might be reduced on the outer aspect of the cytoplasmic membrane.

The hypothesis was recently advanced that the reduction of Fe^{3+} by growing cultures of many bacteria is mediated by the dissimilatory nitrate reductase (462, 463). The finding that the nitrate-reducing site of nitrate reductase is on the outer aspect of the plasmic membrane is interesting in this respect. Fe^{3+} ions very probably cannot enter the cells and must therefore be reduced outside.

Phosphorylation coupled to dissimilatory nitrate reduction: (i) in vivo evidence. *V. alcalescens* has been reported to grow on hydrogen and nitrate as sole energy source (263). Under these conditions, nitrate is reduced only to nitrite. Thus, nitrate reduction to nitrite must be coupled with phosphorylation in this anaerobic bacterium. Molar growth yields of a variety of bacteria grown anaerobically with nitrate as terminal electron acceptor indicate that phosphorylation coupled to nitrate reduction is a general phenomenon in those bacteria, which contain a particle-bound nitrate reductase (Pichinoty type A) and in which a cytochrome *b* and a quinone participate in electron transport to nitrate (Table 10).

(ii) In vitro evidence. Esterification of orthophosphate concomitant with reduction of nitrate to nitrite has been demonstrated in cell-free yet still vesicle-containing preparations of *E. coli* (459), *M. denitrificans* (278, 444), and *P. aeruginosa* (444, 732). DNP inhibited phosphorylation and increased the basal rate of electron transport (278). $P/2e^-$ ratios with NADH of 0.9 (278), with glutamate of 0.65 (459), and with citrate of 1.1 (459) as electron donor were observed.

Nitrite reduction to N_2 .



$$\Delta G^{\circ\prime} = -190 \text{ kcal/mol} \\ (-795 \text{ kJ/mol})$$

A limited number of bacteria can reduce nitrate to elemental nitrogen by a series of anaerobic respiratory processes that, in sum, are called denitrification (for review, see Payne [473], and Pichinoty [491]). Nitrite, nitric oxide, and nitrous oxide have been shown to be free

intermediates (40, 140, 392, 393, 427, 428, 429, 474, 527). *Alcaligenes odorans* does not reduce nitrate but reduces nitrite (104), and *Corynebacterium nephridii* reduces nitrate to nitrous oxide but no further (225, 527). Supplied with unrestricted quantities, nitrous oxide supports good growth of *Pseudomonas denitrificans* (324, 389), *Pseudomonas stutzeri* (12), and *M. denitrificans* (470, 493). Most denitrifiers are nonfermentative facultative bacteria which can grow anaerobically only if supplied with an inorganic substitute for O_2 . The only anaerobe in which denitrification has been observed is *Propionibacterium pentosaceum* (184). Nitrate reduction to nitrite in denitrifying bacteria is linked with phosphorylation (see Table 10). Nitrite reduction to nitrogen is also coupled with phosphorylation, as will be discussed in this section.

Enzymes and electron carriers involved in nitrite reduction to N_2 . Denitrifying bacteria growing at the expense of nitrate- and nitrite-reduction release nitrogen but none of the nitrogenous intermediates, whereas both nitric oxide and nitrous oxide are produced and reduced in anaerobic incubation mixtures containing cell-free extracts of nitrate- or nitrite-grown cells (40). In most organisms, both the soluble and the particulate fraction are required for nitrite reduction to N_2 . The reduction process is catalyzed by an electron transport system involving specific dehydrogenases, electron carriers, and three reductases, i.e., nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Fig. 10).

(i) Dehydrogenases. In cell-free extracts of denitrifying bacteria, reduction of nitrite, nitric oxide, and nitrous oxide was observed with NADH, succinate, and lactate (121, 278, 343, 444, 474) via membrane-bound NADH dehydrogenase, succinate dehydrogenase, and lactate dehydrogenase (425, 470). When solubilized

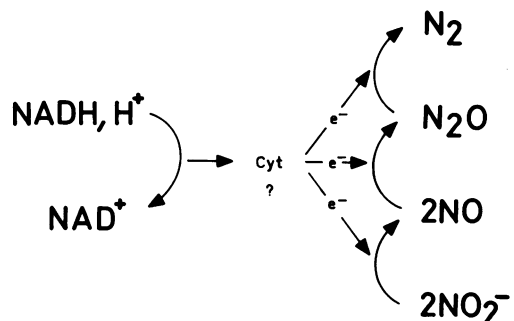


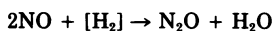
FIG. 10. Scheme of the electron transport system involved in nitrite reduction to N_2 (according to Payne [473]). Symbols: $-e^- \rightarrow$, electron transport chain, components are unknown; cyt, cytochrome.

with deoxycholate, artificial electron carriers such as viologen dyes or free flavins must be added for the reaction to proceed (514). None of the dehydrogenases have been characterized; their physiological electron acceptors are not known. Free flavins, viologen dyes, dichlorophenol indophenol, and phenazine methosulfate can be used as artificial electron acceptors (121, 470, 514). Reduction of nitrite to N_2 in cell-free extracts with malate, pyruvate, and glutamate (444) probably proceed via NADH, the reduction of which is catalyzed by soluble NAD-dependent dehydrogenases (278, 427). Sulfite, sulfide, and thiosulfate are utilizable electron donors in the obligatory chemolithotrophic bacterium *Thiobacillus denitrificans*, which can reduce nitrate to nitrite, nitric oxide, nitrous oxide, and N_2 (4, 15, 16).

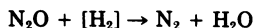
(ii) *Reductases*. It is generally accepted that nitrite reduction to N_2 proceeds via nitric oxide and nitrous oxide.



$$\Delta G^\circ = -35.1 \text{ kcal/mol} \\ (-147.0 \text{ kJ/mol})$$



$$\Delta G^\circ = -73.2 \text{ kcal/mol} \\ (-306.1 \text{ kJ/mol})$$



$$\Delta G^\circ = -81.6 \text{ kcal/mol} \\ (-341.4 \text{ kJ/mol})$$

(H_2 , NO, NO_2 , and N_2 in the gaseous state)

Nitrite reduction to nitric oxide is catalyzed by nitrite reductase (268, 427, 428, 514, 686). The enzyme from *Alcaligenes faecalis* (269), *P. aeruginosa* (730, 731), and *M. denitrificans* (446, 343, 344) was purified and characterized to be a type of cytochrome *cd*. The nitrite reductase from *Achromobacter fischeri* is a *c*-type heme protein which, however, reduces nitrite to NH_3 rather than to nitric oxide (503, 504). The enzyme from *Achromobacter cycloclastes* appears to contain copper (270). There is no evidence that these dissimilatory nitrate reductases contain siroheme as the prosthetic group as do the assimilatory type enzymes (440, 680). Nitrite reductase is generally found in the soluble cell fraction (121, 391). The physiological electron donor is not known. The enzyme can be tested with phenazine methosulfate/ascorbate or reduced viologen dyes (514) as electron-donating systems (392).

The reduction of nitric oxide to nitrous oxide is mediated by nitric oxide reductase (121, 391, 425, 474). The enzyme has not yet been characterized. It is found in the soluble cell fraction of *Pseudomonas perfectomarinus* (121) and is particle bound in *A. faecalis* (391) and in *P. deni-*

trificans (428). Nitric oxide-reducing activity is exhibited by nitrite reductase of *A. faecalis*. It has been established, however, that nitrite reductase and nitric oxide reductase are two distinct enzymes (392). The physiological electron donor of the dissimilatory nitrite reductases are not known; $FADH_2$, reduced phenazine methosulfate, and viologens dyes are effective artificial electron donors (121, 392, 514, 515).

The reduction of N_2O to N_2 is catalyzed by nitrous oxide reductase (121, 388–391, 393, 474). This enzyme is always found in the particulate cell fraction, is very labile, and has not yet been characterized. The enzyme is inhibited by CO (389), and by cyanide and azide (390), which indicates that a transition metal is probably involved in N_2O reduction to N_2 . The physiological electron donor of nitrous oxide reductase is not known. The enzyme can be tested with $FADH_2$, viologen dyes, or dichlorophenol indophenol as artificial electron donors.

(iii) *Electron carriers*. The electron carriers linking the dehydrogenase reactions to the reductase reactions have not been elucidated. Denitrifying bacteria contain *b*- and *c*-type cytochromes and quinones, either menaquinone (gram-positive bacteria) or ubiquinone (gram-negative bacteria). Indirect evidence is available indicating that a *c*-type cytochrome is involved in nitrite reduction (121, 269) and *b*- and *c*-type cytochromes are involved in N_2O reduction to N_2 (389, 390). A *c*-type cytochrome is tightly associated with the membrane fraction containing nitric oxide reductase in *P. perfectomarinus* (474). Whether these cytochromes are the physiological electron carriers remains to be demonstrated. The quinones have been shown to be obligatorily involved in dissimilatory nitrate reduction to nitrite (see subsection, Nitrate reduction to nitrite). There is no indication, however, whether they also participate in nitrite reduction to N_2 .

(iv) *Topography*. A *c*-type cytochrome, which is probably involved in nitrite reduction to N_2 , is localized in the periplasmic space of *M. denitrificans* (573). The significance of this finding is not understood (see sections, Sulfate reduction to sulfide and Nitrate reduction to nitrite).

Phosphorylation coupled to nitrite reduction to N_2 : (i) in vivo evidence. The molar growth yields of *P. denitrificans* for nitrate, nitrite, and nitrous oxide were recently determined in chemostat culture under electron acceptor-limited conditions. The molar growth yields corrected for maintenance energy were shown to be 28.6 g/mol of nitrate, 16.9 g/mol of nitrite, and 8.8 g/mol of nitrous oxide. The energy yield, expressed on an electron basis, was pro-

portional to the oxidation state of the nitrogen. It was concluded that oxidative phosphorylation occurs to a similar extent in each of the electron transport chains associated with the reduction of nitrate to nitrite, nitrite to nitrous oxide, and nitrous oxide to N_2 (324, 325).

(ii) *In vitro* evidence. The *in vivo* evidence indicates that phosphorylation is coupled with each of the three reactions involved in nitrate reduction to N_2 (325). This finding is incompatible with the view presented by Cox and Payne (121), who found that nitrite and nitric oxide reductases of *P. perfectomarinus* are solubilizable, but nitrate and nitrous oxide reductases are particle bound. They inferred from this observation that, throughout the whole process of denitrification, only two steps—the reduction of nitrate and of nitrous oxide—function as energy-yielding systems. Naik and Nicholas (444) did not observe in experiments with ^{32}P that nitrite reduction and nitrous oxide reduction in cell-free extracts of *P. denitrificans* are coupled with phosphorylation. On the other hand, the occurrence of phosphorylation coupled to nitrite reduction has been observed in the Iwasaki strain of *P. denitrificans* (458) and in *M. denitrificans* (444).

(iii) *Thermodynamic and mechanistic considerations*. Theoretically, each of the three reactions involved in nitrite reduction to N_2 could be coupled with phosphorylation. The free energy change of the reactions is large enough to allow the synthesis of at least one ATP. The reduction of nitrite to nitric oxide is a one-electron redox process and therefore deserves special consideration. With the possible exception of sulfite reduction to sulfide in sulfate-reducing bacteria, phosphorylation is always coupled to two-electron redox processes, both in chemotrophic and in phototrophic organisms. This argument does not exclude, however, that nitrite reduction to nitric oxide is not a possible site of energy conservation. The general mechanism of ETP proposed by Mitchell (415) (see section, ATP synthesis via ETP) requires that the electron transport chain be arranged as a series of alternating hydrogen and electron carriers so positioned in the membrane to form loops that proton translocation is an inevitable consequence of the transfer of reducing equivalents from substrates to the terminal electron acceptor (Fig. 3). The free energy change of the redox process is thus conserved in the resulting pH gradient and membrane potential (proton-motive force). The membrane-associated ATPase is then poised by the proton-motive force and the mechanism is such that, per mole of ATP formed, either two, three, or four protons are consumed. If this is the way it works, then

redox processes with any number of electrons involved can be coupled with phosphorylation provided that the free energy change of the redox reaction per proton (=per electron equivalent) is negative enough.

Hydrogenation Reactions Possibly Coupled with Phosphorylation

A number of reductive processes have been postulated to be coupled with phosphorylation. Direct evidence, however, is not available. The reactions will be discussed in the order shown in Table 11.

Note that the free energy yields for the transfer of one electron equivalent from H_2 to CO_2 and S^0 are very small, smaller than for CO_2 reduction to methane which is -3.9 kcal per electron equivalent. Reactions 1 to 4 are catalyzed by strictly anaerobic bacteria, reactions 5 to 6 are usually catalyzed by facultative organisms.

CO_2 reduction to acetate.



$$\Delta G^{0'} = -22.7 \text{ kcal/mol} \\ (-95 \text{ kJ/mol})$$

(CO_2 and H_2 in the gaseous state)

Clostridium acetivum has been reported to grow on H_2 and CO_2 as energy source and to produce acetate rather than CH_4 as an end product (304, 703, 704). The species has been lost. Attempts to reisolate the sporeforming anaerobic bacillus were without success (21, 158) until recently (383a). Nonsporeforming anaerobic bacteria mediating a total synthesis of acetate from CO_2 and H_2 were isolated in the

TABLE 11. Reductive processes postulated to be coupled with phosphorylation

Reductive Process	$-\Delta G^{0'a}$	
	kcal/electron equivalent from H_2	kJ/electron equivalent from H_2
1. CO_2 reduction to acetate	2.8	11.7
2. S^0 reduction to HS^-	3.3	13.9
3. Glycine reduction to acetate	9.3	38.9
4. Acrylyl CoA reduction to propionyl CoA	9.3	38.9
5. Tetrathionate reduction to thiosulfate	10.1	42.3
6. Fe^{3+} reduction to Fe^{2+}	27.3	114.2

^a $G^{0'}$ for the different reactions were calculated from $\Delta G_f^{0'}$ values given in Table 15 (CO_2 in the gaseous state, all other substances in aqueous solution).

laboratories of Wolfe and of Zeikus (personal communications). Biochemical data on this new bacterium are not yet available. The fact that the organisms grow on CO₂ and H₂ as sole energy source indicates, however, that the reduction of CO₂ to acetate should be coupled with phosphorylation.

Clostridium thermacetivum (360), *C. formicoaceticum*, *C. acidurici*, *C. cylindrosporium*, *Butyribacterium rettgeri*, and *Diplococcus glycinophilus* also perform a total synthesis of acetate from CO₂. This was recently confirmed by mass analysis of the different types of acetate formed from ¹³CO₂ (578). These organisms differ from *C. aceticum*, however, in that they are devoid of hydrogenase and thus cannot use H₂ as electron donor for CO₂ reduction. The reactions and enzymes involved in CO₂ reduction to acetate have been studied extensively with *C. thermoaceticum* and *C. formicoaceticum*. They will be discussed in this subsection. For the earlier literature, the reader is referred to the review by Ljungdahl and Wood (377).

Enzymes and electron carriers involved in CO₂ reduction to acetate. *C. thermoaceticum* and *C. formicoaceticum* ferment hexoses to 3 mol of acetate, one of which is formed via total synthesis from CO₂. CO₂ reduction to acetate can be demonstrated in cell-free extracts using pyruvate as electron donor (24, 187). ATP is required for the reaction to proceed. The evidence available indicates that CO₂ reduction to acetate is catalyzed by a solubilizable electron transport system involving dehydrogenases, electron carriers, and four reductases.

(i) *Dehydrogenases.* Glucose is oxidized to 2 mol of acetate and 2 mol of CO₂ in the electron-donating partial reaction. Cell-free extracts of the two organisms contain an NAD-specific glyceraldehyde phosphate dehydrogenase and a ferredoxin-specific pyruvate dehydrogenase (19, 24, 373, 643). Both enzymes are found in the soluble cell fraction (see also Andreesen and Gottschalk [20]).

(ii) *Reductases.* The reduction of CO₂ to acetate is assumed to proceed via free formate, formyltetrahydrofolate, methenyltetrahydrofolate, methylenetetrahydrofolate, and methyltetrahydrofolate (187, 377, 468). All of the enzymes required for this sequence have been demonstrated in extracts of *C. thermoaceticum* (24) and *C. formicoaceticum* (453). The reduction of CO₂ to formate in *C. thermoaceticum* is catalyzed by an NADP-specific formate dehydrogenase (22, 23, 368, 376, 643; see also Thauer [644]). A formate dehydrogenase activity can be demonstrated in cell-free extracts of *C. formicoaceticum* using methylviologen as electron acceptor for the oxidation of formate. The phys-

iological electron donor for this enzyme is not known (19). Methenyltetrahydrofolate is formed from formate via formyltetrahydrofolate synthetase and methenyltetrahydrofolate cyclohydrolase (24, 378, 631). Thus 1 mol of ATP is required per mol of CO₂ reduced to acetate. The reduction of methenyltetrahydrofolate to methylenetetrahydrofolate is mediated by an NADP-specific methylenetetrahydrofolate dehydrogenase in *C. thermoaceticum* (454) and an NAD-specific enzyme in *C. formicoaceticum* (432). The electron donor for methylenetetrahydrofolate reductase, which mediates the reduction of methylenetetrahydrofolate to methyltetrahydrofolate, is not known (468). Reduced free flavins can be used to test the reductase. The formation of acetate from methyltetrahydrofolate plus CO₂ is the last step in the sequence of reactions. It is catalyzed by a corrinoid enzyme (187) and requires two electrons. The electron donor has not been elucidated. Recently, evidence has been presented that the carboxyl group of acetate is derived from the carboxyl group of pyruvate rather than from CO₂ (577). The mechanism of this reaction is not yet understood.

(iii) *Electron carriers.* Cell-free extracts of *C. thermoaceticum* and of *C. formicoaceticum* contain ferredoxin (see Thauer et al. [645]), flavodoxin (368), cytochromes, and menaquinones (193). In *C. thermoaceticum* the reduction of CO₂ to the oxidation level of methylene-FH₄ requires 2 mol of NADPH. NADH and reduced ferredoxin rather than NADPH are regenerated in the electron-donating partial process of the energy metabolism. The electron carriers and enzymes involved in transhydrogenation have not been elucidated. The finding that the two clostridia contain cytochromes of the *b* type (0.19 μmol/g of particle protein) and menaquinone (0.3 μmol/g of particulate protein) is of special interest. The two electron carriers are found in *C. formicoaceticum* only in considerable amounts when the organism is grown on formate plus fumarate as energy source (193). Under these conditions, succinate and CO₂ rather than acetate are formed, indicating that the *b*-type cytochrome and menaquinone are involved in fumarate reduction rather than in the total synthesis of acetate from CO₂. In *C. thermoaceticum*, however, which cannot use fumarate as electron acceptor, and which is devoid of fumarate reductase, cytochromes and menaquinone are also found in glucose-grown cells. Their presence is not understood.

Phosphorylation coupled to the reduction of CO₂ to acetate. CO₂ reduction to acetate must be coupled with phosphorylation in those organisms which can grow on CO₂ and H₂ as sole

energy source, i.e., *C. aceticum* and the non-sporeforming bacteria recently isolated in the laboratories of Wolfe and Zeikus (personal communications). Growth yields of *C. thermoaceticum* on glucose have been reported to be between 40 and 50 g (dry weight) per mol of glucose, suggesting that between 4 and 5 mol of ATP is formed in the homoacetate fermentation of glucose (24). This value is thermodynamically reasonable as the formation of 3 mol of acetate from 1 mol of glucose is an exergonic reaction with a ΔG° of -74.3 kcal/mol (-310.9 kJ/mol). Two of the four to five high-energy phosphates are formed via SLP in the glyceraldehydephosphate dehydrogenase reaction (643) and another two are formed in the acetokinase reaction (562). One mole of ATP is, however, required for the activation of formate to formyltetrahydrofolate. Thus, the formation of 1 to 2 mol of ATP cannot be explained via SLP. It is assumed to be generated via ETP (24). The finding that *C. thermoaceticum* contains cytochrome *b* and menaquinone is in line with this interpretation (193). *C. formicoaceticum* can grow on fumarate and formate as sole energy source, with the concomitant formation of succinate and CO_2 . This clearly indicates that this organism in principle can derive useful energy from hydrogenation reactions via ETP (193).

The reduction of CO_2 to the redox level of formaldehyde (methylenetetrahydrofolate) can be excluded as the possible site of phosphorylation during acetate formation from CO_2 . Both formate dehydrogenase (368) and methylenetetrahydrofolate dehydrogenase (454) in *C. thermoaceticum* are soluble enzymes using NADPH as electron donor; an electron transport chain is not involved. This leaves the reduction of methylenetetrahydrofolate to acetate as the sequence possibly coupled with phosphorylation. The free energy change of this process is sufficient to allow the synthesis of 1 or even 2 mol of ATP (ΔG° = -29.1 kcal/mol [-121.6 kJ/mol]). The physiological electron donors for the reactions are not known.

Elemental sulfur reduction to sulfide.



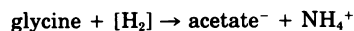
Pfennig and Biebl (488) recently described a new genus and species, *Desulfuromonas acetoxidans*, which obtains energy for growth by oxidizing acetate or ethanol to CO_2 with elemental sulfur as electron acceptor. The organism is devoid of hydrogenase. Organic sulfides, cysteine and oxidized glutathione, can substitute for elemental sulfur as oxidant. From growth yield studies (Y -acetate = 4.21; Y -ethanol = 9.77), it was concluded that

sulfur reduction to sulfide is linked with phosphorylation. The enzymes and electron carriers involved in acetate or ethanol oxidation to CO_2 and in sulfur reduction to sulfide have not yet been elucidated. The cells contain large amounts of cytochrome *c*-551.5 (506a), which has also been found in a *Chloropseudomonas* culture (14a). The findings that *Desulfuromonas* can also grow using fumarate instead of sulfur as electron acceptor and that fumarate-grown cells contain cytochrome *b* suggest that a naphthoquinone may also be present in the new genus (see subsection, Fumarate reduction to succinate).

The most surprising fact about *D. acetoxidans* is its ability to grow under anaerobic conditions with acetate as the sole carbon source and electron donor and with elemental sulfur as sole electron acceptor. Under standard conditions, the free energy change of the reaction is not negative enough to drive the synthesis of 1 mol of ATP from ADP and P_i ($\text{CH}_3\text{COO}^- + 2\text{H}_2\text{O} + 4\text{S}^0 \rightarrow 2\text{CO}_2$ (gaseous) + $4\text{HS}^- + 3\text{H}^+$; ΔG° = -4.0 kcal/mol [-16.7 kJ/mol]). However, taking physiological conditions into account ($\text{HS}^- = 10^{-2}$ M), the free energy change is sufficient for the synthesis of 1 mol of ATP ($\Delta G' = -14.8$ kcal/mol [-61.9 kJ/mol]).

An oxidation of acetate to CO_2 via the tricarboxylic acid cycle in *D. acetoxidans* does not appear to be likely. The redox potential of the S^0/SH^- couple ($E_0' = -270$ mV) is not positive enough to allow the oxidation of succinate to fumarate ($E_0' = +33$ mV), despite the fact that activities of all enzymes of the tricarboxylic acid cycle can be demonstrated in cell-free extracts of the organism.

Glycine reduction to acetate.



$$\Delta G^{\circ} = -18.6 \text{ kcal/mol} \\ (-77.8 \text{ kJ/mol})$$

Clostridium sticklandii and many other clostridia (for a list see a review by Barker [42]) ferment alanine plus two glycine to three acetate, 1 CO_2 , and 3 NH_3 ($\Delta G_0' = -36.6$ kcal/mol [-153.1 kJ/mol]). Alanine is oxidized via pyruvate to acetate and CO_2 , whereby 1 mol of ATP is synthesized via SLP in the acetate kinase reaction (for a scheme see Decker et al. [129]). The electrons liberated in this reaction (NADH, reduced ferredoxin) are used to reduce 2 mol of glycine to 2 mol of acetate and NH_3 . It is assumed that additional ATP is formed in this reaction via ETP (614). Growth yield studies supporting this assumption are not available. *C. sticklandii* is devoid of hydrogenase.

Cell-free extracts of *C. sticklandii* and *Clostridium lentoputrescens* have been reported to

catalyze the reduction of glycine with NAD(P)H as electron donor (615). The reaction has been reported to be coupled with phosphorylation (615, 621, 624). $P/2e^-$ ratios up to 1 were observed (624). Note, however, that in the absence of glycine considerable amounts of ATP were formed (615). Thus, the phosphorylation data must be regarded with reserve. The components of the electron transport system involved have been partially purified. An NADH dehydrogenase (617, 618), ferredoxin (617), a selenoprotein (protein A) (621, 663), and at least two membrane-associated proteins (663) are involved in glycine reduction with NADH. NADH, NADH-dehydrogenase, and ferredoxin can be omitted from the system if either reduced methyl viologen or dimercaptans such as 1,4-dithiothreitol or 1,3-dimercaptopropanol are used as electron donors. An essential component of the system is destroyed when the cell-free extracts are irradiated at 366 nm (614, 615). Furthermore, glycine reduction is inhibited by 2-methyl-1,4-naphthoquinone, indicating that a quinone may be part of the electron transport chain (614).

Acrylyl CoA reduction to propionyl CoA.

acrylyl CoA + $[H_2] \rightarrow$ propionyl CoA

$$\Delta G^{\circ} = -18.5 \text{ kcal/mol} \\ (-77.4 \text{ kJ/mol})$$

Propionate is the end product of fermentation in many anaerobic bacteria. In most bacteria, e.g., propionic acid bacteria (139, 280), *V. alcalescens* (281), and *S. ruminantium* (475), propionate is formed from lactate via pyruvate, oxaloacetate, malate, fumarate, succinate, succinyl CoA, methyl malonyl CoA, and propionyl CoA (fumarate pathway) (11, 192). In at least three bacteria, *Clostridium propionicum* (100, 279), *Peptostreptococcus elsdenii* (366), and *Bacteroides ruminicola* (687), propionate is formed from lactate via lactyl CoA, acrylyl CoA, and propionyl CoA (acrylate pathway [18]). The mechanism of acrylyl CoA formation from lactyl CoA is unknown. Whether phospholactyl CoA is an intermediate is still a matter of controversy (566). The reduction of fumarate to succinate has been shown to be coupled with phosphorylation in many bacteria, including propionic acid bacteria (see Table 9). On the basis of theoretical considerations and the finding that the reduction of lactate to propionate is inhibited by low concentrations of dinitrophenol (342), Anderson and Wood (18) suggested that *P. elsdenii* may be able to synthesize ATP by electron transport-mediated phosphorylation. The redox potential of the propionyl CoA-acrylyl CoA couple is positive enough ($E_0' = -15 \text{ mV}$ [232]) to allow the oxidation by acrylyl

CoA of lactate ($E_0' = -190 \text{ mV}$) and NADH ($E_0' = -320 \text{ mV}$) and to couple these reactions with the synthesis of ATP. But so far it has not been possible to demonstrate unequivocally the coupling of phosphorylation to acrylyl CoA reduction.

P. elsdenii is an obligate anaerobic bacterium which ferments D,L-lactate essentially to acetate, propionate, butyrate, and valerate (159). The bacterium appears to be devoid of cytochromes but has been reported to contain menaquinone (188). The soluble cell fraction contains ferredoxin (36), flavodoxin (395, 397), rubredoxin (396), electron-transferring flavoproteins (77, 700, 701), and all the enzymes required for propionate formation from lactate, including an NAD-independent D-lactate dehydrogenase (78), a pyruvate ferredoxin oxidoreductase (36, 483), and butyryl CoA dehydrogenase (160, 161). Electron transfer from lactate to acrylyl CoA has been shown to proceed via lactate dehydrogenase, an electron-transferring flavoprotein containing an unusual flavin (ETF-lactate) and butyryl CoA dehydrogenase (77). The electron transport from pyruvate to acrylyl CoA proceeds via ferredoxin and NAD.

NADH reduces acrylyl CoA in a reaction requiring butyryl CoA dehydrogenase and another protein fraction (36), which has recently been purified and shown to be an FAD-containing, electron-transferring flavoprotein (ETF-NADH) (700, 701).

The observation that all the enzymes and electron carriers involved in acrylyl CoA reduction are found in the soluble cell fraction does not support the suggestion that this reaction is coupled with phosphorylation in *P. elsdenii*. The analysis of the components indicates that the acrylyl CoA-reducing system is more similar to the crotonyl CoA-reducing system of butyric acid-forming clostridia than to the fumarate-reducing system of propionic acid-forming bacteria containing the fumarate pathway. The reduction of crotonyl CoA to butyryl CoA in *C. kluyveri* has been shown not to be associated with phosphorylation (129, 650).

Tetrathionate reduction to thiosulfate.

$S_4O_6^{2-} + [H_2] \rightarrow 2S_2O_3^{2-} + 2H^+$

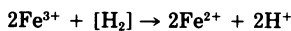
$$\Delta G^{\circ} = -20.2 \text{ kcal/mol} \\ (-84.5 \text{ kJ/mol})$$

Pollock and co-workers (497) discovered that some facultative anaerobic bacteria reduce tetrathionate to thiosulfate (496) and suggested (321) that this bacterial activity may be a form of anaerobic respiration analogous to the reduction of nitrate to nitrite. Indeed, tetrathionate reduction appears to be almost as widespread as nitrate reduction among facultative anaer-

obes (301, 361, 467, 662, 664). The reduction of tetrathionate is catalyzed by a membrane-bound electron transport system consisting of dehydrogenases, electron carrier(s), and tetrathionate reductase (134, 302, 361, 362, 492). Tetrathionate reductase has been shown to be an inducible enzyme, whose synthesis and activity is inhibited by oxygen (302). Little attention has been paid to the energetics of tetrathionate reduction. The reaction appears to be coupled with phosphorylation in *Citrobacter* as indicated by growth yield studies (301; see also Tuttle and Jannasch [664]). On the other hand, DeGroot and Stouthamer (134) showed that tetrathionate in *Proteus mirabilis* did not have a significant influence on anaerobic growth (whether tetrathionate was reduced by the strain in considerable amounts, was, however, not reported).

The reduction of tetrathionate to thiosulfate ($E_0' = +24$ mV) (Table 5) is a reaction very similar to the reduction of trithionate to sulfite and thiosulfate. The latter reaction is discussed as an intermediary step in sulfite reduction to sulfide in sulfate-reducing bacteria and is assumed, on the basis of thermodynamic considerations to be coupled with phosphorylation (see subsection, Sulfate reduction to sulfide). Tetrathionate has been shown to be an excellent electron acceptor for sulfate-reducing bacteria (499). It is not known, however, whether trithionate and tetrathionate reduction in sulfate-reducing bacteria are catalyzed by one enzyme. It will be interesting to see whether the tetrathionate-reducing system in facultative anaerobes is similar to the trithionate system in the obligately anaerobic sulfate-reducing bacteria.

Ferric iron reduction to ferrous iron.



$$\Delta G^{\circ} = -54.6 \text{ kcal/mol} \\ (-228.3 \text{ kJ/mol})$$

The ability to reduce ferric iron to the soluble ferrous state has been observed among a great number of bacteria belonging to the *Enterobacteriaceae*, *Bacillaceae*, and *Pseudomonadaceae* (212, 460-463, 535). This property has not received much attention during the last few decades, although it should be considered of great ecological significance for the iron cycle in nature. The hypothesis that nitrate reductase A (dissimilatory nitrate reductase which reduces chlorate) is responsible for the reduction of ferric iron in most bacteria has recently been advanced (462, 463). Consistent with this notion is the finding that the ability to reduce ferric iron is correlated with the presence of nitrate reductase in many bacteria. Nitrate reductase-

less mutants of several iron-reducing bacteria were shown to reduce very little iron and it was observed that the simultaneous presence of nitrate and ferric iron decreases the amount of iron reduced, possibly by competition (462, 463). Ferric iron thus appears to be an alternative substrate for dissimilatory nitrate reductase. As dissimilatory nitrate reduction to nitrite is coupled with phosphorylation, the reduction of ferric iron to ferrous iron may be also. The redox potential of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple ($E_0' = +772$ mV) (Table 5) makes an ETP with all of the known physiological electron donors thermodynamically feasible.

TRANSPORT OF ENERGY SUBSTRATES AND PRODUCTS, AND THE PROTON-MOTIVE FORCE

In all catabolic processes the first and the last steps are of course the translocations of the substrates into, and of the end products out of, the cell. At first glance one could suspect that both steps may be "passive": high concentrations of substrate are offered—especially with unicellular organisms held in laboratory culture—and high concentrations of products accumulate, so that both substrates and products may pass by facilitated diffusion with their concentration gradients over the membrane barrier. Intensive investigations on *substrate uptake* by microorganisms, that have been critically reviewed (62, 213, 219, 298, 595) have revealed, however, that this process is not passive at all (in contrast to, for example, glucose uptake by erythrocytes). This surely reflects the fact that, in the natural habitat, substrate concentrations may be very low so that "active" accumulation within the cell should be required for an effective catabolism. Studies on *end product excretion* appear to be scarce. This might be due to the fact that, in the most widely studied aerobic respiration, the end product is CO_2 which can leave the cell by nonionic diffusion. In anaerobic fermentations, however, the situation is quite different; lactic, acetic, butyric, or formic acids are the major end products. The disposition of lactic acid was clarified in an investigation that will be discussed below (220).

Substrate Accumulation

The active translocations of substrates and electrolytes, which have been repeatedly reviewed (62, 213, 416, 595), may be divided into two major classes—primary translocations and secondary translocations (416).

Primary translocations are energized directly by *chemical* reactions involving the breaking and making of covalent bonds (416).

There are two subclasses. In *solute modification* (group translocation), the substrate is transported by being chemically modified (595). The best studied example is the phosphotransferase system, which catalyzes the conversion of glucose and other sugars with the energy-rich phosphoenolpyruvate to glucose-6-phosphate plus pyruvate (542). This transport system has been found in several facultative and anaerobic bacteria, including photosynthetic organisms, whereas it appears to be absent from strict aerobes (62). In *carrier modification*, the solute is accumulated unaltered, whereas the carrier is chemically modified so that it has differential solute-binding properties (595). Examples are the ion-specific ATPases, particularly the Na^+/K^+ -ATPase of animal plasma membranes and the Ca^{2+} -ATPase from sarcoplasmic reticulum; this type of transport has so far not been found in prokaryotes.

Secondary translocations are not energized directly by the chemical reactions of metabolism but by the *physical* energy of a chemiosmotic potential (chemiosmotic transport) (416). The solute is translocated as such without chemical modification; this transport is driven in bacteria by a proton-motive force consisting of a chemical (ΔpH , interior alkaline) and an electrical ($\Delta \psi$, interior negative) potential difference, and in animal cells by a sodium-motive force (190) composed of a sodium gradient (ΔpNa , interior low sodium) and a membrane potential ($\Delta \psi$, interior negative). Three subclasses may be distinguished. In *symport* processes the translocation of one solute is coupled to the translocation of the other in the same direction (416). Proton-symport is most common in bacteria; uncharged species such as galactose or neutral amino acids are taken up electrogenically by utilizing both the ΔpH and $\Delta \psi$ components, and negatively charged species such as gluconate, glutamate, or H_2PO_4^- are absorbed electroneutrally by employing the ΔpH component only. In *antiport* reactions the translocation of one solute is coupled to the translocation of the other in the opposite direction (416). Sodium extrusion is accomplished in prokaryotes via the electroneutral H^+/Na^+ -antiport utilizing the ΔpH component. In *uniport* processes the translocation of a solute is not coupled to the translocation of another solute (416). Positively charged species such as lysine and potassium are accumulated electrogenically by employing the $\Delta \psi$ component (213) (Fig. 11).

Aerobic and facultative organisms can generate the proton-motive force by the redox reactions of the electron transport chain. Proton extrusion was observed in *M. denitrificans* (570, 571) and in *E. coli* vesicles (524), and the electrical potential (interior negative) was

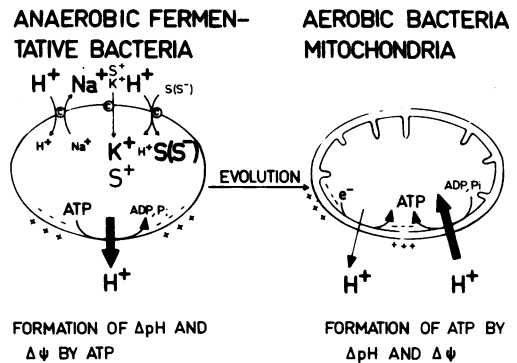


FIG. 11. *Substrate accumulation and ion transport as processes dependent on an electrogenic H^+ -translocating ATPase in anaerobic fermentative bacteria; hypothetical change of the H^+ -translocating ATPase to an H^+ -retranslocating ATP synthase during evolution. Symbols: ΔpH , pH gradient (interior alkaline); $\Delta \psi$, electrical potential difference (interior negative); S , neutral substrate; S^- , anionic substrate; S^+ , positively charged substrate; e^- , electron transport in respiratory chain generating ΔpH and $\Delta \psi$; C , carrier.*

measured in *E. coli* cells by the distribution of permeant cations to be -140 mV (197, 198) and in *E. coli* vesicles to be -100 mV (13, 244). Accumulation of neutral amino acids or sugars occurred in response to an electrical potential generated physiologically by respiration (469) or artificially by induction of electrogenic efflux of K^+ with the aid of K^+ ionophores (244, 245).

Anaerobic fermentative bacteria and facultative organisms in the absence of oxygen generate the proton-motive force via an electrogenic H^+ -translocating ATPase. A transmembrane pH gradient was first observed in glycolyzing, yet non-growing suspensions of *S. faecalis* (224). The cell interior was found, using an acid-base distribution method, to be maintained more alkaline than the surrounding medium by 0.5 to 1.2 pH units, depending on the external pH. Under similar conditions, using a permeant cation distribution method, an electrical potential (interior negative) of -155 to -195 mV was measured (221, 222); fluorescent probes indicated -130 to -140 mV (348). pH gradient and electrical potential difference were sensitive to dicyclohexylcarbodiimide, an ATPase inhibitor, indicating that the proton-motive force was indeed produced by ATP splitting. Accumulation of neutral amino acids was driven physiologically by the ATP-generated proton-motive force or artificially by an electrical potential imposed by valinomycin-induced K^+ efflux; substrate uptake could be blocked by proton-conducting uncouplers (26).

Since *S. faecalis* may be an atypical anaerobe reverted from aerobiosis (702), the question was

to be clarified whether the generation of a proton-motive force by ATPase action was retained as a relict during evolutionary reversion from aerobiosis to anaerobiosis, or whether it is in general an intrinsic property of all procaryotes, thus already occurring in the strictly anaerobic fermentative clostridia, which resemble best in their biological properties (e.g., type of energy metabolism) the earliest forms of microorganisms in evolution (129, 434). It was found, by using an acid-based distribution method with [^{14}C]dimethylloxalidinedione or [^{14}C]acetic acid, that in growing *C. pasteurianum* the intracellular pH was more alkaline than the extracellular pH by 0.4 to 0.8 pH units (529). During growth the extracellular pH decreased from 7.1 to 5.1; simultaneously the intracellular pH changed from 7.5 to 5.9. This pH gradient (interior alkaline) was abolished by a proton conductor and an ATPase inhibitor; it could not be demonstrated in cells depleted of an energy substrate. The pH gradient must therefore be formed by an ATPase-driven extrusion of protons from the cells rather than by a Donnan potential. Growth of the organism was inhibited by low concentrations of both proton conductor or ATPase inhibitor, suggesting that the pH gradient is essential for the growing cell. The results make it appear likely that an ATPase-dependent pH gradient (interior alkaline) and a concomitant electrical potential difference (interior negative) are intrinsic properties of procaryotic cells and as such phylogenetically very old (529). In *C. pasteurianum* suspensions, uptake of galactose and gluconate via inducible transport systems was energized by a proton-motive force generated physiologically by the action of an ATPase or artificially by acidification of the medium from 7.1 to 6.2 (ΔpH component) or by valinomycin-induced K^+ efflux ($\Delta\psi$ component) (63). Galactose accumulation was accomplished by electrogenic H^+ symport motivated only by the pH gradient. The membrane potential, whereas gluconate accumulation occurred by electroneutral H^+ symport motivated only by the pH gradient. The translocation of galactose and of gluconate was inhibited by proton conductors and by an ATPase inhibitor in contrast to the transport of glucose or fructose which is performed via the phosphotransferase system (63, 254). The H^+ -translocating ATPase is a key component for the functioning of the chemiosmotic transport mechanism. The enzyme (695; see also review by Abrams and Smith [2]) has been demonstrated in a number of aerobic and facultative bacteria, in which it should be involved in ETP; it has been thoroughly studied in the atypical anaerobe *S. faecalis*, in which it supports sub-

strate and ion transport (1-3, 46, 567, 568). Recently the enzyme was detected also in the strict anaerobe *C. pasteurianum* (111, 528). The polypeptide composition, the inhibition, and the allotropic properties of the membrane-bound versus the solubilized enzyme point to similarities with the other bacterial and mitochondrial ATPase. The ATP-consuming electrogenic proton translocation appears to be a general prerequisite for the transmembrane movement of substrates and ions and thus for the life of an anaerobic fermentative bacterium.

It has been speculated, therefore, that this phylogenetically old "ATP consumption for the formation of a pH gradient and an electrical potential difference," already realized in strictly anaerobic fermentative bacteria, may have been reversed in the course of evolution to the phylogenetically younger "ATP synthesis by utilization of a pH gradient and an electrical potential difference," probably realized in anaerobic respiratory, anaerobic phototrophic, and aerobic bacteria, and in mitochondria and chloroplasts. As preserved in fermentative anaerobic procaryotes, the proton-motive force had originally the generalized function to link metabolism to transport. In eucaryotes this generalized transport function appears to have been taken over in part by a sodium-motive force (190), since the original proton-motive force was simultaneously specialized on ATP generation and confined to mitochondria and chloroplasts (518, 529) (Fig. 11).

End Product Excretion

The streptococci obtain metabolic energy by conversion of 1 mol of glucose to 2 mol of lactic acid ($\text{pK}_a = 3.86$). Under physiological conditions, lactic acid is present in the cytosol predominantly as the anion lactate, because the intracellular pH is more than 3 units above the pK_a (224). Lactate then may pass across the membrane by electrogenic uniport as the anion alone or by electroneutral symport as the anion plus a proton (or another cation). In cell suspension experiments without external substrate, it was found that the protonated species is translocated, whereas the anion is largely impermeable (220). This was inferred from two lines of evidence. (i) Lactic acid was distributed between cells and medium as a function of the pH gradient between the two compartments; lactate concentration and the pH were higher inside than outside (Fig. 12). (ii) The distribution of lactic acid was affected by the ionophores valinomycin, nigericin, and CCCP in a manner consistent with the transport of the protonated form. The specificity and kinetic properties of the translocation pointed to a carrier-mediated

ANAEROBIC FERMENTATIVE BACTERIA

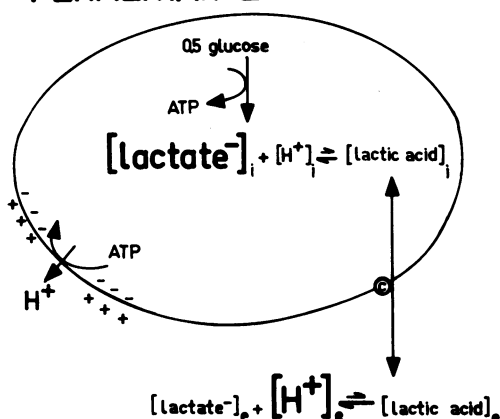


FIG. 12. Lactic acid excretion as a process independent of an electrogenic H^+ -translocating ATPase in anaerobic fermentative bacteria. If (i) the undissociated form of a weak acid passes rapidly and reversibly through the membrane, so that equal concentrations are reached internally and externally, if (ii) the dissociated form is practically nonpermeant, and if (iii) the internal and external dissociation constants are equal, then the distribution of the acid anion becomes a function of the pH gradient between interior and exterior: proton concentration and acid anion concentrations (activities) are inversely proportional, i.e., pH and anion concentrations are proportional. Abbreviations: C, carrier; i, internal; e, external.

$$\begin{aligned}
 [AH]_e &= [AH]_i \\
 K_e &= \frac{[H^+]_e \cdot [A^-]_e}{[AH]_e} = \frac{[H^+]_i \cdot [A^-]_i}{[AH]_i} = K_i \\
 \frac{[H^+]_e}{[H^+]_i} &= \frac{[A^-]_i}{[A^-]_e}
 \end{aligned}$$

process. Extrusion to lower concentrations was independent, uptake to higher concentrations was dependent on the proton-motive force (220). This was in agreement with earlier findings that glycolysis, i.e., lactate extrusion in non-growing cell suspensions, was not inhibited by an uncoupler or an ATPase inhibitor (224).

A similar situation is given for clostridia, in which acetic acid is a major end product. Since in *C. pasteurianum* the pH gradient could also be determined with acetic acid (529), it can be deduced that acetic as well as lactic acid passes through the membrane in the undissociated form, whereas the acetate anion is nonpermeant. Extrusion of acetic acid should therefore also be independent of the proton-motive force.

This independence of lactic or acetic acid excretion from metabolic energy may be surprising. Must not all the protons formed during

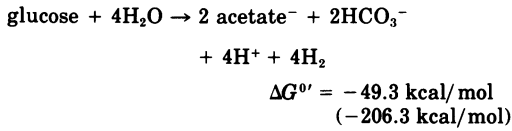
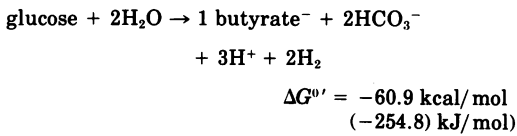
glucose catabolism (Fig. 12) [glucose \rightarrow 2 lactate $^-$ + 2H $^+$ (+ 2 ATP)] [glucose + 4H $_2$ O \rightarrow 2 acetate $^-$ + 2HCO $_3^-$ + 4H $^+$ + 4H $_2$ (+ 4 ATP)] be expelled through the ATPase? On closer inspection it becomes clear that this should not be the case. (i) Any end product, as long as it is formed in the cell, can easily reach concentrations at least slightly higher than those outside the cells, so that it may leave the cell with its concentration gradient provided a suitable membrane carrier is available. The proton-motive force is "accidentally" superposed to this facilitated diffusion process with the effect that the acid anion concentration is maintained higher in the cell than in the medium. If the proton-motive force was to be dissipated, the acid anion concentration would be equal on both sides, but a net extrusion would still be possible. (ii) Physiologically, the end product cannot be excreted by the H $^+$ -translocating ATPase. With one proton formed per ATP generated (see Table 3), the extrusion of end-product protons with ATP would consume all or most of the metabolic energy; such a cell cannot be viable. It thus appears that the excretion of acid end products occurs via a carrier which is not linked to metabolic energy.

REGULATION OF THE THERMODYNAMIC EFFICIENCY OF ATP SYNTHESIS

The catabolic redox processes of many anaerobic bacteria is branched, and thus the ATP and thermodynamic efficiency of ATP synthesis are variable (Fig. 2) (see section, Thermodynamic efficiencies of energy transformation). The fluxes in the different branches are adjusted such that the ATP gain and the thermodynamic efficiency are optimal for the respective growth conditions. The glucose fermentation to butyrate, acetate, CO $_2$, and H $_2$ by saccharolytic clostridia, the ethanol-acetate fermentation of *C. kluveri*, and the glucose fermentation to ethanol, acetate, CO $_2$, and H $_2$ by *Ruminococcus albus* are examples for this kind of regulatory adjustment of the ATP/entropy quotient (= thermodynamic efficiency); they will be discussed in some detail.

Glucose Fermentation of *C. Pasteurianum*

The saccharolytic clostridia derive their energy mainly from the conversion of glucose to butyrate, acetate, CO $_2$, and H $_2$. The fermentation balance of *C. pasteurianum* and also of *C. butyricum* (126, 296) cannot easily be expressed in a simple stoichiometry; the underlying metabolic process is branched rather than linear. It can formally be represented as the weighted sum of two partial sequences (Fig. 13):



Acetyl CoA occupies in the overall fermentation the position of a branch point, which is stoichiometrically coupled to another, the NADH branch point (Fig. 13). Acetyl CoA is either converted to acetyl phosphate by phosphotransacetylase and then to acetate by acetate kinase, yielding 1 mol of ATP per acetyl CoA, or condensed with another mole of acetyl CoA to acetoacetyl CoA, reduced to butyryl CoA, and converted to butyrate via phosphotransbutyrylase and butyrate kinase or thio-phorase, yielding only 0.5 mol of ATP per acetyl CoA. It has been shown that, in *C. pasteurianum*, NADH is formed exclusively by an NAD-specific glyceraldehydephosphate dehydrogenase, whereas during pyruvate dehydrogenation to acetyl CoA and CO₂ electrons are

transferred to ferredoxin (296). In the reaction sequence to butyrate, the NADH generated is quantitatively consumed in butyrate formation and all the reducing equivalents required for H₂ formation can be accounted for by pyruvate dehydrogenation. In the sequence leading to acetate, however, more hydrogen is evolved than is pyruvate oxidized. In this sequence, NADH generated during triose phosphate dehydrogenation should be able to reduce ferredoxin and to give rise to molecular hydrogen. This thermodynamically unfavorable mechanism of H₂ formation (for thermodynamic data see section, Energy Conservation via SLP) has been clearly demonstrated in cell-free lysates of different clostridia (292-296, 486, 651) (for a discussion of the thermodynamic problems associated with H₂ formation from NADH see Jungermann et al. [296] and Decker and Pfitzer[130]).

The following control system of ATP and entropy generation involving both acetyl-CoA and NADH as regulating effectors emerged from the studies of Decker, Jungermann, and Thauer (130, 292-294, 296) (Fig. 13). The conversion of glucose to butyrate, CO₂, and H₂ yields only 3 mol of ATP per mol of glucose with a thermodynamic efficiency of approximately

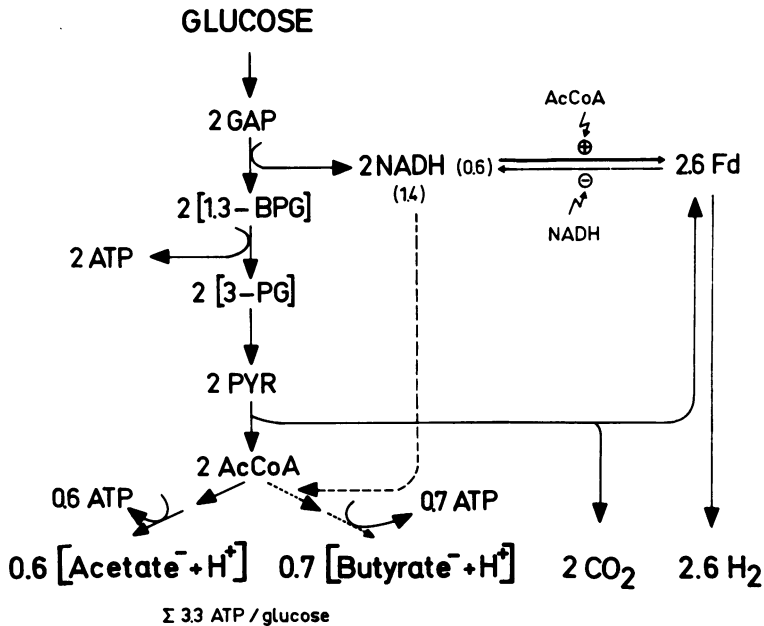
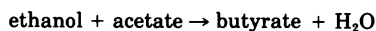


FIG. 13. Variable thermodynamic efficiency in a branched glycolytic fermentation (*Clostridium pasteurianum*). The ATP gain is not stoichiometrically coupled to glucose catabolism; it may be shifted between 3 ATP/glucose, when glucose is fermented solely to butyrate (NADH:ferredoxin oxidoreductase inactive), and 4 ATP/glucose, when glucose is catabolized solely to acetate (NADH:ferredoxin oxidoreductase fully active). The observed ATP gain is 3.3 ATP/glucose. Abbreviations: GAP, glyceraldehydephosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PYR, pyruvate; AcCoA, acetyl coenzyme A; Fd, ferredoxin.

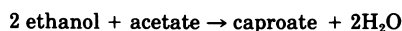
52%; the conversion of glucose to acetate, CO₂, and H₂, however, yields 4 mol of ATP per mol of glucose and is intrinsically connected with H₂ formation via NADH:ferredoxin oxidoreductase (EC 1.6.7.?) plus ferredoxin hydrogenase (EC 1.12.7.1); the NADH:ferredoxin oxidoreductase requires acetyl CoA as obligatory allosteric activator whereas CoA is competitively antagonistic. Thus, the acetyl CoA/CoA quotient regulates H₂ formation from NADH and, concomitantly, the extra ATP generated in the acetate kinase reaction. The thermodynamic efficiency of ATP synthesis in this reaction sequence is 85%. It appears that such a high efficiency is incompatible with the entropy requirements of clostridial metabolism since glucose fermentation solely to acetate, CO₂, and H₂ has never been observed in these anaerobic bacteria. Therefore butyrate is always formed. The acetyl CoA/CoA ratio adjusts the fraction of the acetyl CoA being converted to acetate with mainly ATP generation and to butyrate with mainly entropy generation so that 3.3 mol of ATP is gained with a thermodynamic efficiency of approximately 62%.

Ethanol-Acetate Fermentation of *C. kluyveri*

In the fermentation of *C. kluyveri*, ethanol and acetate are converted to butyrate and caproate:



$$\Delta G^{\circ} = -9.2 \text{ kcal/mol} \\ (-38.6 \text{ kJ/mol})$$



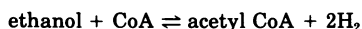
$$\Delta G^{\circ} = -18.5 \text{ kcal/mol} \\ (-77.4 \text{ kJ/mol})$$

Since ETP coupled to enoyl CoA reduction could be definitely excluded (650), the reaction sequences do not include ATP formation and cannot be representative of the total fermentation. The missing partial process was found to be associated with H₂, H⁺, and acetate production (650):

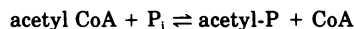


$$\Delta G^{\circ} = +2.3 \text{ kcal/mol} \\ (+9.7 \text{ kJ/mol})$$

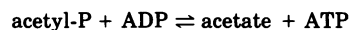
In its course, acetyl CoA is converted to acetyl phosphate which in turn yields acetate and ATP:



$$\Delta G^{\circ} = +10.9 \text{ kcal/mol} \\ (+45.6 \text{ kJ/mol})$$



$$\Delta G_{obs}^{\circ} = +2.2 \text{ kcal/mol} \\ (+9.0 \text{ kJ/mol})$$

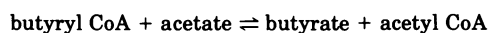


$$\Delta G_{obs}^{\circ} = -3.1 \text{ kcal/mol} \\ (-13 \text{ kJ/mol})$$

[ΔG° (ethanol \rightarrow acetyl CoA) has been calculated from ΔG° values (Table 15) and ΔG_{obs}° for the hydrolysis of acetyl CoA (Table 4); the ΔG_{obs}° values for the phosphotransacetylase reaction and for the acetate kinase reaction have been calculated from ΔG_{obs}° for the hydrolysis of acetyl CoA and acetyl phosphate (Table 4) and ΔG_{obs}° (ATP \rightarrow ADP + P_i) = -7.6 kcal/mol (-31.8 kJ/mol)].

The endergonic formation of acetate, H₂ and H⁺ from ethanol is nonstoichiometrically coupled to the exergonic fatty acid synthesis, *acetyl CoA being the branch point* of the two partial processes. In contrast to the saccharolytic clostridia described in the preceding chapter, both the ATP- and the entropy-producing sequences start after the branch point and, therefore, can be regulated quite independently (Fig. 14). It is a rather unique metabolic situation where one pathway produces entropy and allows the substrate flux necessary to maintain the open system while the route which includes the only SLP of the entire cell metabolism operates at near-equilibrium and must be driven at the expense of fatty acid formation.

The mechanistic coupling of the two partial processes is accomplished by the enzyme acetate CoA transferase (EC 2.8.3.8):



$$G^{\circ} \approx 0 \text{ kcal/mol} \\ (0 \text{ kJ/mol})$$

In the transferase reaction the higher group potential of butyryl CoA built up by the large flux through fatty acid synthesis is transferred to the acetyl CoA pool; the resulting steady-state concentration of "active acetate" is sufficient to allow for the necessary ATP regeneration. In growing *C. kluyveri* cultures, the levels of acetyl CoA and butyryl CoA are about the same (Table 12), indicating that the acetate CoA transferase activity in intact cells is capable of coupling the exergonic and the endergonic part of catabolism. It follows from the observed thermodynamic efficiency of the energy metabolism of *C. kluyveri* (about 27%) that 5 to 6 mol of acetyl CoA must be reduced to medium-chain fatty acids for every mole of ATP regenerated (569a, 642, 650). The necessary ex-

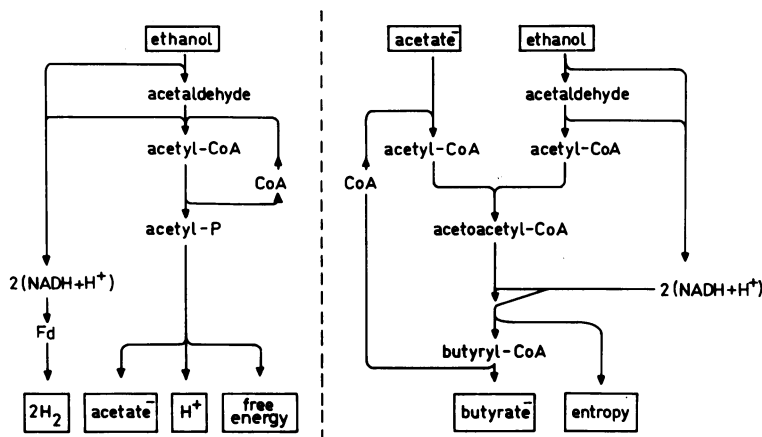


FIG. 14. Endergonic and exergonic pathways of the energy metabolism of *Clostridium kluyveri*.

TABLE 12. Steady-state levels of CoA esters and of pyridine nucleotides in growing *Clostridium kluyveri*^a

CoA ester or pyridine nucleotide	mM ^b	Ratio
CoASH	0.24	
CoAS-SCoA	0.02	
Dephospho-CoA	0.02	
Acetyl CoA	0.92	
Other acyl CoA	0.96	
Total CoA derivatives	2.12	
Acetyl CoA/CoA		3.8
NAD ⁺ ^c	12.56	
NADH ^c	3.36	
NADP ⁺	0.98	
NADPH	1.40	
NADH/NAD ⁺		0.27
NADPH/NADP ⁺		1.42

^a From Decker et al. (130, 131).

^b Calculated from original data (moles per gram of dry cells) assuming 2.5 ml of cytoplasmic space per g of dry cells (see Riebeling et al. [529]).

^c For levels of nicotinamide adenine dinucleotides in *Clostridium welchii* and in facultative bacteria growing under anaerobic and aerobic conditions see Wimpenny and Firth (711) and London and Knight (379).

cess butyrate and caproate synthesis also explains the acetate requirement for growth of this organism (Fig. 14).

Besides its role as a key intermediate and branch point of the carbon flow in the energy metabolism, acetyl CoA plays an important part as allosteric activator of NADH:ferredoxin oxidoreductase. The regulatory properties of the *C. kluyveri* enzyme correspond closely to the one described for saccharolytic clostridia (see above). Since ferredoxin-mediated H₂ formation from reduced pyridine nucleotides is

intrinsically coupled to ATP formation (Fig. 14), any control of ferredoxin reduction also influences the rate of energy transformation. The physiological feasibility of regulation of hydrogen evolution by the acetyl CoA/CoA ratio as studied in *C. kluyveri* (295, 651) and in *C. pasteurianum* (292-294, 296) is supported by the content of CoA derivatives measured in growing *C. kluyveri* cells (Table 12). Assuming an aqueous space of 2.5 ml/g of dry matter (529) and a homogeneous acetyl CoA pool within the cell (642), the concentration of acetyl CoA is 0.92 mM and that of CoA is 0.24 mM. K_A for acetyl CoA activation of NADH:ferredoxin oxidoreductase has been determined as 0.28 mM (295); assuming a K_I for CoASH of 0.25 mM, an acetyl CoA level of about 0.6 mM is required for half-maximal activation of ferredoxin reduction. It appears, therefore, that the actual concentrations of acetyl CoA and CoASH are in a range which allows most efficient regulation of hydrogen production and, consequently, of ATP regeneration.

The ratios of the reduced and the oxidized forms of the pyridine nucleotides (NADH/NAD = 0.27; NADPH/NADP = 1.42) also contribute to the regulation of the energy metabolism by influencing the electron transfer between ethanol dehydrogenation and acetaldehyde dehydrogenation on the one hand and the acetyl CoA reduction to medium-chain fatty acids on the other (297, 652).

The adjustment of ATP regeneration and entropy production, $d(ATP)/TdS$, to a level of optimal thermodynamic efficiency appears to be accomplished in *C. kluyveri* largely by nucleotide ratios. These parameters can be incorporated into a regulatory circuit (Fig. 15) in which $d(ATP)/TdS$ is the term to be controlled (131). The acetyl CoA/CoA ratio functions as a

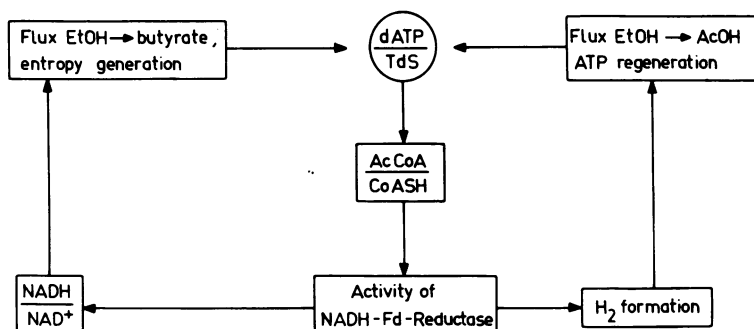


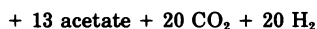
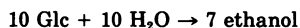
FIG. 15. Nucleotide-dependent regulation of the energy metabolism of *Clostridium kluyveri* (according to Decker et al [131]).

sensor for both ATP regeneration coupled to hydrogen evolution and fatty acid synthesis which provides the necessary entropy to drive the catabolic system. In addition, the acetyl CoA/CoA quotient adjusts the activity of NADH:ferredoxin oxidoreductase which in turn regulates the NADH/NAD system by consumption of NADH and the flux of acetyl CoA towards ATP and entropy production, respectively.

Glucose Fermentation of *R. albus*

Although a great number of rumen microorganisms produces H_2 in monoculture, very little hydrogen gas is evolved during fermentation in the rumen or by nonselectively isolated rumen bacteria in mixed culture. Hungate (256) found the partial pressure of hydrogen in the rumen of the order of 3×10^{-4} atm. This indicates that almost all of the hydrogen produced by H_2 -evolving cells is avidly taken up by other organisms; quantitatively, methanogenic bacteria appear to be the most important H_2 consumers (255). Since interspecies hydrogen transfer efficiently reduces the H_2 concentration and raises the redox potential of the H^+/H_2 couple, saccharolytic anaerobes are no longer obliged to recycle the electrons generated during glycolysis to carbon compounds but are free to produce H_2 from NADH and thereby to generate more ATP per mole of substrate.

A telling example of this kind of metabolic cooperation was given by Bryant, Wolin, and co-workers (83, 262) with a mixed culture of *R. albus* and *V. succinogenes*. In a chemostat monoculture, *R. albus* ferments glucose to ethanol, acetate, CO_2 , and H_2 (262):



Under these conditions ethanol formation traps an appreciable portion of the electrons

generated during the glucose \rightarrow acetyl CoA conversion and prevents concomitantly the ATP regeneration from acetyl CoA (Fig. 16). As with *C. pasteurianum* (Fig. 13), only 3.3 mol of ATP per mol of glucose fermented rather than 4 mol of ATP is formed. *V. succinogenes* cannot use substrates such as glucose, ethanol, or acetate for its energy metabolism but can obtain energy for growth by reducing fumarate with hydrogen to succinate in a cytochrome-dependent reaction (715) and vice versa; *R. albus* does not reduce fumarate. A chemostat coculture of *R. albus* and *V. succinogenes* produced good growth of both organisms but yielded a different pattern of products of the carbohydrate fermentation: ethanol was no longer formed, more acetate accumulated instead and the corresponding electron equivalent were found as succinate; it indicates that *R. albus* produced in coculture a greater amount of H_2 which was taken up by *V. succinogenes* and used for the reduction of fumarate (Fig. 16).

The interspecies cooperation allows *V. succinogenes* to grow at the expense of hydrogen produced by *R. albus*. But it also offers a bonus to the latter in the form of a higher ATP gain (4 mol of ATP instead of 3.3 per mol of glucose fermented [Fig. 16]) and, consequently, of a better growth yield (Y_p). For every acetyl CoA shifted from ethanol to acetate formation, an extra ATP is generated. In the case of the chemostat coculture with *V. succinogenes*, this amounts to an ATP generation of 121% as compared to the *R. albus* monoculture (83).

APPENDIX

List of Dehydrogenation and Hydrogenation Partial Reactions and Their Free Energy Changes

The "dehydrogenation," formally H_2 -forming reactions of energy metabolism are summarized in Table 13. The "hydrogenation," for-

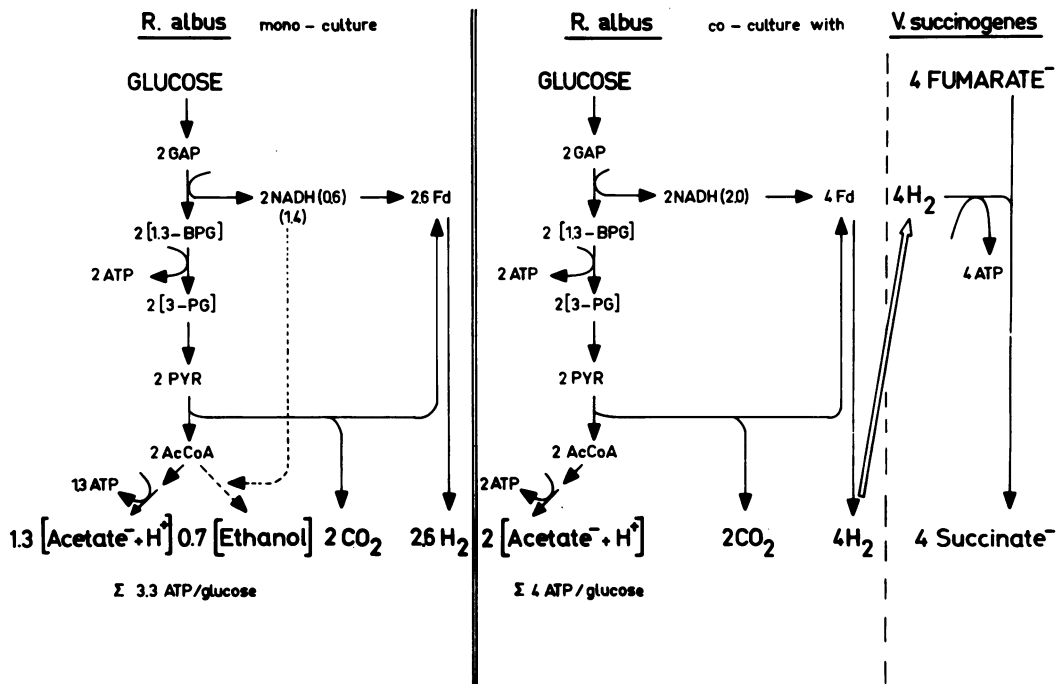


FIG. 16. Variation of the ATP gain in the branched glucose fermentation of *Ruminococcus albus* by interspecies hydrogen transfer to *Vibrio succinogenes* (for comparison see Fig. 13). Abbreviations: GAP, glyceraldehydphosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PYR, pyruvate; AcCoA, acetyl coenzyme A; Fd, ferredoxin.

mally hydrogen-consuming reactions of energy metabolism are included in Table 14 (see section, Energy-providing processes and the synthesis of ATP). All of the energy values given in the tables are expressed in terms of thermochemical calories and of joules. One calorie is equal to 4.1840 joules.

The standard free energy changes associated with the partial reactions of energy metabolism (Tables 13 and 14) have been approximately evaluated from free energy of formation data and the relationship $\Delta G^0 = \Sigma \Delta f^0$ (products) - $\Sigma \Delta f^0$ (substrates). ΔG^0 is the increment of free energy for the reaction under standard conditions, which are 25°C and a pressure of 1 atm. In aqueous solution, the standard condition of all solutes is 1 mol/kg activity, that of water is the pure liquid. ΔGf^0 refers to the standard free energy of formation from the elements of the substrates and the products.

Under physiological conditions the pH is near 7 rather than 1 mol/kg activity (=pH 0). This is considered in $\Delta G^{0'}$ values. $\Delta G^{0'}$ is identical with ΔG^0 except that the standard conditions of H⁺ ion is that of pH 7, and can be calculated using the following equation: $\Delta G^{0'} =$

$\Delta G^0 + m \Delta Gf'(H^+)$ where m is the net number of protons in the reaction (m is negative when more protons are consumed than formed). $\Delta Gf'(H^+)$ is the free energy of formation of a proton at pH 7. It is equal to $2.3 \times RT \log 10^{-7} = -9.534$ kcal/mol, where R is the gas constant ($1.98717 \text{ cal mol}^{-1} T^{-1}$) and T the absolute temperature ($298^\circ\text{K} = 25^\circ\text{C}$).

Under physiological conditions the concentrations of substrates and of products are not 1 mol/kg. This is considered in $\Delta G'$ values, which are calculated using the following equation:

$$\begin{aligned} \Delta G' &= \Delta G^{0'} + RT \ln \frac{[C][D]}{[A][B]} \\ &= \Delta G^{0'} + 1.36 \log \frac{[C][D]}{[A][B]} \end{aligned}$$

where [A] and [B] are the physiological concentrations of the substrates and [C] and [D] are the physiological concentrations of the products ($RT \cdot 2.3$ is 1.42 rather than 1.36 when the temperature is 37°C rather than 25°C).

In cultures of non-H₂-forming bacteria, the concentration ratio of products to exogenous substrates is generally $\geq 10^{-2}$ at the beginning

TABLE 13. Dehydrogenations: electron-donating, formally hydrogen-forming reactions of energy metabolism^a

Equation no.	Substrates ^b	Products ^b	ΔG°		Route ^c
			kcal/reaction	kJ/reaction	
Carboxylic acids					
1	Formate ⁻ + H ₂ O	HCO ₃ ⁻ + H ₂	+0.3	+1.3	
2	Acetate ⁻ + 4H ₂ O	2 HCO ₃ ⁻ + 4H ₂ + H ⁺	+25.0	+104.6	CC
3	2 Acetate ⁻ + 2H ₂ O	1 HCO ₃ ⁻ + pyruvate ⁻ + 3H ₂	+36.3	+151.7	GC
4	Acetate ⁻ + H ₂ O	1 HCO ₃ ⁻ + CH ₄	-7.4	-31.0	
5	Propionate ⁻ + 3H ₂ O	1 HCO ₃ ⁻ + acetate ⁻ + H ⁺ + 3H ₂	+18.2	+76.1	
6	Propionate ⁻ + 7H ₂ O	3 HCO ₃ ⁻ + 2H ⁺ + 7H ₂	+43.3	+181.1	CC/OD
7	Butyrate ⁻ + 10H ₂ O	4 HCO ₃ ⁻ + 3H ⁺ + 10H ₂	+61.5	+257.3	CC
8	Succinate ²⁻ + 8H ₂ O	4 HCO ₃ ⁻ + 2H ⁺ + 7H ₂	+38.3	+160.2	CC/OD
9	Butyrate ⁻ + 2H ₂ O	2 Acetate ⁻ + H ⁺ + 2H ₂	+11.5	+48.1	BO
10	Caproate ⁻ + 4H ₂ O	3 Acetate ⁻ + 2H ⁺ + 4H ₂	+23.0	+96.2	BO
α-Keto acids					
11	Pyruvate ⁻ + 2H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + H ₂	-11.3 ^d	-47.3	OD
12	Pyruvate ⁻ + 6H ₂ O	3 HCO ₃ ⁻ + 2H ⁺ + 5H ₂	+13.7	+57.3	OD/CC
13	Glyoxylate ⁻ + 2H ₂ O	Formate ⁻ + HCO ₃ ⁻ + H ⁺ + H ₂	-8.3	-34.7	OD
14	Glyoxylate ⁻ + 3H ₂ O	2 HCO ₃ ⁻ + H ⁺ + 2H ₂	-8.0	-33.5	OD/GC
15	α -Ketobutyrate ⁻ + 2H ₂ O	Propionate ⁻ + HCO ₃ ⁻ + H ⁺ + H ₂	-11.3	-47.3	OD
16	α -Ketoglutarate ²⁻ + 2H ₂ O	Succinate ²⁻ + HCO ₃ ⁻ + H ⁺ + H ₂	-10.8	-45.2	OD
17	Oxalacetate ²⁻ + 3H ₂ O	Acetate ⁻ + 2HCO ₃ ⁻ + H ⁺ + H ₂	-17.8	-74.5	OD
α, β-Unsaturated acids, hydroxy acids					
18	Lactate ⁻ + 2H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + 2H ₂	-1.0	-4.2	OD
19	Malate ²⁻ + 3H ₂ O	Acetate ⁻ + 2HCO ₃ ⁻ + H ⁺ + 2H ₂	-6.3	-26.4	OD
20	Citrate ³⁻ + 3H ₂ O	Succinate ²⁻ + 2HCO ₃ ⁻ + H ⁺ + 2H ₂	-5.7	-23.8	CC
21	Isocitrate ³⁻ + 3H ₂ O	Succinate ²⁻ + 2HCO ₃ ⁻ + H ⁺ + 2H ₂	-7.3	-30.5	CC
22	β -Hydroxybutyrate ⁻ + H ₂ O	2 Acetate ⁻ + H ⁺ + H ₂	-8.4	-35.1	BO
23	Crotonate ⁻ + 2H ₂ O	2 Acetate ⁻ + H ⁺ + H ₂	-6.4	-26.9	BO
24	Acrylate ⁻ + 3H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + 2H ₂	+0.4	+1.7	OD
25	Fumarate ²⁻ + 4H ₂ O	Acetate ⁻ + 2HCO ₃ ⁻ + H ⁺ + 2H ₂	-7.2	-30.1	CC/OD
26	Glycollate ⁻ + 2H ₂ O	Formate ⁻ + HCO ₃ ⁻ + H ⁺ + 2H ₂	+6.6	+27.6	OD
Aldehydes (aldoses, ketoses)					
27	Formaldehyde + H ₂ O	Formate ⁻ + H ⁺ + H ₂	-5.6	-23.4	
28	Acetaldehyde + H ₂ O	Acetate ⁻ + H ⁺ + H ₂	-7.7	-32.2	
29	Glyceraldehyde + H ₂ O	Glycerate ⁻ + H ⁺ + H ₂	-5.4	-22.6	EM
30	Glyceraldehyde	Pyruvate ⁻ + H ⁺ + H ₂	-18.4	-76.4	EM
31	Glyceraldehyde + 2H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + 2H ⁺ + 2H ₂	-29.5	-123.6	EM/OD

TABLE 13—Continued

Equation no.	Substrates ^b	Products ^b	ΔG°		Route ^c
			kcal/reaction	kJ/reaction	
32	Glyceraldehyde + 6H ₂ O	3HCO ₃ ⁻ + 3H ⁺ + 6H ₂	-4.7	-19.7	EM/CC
33	3 Ribose	5 Pyruvate ⁻ + 5H ⁺ + 5H ₂	-71.9	-300.8	TT/EM
34	Ribose	Acetate ⁻ + pyruvate ⁻ + 2H ⁺ + H ₂	-39.8	-166.5	PK
35	Glucose	2 Pyruvate ⁻ + 2H ⁺ + 2H ₂	-26.8	-112.1	EM
36	Glucose + 4H ₂ O	2 Acetate ⁻ + 2HCO ₃ ⁻ + 4H ⁺ + 4H ₂	-49.3	-206.3	EM/OD
37	Glucose + 2H ₂ O	Acetate ⁻ + pyruvate ⁻ + HCO ₃ ⁻ + 3H ⁺ + 3H ₂	-38.0	-159.0	PK/EM
38	Glucose + 12H ₂ O	6 HCO ₃ ⁻ + 6H ⁺ + 12H ₂	+0.8	+3.2	EM/CC
39	3 Heptose	7 Pyruvate ⁻ + 7H ⁺ + 7H ₂	-88.6	-370.7	TT/EM
40	Gluconate ⁻ + H ₂ O	Acetate ⁻ + pyruvate ⁻ + HCO ₃ ⁻ + 2H ⁺ + 2H ₂	-34.6	-144.9	PK/EM
41	Gluconate ⁻	2 Pyruvate ⁻ + H ⁺ + H ₂ + H ₂ O	-24.1	-100.8	ED
42	3 Gluconate ⁻ + 3H ₂ O	5 Pyruvate ⁻ + 3HCO ₃ ⁻ + 5H ⁺ + 8H ₂	-56.3	-235.7	TT/EM
43	6 Gluconate ⁻	11 Pyruvate ⁻ + 3HCO ₃ ⁻ + 8H ⁺ + 11H ₂	-126.4	-528.9	
Alcohols					
44	Methanol + H ₂ O	Formate ⁻ + H ⁺ + 2H ₂	+5.2	+21.8	
45	Methanol + 2H ₂ O	HCO ₃ ⁻ + H ⁺ + 3H ₂	+5.5	+23.5	
46	Ethanol + H ₂ O	Acetate ⁻ + H ⁺ + 2H ₂	+2.3	+9.6	
47	Ethylene glycol	Acetate ⁻ + H ⁺ + H ₂	-18.8	-78.7	
48	Glycerol	Pyruvate ⁻ + H ⁺ + 2H ₂	-6.2	-25.9	EM
49	Glycerol + 2H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + 2H ⁺ + 3H ₂	-17.5	-73.2	EM/OD
Amino acids					
50	2 Glycine + 4H ₂ O	Acetate ⁻ + 2HCO ₃ ⁻ + H ⁺ + 2NH ₄ ⁺ + 2H ₂	-12.3	-51.5	OD
51	Glutamate ⁻ + 3H ₂ O	2 Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + NH ₄ ⁺ + H ₂	-8.1	-33.9	OD
52	Alanine + 3H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + NH ₄ ⁺ + 2H ₂	+1.8	+7.5	St
53	Leucine + 3H ₂ O	Isovalerate ⁻ + HCO ₃ ⁻ + H ⁺ + NH ₄ ⁺ + 2H ₂	+1.0	+4.2	St
54	Choline ⁺ + H ₂ O	Acetate ⁻ + H ⁺ + (CH ₃) ₃ NH ⁺ + H ₂			
Sulfonium compounds					
55	Propiothetine + 3H ₂ O	Acetate ⁻ + HCO ₃ ⁻ + 2H ⁺ + (CH ₃) ₂ S + 2H ₂ (618)	-10.1	-42.3	OD
Inorganic electron donors					
56	2 NH ₄ ⁺	N ₂ + 2H ⁺ + 3H ₂	+18.8	+78.7	
57	NH ₄ ⁺ + 2H ₂ O	NO ₂ ⁻ + 2H ⁺ + 3H ₂	+104.3	+436.4	
58	NH ₄ ⁺ + 3H ₂ O	NO ₃ ⁻ + 2H ⁺ + 4H ₂	+143.3	+599.6	
59	NO ₂ ⁻ + H ₂ O	NO ₃ ⁻ + H ₂	+39	+163.2	
60	HS ⁻ + H ⁺	S + H ₂	+6.7	+28.8	
61	HS ⁻ + 4H ₂ O	SO ₄ ²⁻ + H ⁺ + 4H ₂	+36.4	+152.2	
62	S + 4H ₂ O	SO ₄ ²⁻ + 2H ⁺ + 3H ₂	+29.7	+124.3	
63	S ₂ O ₃ ²⁻ + 5H ₂ O	2 SO ₄ ²⁻ + 5H ₂	+50.2	+210.0	
64	S ₂ O ₃ ²⁻ + 3H ₂ O	2 SO ₃ ²⁻ + 3H ₂	+60.2	+251.9	
65	2 Fe ²⁺ + 2H ⁺	2 Fe ³⁺ + H ₂	+54.6	+228.5	

^a According to Decker et al. (129). The free energy data have been recalculated using the free energies of formation from the elements listed in Table 15, and are given to the first decimal place, which is, however, not significant in most of the values. The data do not include formation or consumption of ATP.

^b H₂, N₂, CH₄, and C₂H₆ in the gaseous state; all other substances in aqueous solution at 1 mol/kg activity.

^c Abbreviations: EM, Embden-Meyerhof pathway; OD, oxidative decarboxylation; CC, citric acid cycle; GC, glyoxylate cycle; BO, β -oxidation of fatty acids; ED, Entner-Doudoroff pathway; PK, phosphoketolase pathway; TT, transaldolase-transketolase pathway; St, Stickland reaction.

^d In analogy with equation II.

TABLE 14. Hydrogenations: electron-accepting, formally hydrogen-consuming reactions of energy metabolism^a

Equation no.	Substrates ^b	Products ^b	ΔG°	
			kcal/reaction	kJ/reaction
Carboxylic acids				
1	Formate ⁻ + H ⁺ + H ₂	Formaldehyde + H ₂ O	+5.6	+23.4
2	Formate ⁻ + H ⁺ + 2H ₂	Methanol + H ₂ O	-5.2	-21.8
3	Formate ⁻ + H ⁺ + 3H ₂	Methane + 2H ₂ O	-32.1	-134.3
4	Acetate ⁻ + H ⁺ + 2H ₂	Ethanol + H ₂ O	-2.3	-9.6
5	2 Acetate ⁻ + H ⁺ + 2H ₂	Butyrate ⁻ + 2H ₂ O	-11.5	-48.1
6	Acetate ⁻ + propionate ⁻ + H ⁺ + 2H ₂	Valerate ⁻ + 2H ₂ O	-11.5	-48.1
7	Acetate ⁻ + butyrate ⁻ + H ⁺ + 2H ₂	Caproate ⁻ + 2H ₂ O	-11.5	-48.1
8	Butyrate ⁻ + H ⁺ + 2H ₂	Butanol + H ₂ O	-3.9	-16.3
9	Propionate ⁻ + H ⁺ + 2H ₂	Propanol + H ₂ O	-3.0	-12.1
10	2 Acetate ⁻ + 2H ⁺ + 2H ₂	Acetoin + 2H ₂ O (129)	+12.5	+52.3
11	2 Acetate ⁻ + 2H ⁺ + 3H ₂	2,3-Butanediol + 2H ₂ O (129)	+4.0	+16.7
12	2 Acetate ⁻ + H ⁺	Acetone + HCO ₃ ⁻	+7.4	+31.0
13	2 Acetate ⁻ + H ⁺ + H ₂	Isopropanol + HCO ₃ ⁻	+1.4	+5.9
α-Keto acids				
14	Pyruvate ⁻ + H ₂	Lactate ⁻	-10.3	-43.1
15	Pyruvate ⁻ + H ₂ O + H ₂	Ethanol + HCO ₃ ⁻	-13.6	-56.9
16	Pyruvate ⁻ + H ₂	Acrylate ⁻ + H ₂ O	-11.6	-48.7
17	Pyruvate ⁻ + 2H ₂	Propionate + H ₂ O	-29.4	-123.0
18	2 Pyruvate ⁻ + 2H ₂ O + H ₂	2,3-Butanediol + 2HCO ₃ ⁻ (129)	-20.2	-84.5
19	2 Pyruvate ⁻ + H ₂ O + 2H ₂	2,3-Butanediol + HCO ₃ ⁻ + formate ⁻ (129)	-20.4	-85.4
20	2 Pyruvate ⁻ + H ₂ O + 2H ₂	Butanol + 2HCO ₃ ⁻	-38.0	-159.0
21	Pyruvate ⁻ + acetate ⁻ + H ₂	Butyrate ⁻ + HCO ₃ ⁻	-22.8	-95.4
22	Pyruvate ⁻ + HCO ₃ ⁻ + H ₂	Malate ²⁻ + H ₂ O	-5.0	-20.9
23	Pyruvate ⁻ + HCO ₃ ⁻ + H ₂	Fumarate ²⁻ + 2H ₂ O	-4.1	-17.2
24	Pyruvate ⁻ + HCO ₃ ⁻ + 2H ₂	Succinate ²⁻ + 2H ₂ O	-24.6	-102.9
25	Acetoacetate ⁻ + 2H ₂	Butyrate ⁻ + H ₂ O	-23.0	-96.2
26	Oxalacetate ⁻ + 2H ₂	Succinate ²⁻ + H ₂ O	-31.1	-130.1
27	Acetoacetate ⁻ + H ₂	β -Hydroxybutyrate ^{-c}	-7.0	-29.3
α,β-Unsaturated acids, hydroxy acids				
28	Acrylate ⁻ + H ₂	Propionate ⁻	-17.8	-74.5
29	Crotonate ⁻ + H ₂	Butyrate ⁻	-18.0	-75.2
30	Fumarate ²⁻ + H ₂	Succinate ²⁻	-20.6	-86.2
31	Lactate ⁻ + H ₂	Propionate ⁻ + H ₂ O	-19.1	-79.9
32	β -Hydroxybutyrate ⁻ + H ₂	Butyrate ⁻ + H ₂ O	-20.0	-83.7
33	Malate ²⁻ + H ₂	Succinate ²⁻ + H ₂ O	-19.7	-82.4
34	Glycollate ⁻ + H ₂	Acetate ⁻ + H ₂ O	-18.1	-75.7
35	Propiothetine + H ₂	Propionate ⁻ + H ⁺ + (CH ₃) ₂ S (618)	-27.9	-116.7
Aldehydes				
36	Formaldehyde + H ₂	Methanol	-10.7	-44.8
37	Acetaldehyde + H ₂	Ethanol	-10.0	-41.8
38	Acetone + H ₂	Isopropanol	-5.9	-24.7
39	Glyceraldehyde + H ₂	Glycerol	-12.2	-51.0
Alcohols				
40	Methanol + H ₂	Methane + H ₂ O	-26.9	-112.5
41	Ethanol + H ₂	Ethane + H ₂ O	-21.1	-88.3
42	Ethylene glycol + H ₂	Ethanol + H ₂ O	-21.1	-88.3
Amino acids				
43	Glycine + H ₂	Acetate ⁻ + NH ₄ ⁺	-18.6	-77.8
Inorganic electron acceptors				
44	HCO ₃ ⁻ + H ₂	Formate ⁻ + H ₂ O	-0.3	-1.3
45	HCO ₃ ⁻ + 2H ₂ + H ⁺	Formaldehyde + 2H ₂ O	+5.2	+21.8
46	HCO ₃ ⁻ + 3H ₂ + H ⁺	Methanol + 2H ₂ O	-5.5	-23.0
47	HCO ₃ ⁻ + 4H ₂ + H ⁺	Methane + 3H ₂ O	-32.4	-135.6
48	2 HCO ₃ ⁻ + 4H ₂ + H ⁺	Acetate ⁻ + 4H ₂ O	-25	+104.6

TABLE 14—Continued

Equation no.	Substrates ^b	Products ^b	$\Delta G^{0'}$	
			kcal/reaction	kJ/reaction
49	S + H ₂	HS ⁻ + H ⁺	-6.7	-28.0
50	SO ₃ ²⁻ + 2H ⁺ + 2H ₂	S + 3H ₂ O	-34.7	-145.2
51	SO ₃ ²⁻ + 2H ⁺ + 3H ₂	H ₂ S + 3H ₂ O	-41.3	-172.8
52	SO ₄ ²⁻ + H ₂	SO ₃ ²⁻ + H ₂ O	+5.0	+20.9
53	SO ₄ ²⁻ + 2H ⁺ + 3H ₂	S + 4H ₂ O	-29.7	-124.3
54	SO ₄ ²⁻ + H ⁺ + 4H ₂	HS ⁻ + 4H ₂ O	-36.3	-151.9
55	S ₂ O ₃ ²⁻ + 4H ₂	2 HS ⁻ + 3H ₂ O	-41.6	-174.1
56	S ₂ O ₃ ²⁻ + 2H ⁺ + 2H ₂	2 S + 3H ₂ O	-28.3	-118.4
57	3SO ₃ ²⁻ + H ₂ + 4H ⁺	S ₃ O ₆ ²⁻ + 3H ₂ O	-12.0	-50.2
58	S ₂ O ₆ ²⁻ + H ₂	S ₂ O ₃ ²⁻ + SO ₃ ²⁻ + 2H ⁺	-29.1	-121.8
59	S ₂ O ₃ ²⁻ + H ₂	HS ⁻ + SO ₃ ²⁻ + H ⁺	-0.3	-1.1
60	S ₁ O ₆ ²⁻ + H ₂	2 S ₂ O ₃ ²⁻ + 2H ⁺	-20.2	-84.5
61	N ₂ + 2H ⁺ + 3H ₂	2 NH ₄ ⁺	-18.8	-80.0
62	2 NO ₂ ⁻ + 2H ⁺ + 3H ₂	N ₂ + 4H ₂ O	-189.8	-794.1
63	NO ₂ ⁻ + 2H ⁺ + 3H ₂	NH ₄ ⁺ + 2H ₂ O	-104.3	-436.4
64	NO ₃ ⁻ + H ₂	NO ₂ ⁻ + H ₂ O	-39.0	-163.2
65	NO ₃ ⁻ + 2H ⁺ + 4H ₂	NH ₄ ⁺ + 3H ₂ O	-143.3	-599.6
66	2 NO ₃ ⁻ + 2H ⁺ + 5H ₂	N ₂ + 6H ₂ O	-267.8	-1120.5
67	NO ₂ ⁻ + 1/2 H ₂ + H ⁺	NO + H ₂ O	-17.5	-73.2
68	2 NO + H ₂	N ₂ O + H ₂ O	-73.2	-306.3
69	N ₂ O + H ₂	N ₂ + H ₂ O	-81.6	-341.4
70	2 O ₂ + H ₂	2 O ₂ ⁻ + 2 H ⁺	-5.2	-21.9
71	O ₂ + H ₂	H ₂ O ₂	-32.1	-134.3
72	H ₂ O ₂ + H ₂	2H ₂ O	-81.3	-340.2
73	O ₂ + 2H ₂	2H ₂ O	-113.4	-474.5

^a According to Decker et al. (129). The free energy data have been recalculated using the free energies of formation from the elements listed in Table 15, and are given to the first decimal place, which is, however, not significant in most of the values. The data do not include the formation or consumption of ATP.

^b H₂, O₂, CH₄, C₂H₆, N₂, NO, and N₂O are in the gaseous state; all other substances at 1 mol/kg activity.

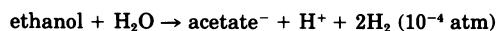
^c See footnote o, Table 15.

of a growth phase and $\leq 10^2$ towards the end. The effectively utilizable energy of a metabolic process $\Delta G'$ thus deviates by at most 2.8 kcal/mol (11.7 kJ/mol) from its standard free energy $\Delta G^{0'}$. It is therefore permissible to use $\Delta G^{0'}$ as the mean of $\Delta G'_{\text{initial}}$ and $\Delta G'_{\text{final}}$ for the assessment of the thermodynamics of the overall process.

In cultures of H₂-forming bacteria the utilizable free energy $\Delta G'$ can, however, differ considerably from $\Delta G^{0'}$. This is the result of the fact that in nature the H₂ partial pressure is generally kept very low ($< 10^{-3}$ atm) by hydrogen-utilizing microorganisms. Thus both $\Delta G'_{\text{initial}}$ and $\Delta G'_{\text{final}}$ are more negative than $\Delta G^{0'}$. An example is the fermentation of the S-organism isolated from *Methanobacterium omelianskii* (84, 519–521):



$$\Delta G^{0'} = +2.3 \text{ kcal/mol} \\ (+9.6 \text{ kJ/mol})$$



$$\Delta G^{0'} = -8.6 \text{ kcal/mol} \\ (-35.9 \text{ kJ/mol})$$

List of Gibbs Free Energies of Formation from the Elements for Compounds of Biological Interest

The standard free energy of formation at present available for compounds of biological interest are listed in Table 15. Where possible, values for aqueous solutions at unit activity are given. Although the first decimal place is not significant in most of the values, two or three decimal places are retained for calculation purposes. It should be recognized in this respect that calculation of free energy changes from standard free energy of formation frequently involves obtaining a small difference between two large numbers. Very small percentage errors in the free energies of formation, therefore, often lead to large percentage errors in the calculation of free energy changes. Consequently, the results of such calculations must be interpreted with reasonable caution.

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TABLE 15. Gibbs free energies of formation from the elements for compounds of biological interest^a

Substance	State	$-\Delta G_f^\circ$ (25°C)		References
		kcal/mol	kJ/mol	
H ₂	g	0	0	683
H ⁺	aq	0	0	683
H ⁺ (pH 7)	aq	9.53	39.87	See text
H ₂ O	liq	56.687	237.178	683
HO ⁻	aq	37.594	157.293	683
H ₂ O ₂	aq	32.05	134.097	683
O ₂ ⁻	aq	[-6.9]	[-28.9]	- ^b
<hr/>				
C graphite	c	0		683
CO	g	32.78	137.15	683
CO ₂	g	94.254	394.359	683
	aq	92.26	386.02	683
H ₂ CO ₃	aq	148.94	623.16	683
HCO ₃ ⁻	aq	140.26	586.85	683
CO ₃ ²⁻	aq	126.17	527.90	683
<hr/>				
Hydrocarbons				
CH ₄	g	12.13	50.75	683
C ₂ H ₆	g	7.86	32.89	683
C ₂ H ₄	g	-16.28	-68.12	683
C ₂ H ₂	g	-50.00	-209.2	683
<hr/>				
Alcohols				
Methanol	aq	41.92	175.39	683
Ethanol	aq	43.44	181.75	683
<i>n</i> -Propanol	aq	42.02	175.81	91
<i>iso</i> -Propanol	aq	44.44	185.94	91, 94
<i>n</i> -Butanol	aq	41.07	171.84	91, 92
Ethylene glycol	liq	77.25	323.21	683
	aq	[79]	[330.5]	618
Glycerol	liq	114.02	477.06	91, 92
	aq	116.76	488.52	91, 92
Mannitol	aq	225.29	942.61	91
Sorbitol	aq	225.31	942.70	91
<hr/>				
Aldehydes:				
Formaldehyde	aq	31.2	130.54	91
	g	27	112.97	683
Acetaldehyde	aq	33.4	139.9	- ^c
	g	30.81	128.91	683
Butyraldehyde	liq	28.6	119.67	303
<hr/>				
Ketones:				
Acetone	aq	38.52	161.17	91, 92
<hr/>				
Monocarboxylic acids				
Formate ⁻	aq	83.9	351.04	683 ⁿ
Acetate ⁻	aq	88.29	369.41	683
Propionate ⁻	aq	[86.3]	[361.08]	618
Butyrate ⁻	aq	84.28	352.63	91, 92
Valerate ⁻	aq	[82.3]	[344.34]	- ^d
Caproate ⁻	aq	[80.3]	[335.96]	- ^d
Palmitic acid	c	72.9	305.0	345
Acrylate ⁻	aq	[68.4]	[286.19]	- ^e
Crotonate ⁻	aq	66.3	277.4	- ^f
Glycollate ⁻	aq	126.9	530.95	91
Lactate ⁻	aq	123.6	517.81	91, 92
β -Hydroxypropionate	aq	123.9	518.4	618
β -Hydroxybutyrate	aq	[121]	[506.3]	91
Glycerate ⁻	aq	[157.3]	[658.1]	- ^g
<i>D</i> -Gluconate ⁻	aq	[269.7]	[1128.3]	- ^h
Glyoxylate ⁻	aq	112.0	468.6	91, 282
Pyruvate ⁻	aq	113.44	474.63	91, 92
β -Ketobutyrate ⁻	aq	118	493.7	91 ^o
<hr/>				
Dicarboxylic-acid				
Oxalate ¹⁻	aq	166.93	698.44	683

TABLE 15—Continued

Substance	State	-Δ <i>G</i> ⁰ (25°C)		References
		kcal/mol	kJ/mol	
Oxalate ²⁻	aq	161.1	674.04	683
Succinic acid	aq	178.39	746.38	91, 92
Succinate ²⁻	aq	164.97	690.23	91, 92
Fumaric acid	aq	154.67	647.14	91, 92
Fumarate ²⁻	aq	144.41	604.21	91, 92
L-Malate ²⁻	aq	201.98	845.08	91, 92
Oxalacetate ²⁻	aq	190.53	797.18	91, 92
α-ketoglutarate	aq	190.62	797.55	91, 92
Tricarboxylic acids				
Citrate ³⁻	aq	279.24	1,168.34	91, 90
Isocitrate ³⁻	aq	277.65	1,161.69	91, 90
cis-Aconitate ³⁻	aq	220.51	922.61	91, 90
Carbohydrates:				
Glyceraldehyde	aq	[104.6]	[437.65]	— ⁱ
Dihydroxyacetone	aq	[106.5]	[445.18]	— ^j
D-Erythrose	aq	[143.]	[598.3]	— ^k
D-Ribose	aq	[181]	[757.3]	— ^k
α-D-Glucose	aq	219.22	917.22	91, 92
α-D-Galactose	aq	220.73	923.53	91
D-Fructose	aq	218.78	915.38	91
D-Heptose	aq	[257]	[1,077]	— ^k
α-Lactose	aq	362.15	1,515.24	91, 92
β-Lactose	aq	375.26	1,570.09	91, 92
β-Maltose	aq	357.80	1,497.04	91, 92
Sucrose	aq	370.90	1,551.85	91, 92
Glycogen (per unit of glucose)	aq	158.3	662.33	91
Amino acids				
L-Alanine	aq	88.8	371.54	92, 260
L-Arginine	c	57.4	240.2	91, 260
L-Asparagine × H ₂ O	aq	182.6	763.998	92, 260
L-Aspartic acid	aq	172.4	721.3	92, 260
L-Aspartate ⁻	aq	167.14	700.4	— ⁱ
L-Cysteine	aq	81.21	339.78	91
L-Cystine	aq	159.4	666.93	260
L-Glutamic acid	aq	173.0	723.8	260
L-Glutamate ⁻	aq	167.2	699.6	— ^m
L-Glutamine	aq	126.6	529.7	260
Glycine	aq	88.618	370.788	683
Glycine ⁺	aq	91.824	384.192	683
Glycine ⁻	aq	75.278	314.963	683
L-Leuine	aq	82.0	343.1	91, 260
L-Isoleucine	aq	82.2	343.9	260
L-Methionine	aq	120.2	502.92	260
L-Phenylalanine	aq	49.5	207.1	260
L-Serine	aq	122.1	510.87	260
L-Threonine	aq	[123]	[514.63]	260
	c	131.5	550.2	
L-Tryptophane	aq	26.9	112.6	260
L-Tyrosine	aq	88.6	370.7	260
L-Valine	aq	85.3	356.9	260
Purines				
Hypoxanthine	aq	-21.4	-89.5	91
Guanine	c	-11.23	-46.99	91
Xanthine	c	39.64	165.85	91
Urate ⁻	aq	77.9	325.9	91
Uric acid	aq	85.3	356.9	91
Other N-containing compounds				
Urea	c	47.04	196.82	683
	aq	48.7	203.76	683
Creatine	aq	63.17	264.30	91

TABLE 15—Continued

Substance	State	-ΔGf° (25°C)		References
		kcal/mol	kJ/mol	
Creatinine	aq	6.91	28.91	91
Allantoin	c	106.62	446.098	91
NH ₂ -CH ₂ -CH ₂ -SO ₃ ⁻	aq	121.76	509.4	683
CH ₃ NH ₃ ⁺	aq	9.55	40.0	683
(CH ₃) ₂ NH ₂ ⁺	aq	0.8	3.3	683
(CH ₃) ₃ NH ⁺	aq	-8.9	-37.2	683
Pyridine		-42.33	-177.1	303
Aromatic compounds				
Benzene		-29.76	-124.5	303
Phenol	c	11.38	47.6	303
		12.45	52.1	
<i>p</i> -Quinone	c	20.0	83.7	303
<i>p</i> -Hydroquinone	c	49.48	207.0	303
Resorcinol	c	50.00	209.2	303
Pyrocatechol	c	50.20	210.0	303
<i>o</i> -Cresol	g	8.86	37.1	303
<i>m</i> -Cresol	g	9.69	40.54	303
<i>p</i> -Cresol	g	7.67	32.09	303
Toluene	liq	-27.30	-114.22	303
Benzoic acid	c	58.7	245.6	303
		59.4	248.5	
<i>o</i> -Hydroxybenzoic acid	c	100.7	421.33	303
<i>m</i> -Hydroxybenzoic acid	c	101.0	422.58	303
<i>p</i> -Hydroxybenzoic acid	c	101.1	423.00	303
Benzyl alcohol	liq	7.47	31.25	303
		6.6	27.6	
N ₂	g	0		683
NH ₃	aq	6.35	26.57	683
NH ₄ ⁺	aq	18.97	79.37	683
NO	g	-20.69	-86.57	683
NO ₂ ⁻	aq	8.9	37.2	683
NO ₃ ⁻	aq	26.61	111.34	683
N ₂ O	g	-24.90	-104.18	683
N ₂ H ₄	aq	-30.6	-128.03	683
ClO ₂ ⁻	aq	-4.1	-17.2	683
ClO ₃ ⁻	aq	0.8	3.35	683
S rhombic	c	0		683
S ²⁻	aq	-20.5	-85.8	683
SH ⁻	aq	-2.88	-12.05	683
SH ₂	g	8.02	33.56	683
	aq	6.66	27.87	683
SO ₃ ²⁻	aq	116.3	486.6	683
HSO ₃ ⁻	aq	126.15	527.81	683
H ₂ SO ₃	aq	128.56	537.895	683
SO ₂	g	71.748	300.194	683
	aq	71.871	300.708	683
SO ₄ ²⁻	aq	177.97	744.63	683
SO ₄ H ⁻	aq	180.69	756.01	683
S ₂ O ₃ ²⁻	aq	122.7	513.4	406
S ₂ O ₄ ²⁻	aq	143.5	600.4	683
HS ₂ O ₄ ⁻	aq	146.9	614.6	683
S ₂ O ₆ ²⁻	aq	229	958.1	349, 546
S ₄ O ₆ ²⁻	aq	244.3	1,022.2	349
H ₂ S ₂ O ₄	aq	147.4	616.7	683
Fe ²⁺	aq	18.85	78.87	684
Fe ³⁺	aq	1.1	4.6	684

^a The table is a revision of the table published by Burton in 1957 (91). It contains the revised free energies of formation for C₁ and C₂ compounds of Technical Note 270-3 of the National Bureau of Standards issued in 1968 (683), and the newly determined values for amino acids of Hutchens of 1970 (260). In addition, this table contains free energies of formation of a series of inorganic compounds previously not thought to be of biological interest. The physical state of each substance is indicated in the column headed "State" as crystalline solid (c), liquid (liq), or gaseous (g). Solutions in water are listed as aqueous (aq). The values in parentheses have been obtained by approximate calculation.

TABLE 15—Continued

- ^b Calculated from E_0 (O_2/O_2^-) = -300 mV (559).
^c Calculated from ΔGf^0 for ethanol and E_0' (acetaldehyde/ethanol) = -200 mV (91).
^d Calculated assuming that per CH_2 group, ΔGf^0 decreases by 2 kcal/mol from acetate to caproate.
^e Calculated from ΔGf^0 for propionate and E_0' (crotonate/butyrate) = -25 mV (232).
^f Calculated from ΔGf^0 for butyrate and E_0' (crotonate/butyrate) = -25 mV (232).
^g Calculated from ΔGf^0 for glyceraldehyde and ΔG^0 (glyceraldehyde-phosphate + $H_2O \rightarrow$ phosphoglycerate⁻ + H^+ + H_2) = -5.4 kcal/mol (679; see also 91, 334).
^h Calculated from ΔGf^0 for glucose and ΔG^0 (glucose + $H_2O \rightarrow$ gluconate⁻ + H^+ + H_2) = -3.36 kcal/mol (384).
ⁱ Calculated from ΔGf^0 for dihydroxyacetone and ΔG^0 (dihydroxyacetone-phosphate \rightarrow glyceraldehyde-phosphate) = +1.83 kcal/mol (91).
^j Calculated from ΔGf^0 for glycerol and E_0' (dihydroxyacetone phosphate/glycerol phosphate) = -190 mV (91).
^k Calculated assuming that per CH_2OH group, ΔGf^0 increases by 38.2 kcal/mol.
^l Calculated from DGf^0 and pK_a (=3.65) for aspartic acid (ΔGf^0 [dissociation] = 2.3 RT pK).
^m Calculated from ΔGf^0 and pK_a (=4.25) for glutamic acid.
ⁿ A ΔGf^0 of -80 kcal/mol has been reported for formate by Latimer (349).
^o A ΔGf^0 of 114.1 kcal/mol is obtained if an E_0' (β -keto butyrate/ β -hydroxybutyrate) of -270 mV (384) instead -349 mV (91) is used to calculate ΔGf^0 for β -ketobutyrate from ΔGf^0 for β -hydroxybutyrate.

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