The Handbook of

Water and Wastewater Microbiology






Edited by Duncan Mara and Nigel Horan

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Preface

Some 2300 years ago Hippocrates wrote: 'My other topic is water, and I now wish to give an account both about waters that cause disease and about those that are healthy, and what bad things arise from water and what good things. For water contributes very much to health.' So our appreciation of a relationship between the water we use and our health has been with us for a very long time. Hippocrates was unlikely to have been the first person to realise the existence of this relationship and we have probably known since our species evolved that water of adequate quantity and quality is essential for our survival and our health.

We now know (and have known for just over 100 years) that water quality is governed by (but, of course, not only by) microorganisms – the viruses, bacteria and parasites that can infect us and may (and very often do) make us ill. Microorganisms are also central to wastewater treatment and the reuse of treated wastewaters – we exploit them to treat our wastes biologically (actually, microbiologically), and we must ensure that pathogenic microorganisms are removed in the treatment processes to a level at which they do not cause any excess disease resulting from wastewater use in agriculture or aquaculture.

Water disinfection, usually with chlorine, has been practised in many parts of the world (but regrettably not all) for over 100 years. Water chlorination is a very efficient process: it kills bacteria very quickly (but viruses more slowly, and protozoa such as *Giardia* and *Cryptosporidium* hardly at all). Faecal bacterial numbers, in particular, are reduced to zero, and thus early water engineers judged the quality of chlorinated water supplies quite simply on whether faecal indicator bacteria – principally coliform bacteria – were present in the disinfected water or not. Zero coliforms, and zero faecal coliforms, quickly became *the* microbiological goal of drinking water quality. No-one would really question the general sense of this goal – chlorinate your water and you get zero coliforms per 100 ml, so everything's OK. End of story.

Life is rarely this simple, and water and wastewater microbiology is no exception. Emerging water-borne pathogens (*Cryptosporidium*, for example) require us to have a deeper understanding of water microbiology. Optimizing (really, maximizing) microbiological wastewater treatment also requires a knowledge of microbiology greater than that possessed by many design engineers. Structural engineers have a pretty good understanding of concrete, for example – so why shouldn't those who design activated sludge plants or waste stabilization ponds have an equal appreciation of the microorganisms whose activities are essential to the treatment process they are designing?

The purpose of this Handbook is to provide an introduction to modern water and wastewater microbiology, especially for water and wastewater engineers. The study of water and wastewater microbiology is very rewarding: better water treatment, better wastewater treatment, safer wastewater reuse, and thus healthier people – in all parts of our world.

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Part I Basic Microbiology

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Microbial nutrition and basic metabolism

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1 INTRODUCTION

In nature, of all living organisms, microorganisms are the most versatile and diversified with regard to their nutritional requirements. For example, microorganisms, such as bacteria, can be found that represent the entire spectrum of nutritional types. Some microbes have an unconditional need for preformed complex organic compounds while others can thrive with just a few inorganic substances as their sole nutritional requirements. Most microorganisms fall between these two extremes.

Although great variation is found in the specific requirements for growth of the diverse species of microorganisms, in general, the nature and functions of growth substances are common for all cells. In part, this is because the chemical composition of microbial cells is more or less similar. For instance, all microbes require a carbon source because carbon is a component of protoplasm; nitrogen is a component of many major macromolecules, such as protein and nucleic acids. Over 95% of a cell's dry weight is made up of a few major elements, such as C, O, H, S, P, K, Ca, Mg, and Fe. All these substances must be put together by biosynthesis to form cellular material. However, not all growth substances are incorporated into cell material. Some are used instead as energy sources. For example, carbon compounds are frequently used as energy sources by many microorganisms; inorganic sulphur compounds are used in energy metabolism by some microbes.

Assimilated nutrient substances need to be metabolized. *Metabolism* refers to the totality of organized biochemical activities carried out by an organism. Such activities, usually catalysed by enzymes, are of two kinds: those involved in generating energy; and those involved in using energy. Some microorganisms use nutrients absorbed by the cell as the source of energy in a process called *catabolism*, which is the reverse of biosynthesis (*anabolism*). In catabolism, large molecules are degraded in sequential stepwise reactions by enzymes and a portion of the energy released is trapped in the form of chemical energy. Other microorganisms derive their energy from the trapping of light and also convert it into chemical energy. This chemical energy is then harnessed to do work for the cell. A microorganism must perform many different types of work, such as synthesis of physical parts of the cell, repair or maintenance of cell components, and growth. Thus metabolism may be viewed as the coupling of energy generation and energy utilization. The kinds of nutrients and how they are assimilated and fed into the various metabolic pathways for energy production and utilization in microorganisms form the subject matter of this chapter.

2 CHEMICAL ELEMENTS AS NUTRIENTS

It may be generalized that there are three essential nutritional needs of a cell: water,

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energy, and chemical compounds, which may be used as building blocks. Most microorganisms, except for the phagocytic protozoa, have an absorptive type of nutrition. Thus chemical energy sources and chemical compounds must be dissolved in water. Water carries the solutes by transport mechanisms into the cell. Within the cell, water is the solvent in which the cell's biochemical reactions occur. It is also the medium for elimination of soluble waste substances from the cell. It is not surprising then that 70–80% of the microbial cell is water and that it constitutes the major portion of a cell's weight.

Required nutritional chemical compounds include chemical elements. These elements are necessary for both the synthesis of cell material and the normal functioning of cellular components such as enzymes. In order to grow, microorganisms must have proper essential chemical elements. The main chemical elements for cell growth include C, N, H, O, S and P – the same elements that form the chemical composition of the cells.

2.1 Macroelements

The major elements (macroelements), like C, H, N, O, S and P, are used in large amounts by microorganisms. The minor elements (microelements), like K, Ca, Mg, Na and Fe, are used in smaller quantities, while the trace elements, such as Mn, Zn, Co, Mo, Ni, Cu, are used in relatively very much smaller amounts. The amount of an element used by a microbe does not correlate with its relative importance; even one used in a trace amount may be essential to the growth or life of a microbial cell.

2.1.1 Carbon

Carbon is one of the most important chemical elements required for microbial growth. Fifty per cent of the dry weight of any cell is carbon; thus all organisms require carbon in some form. Carbon forms the backbone of three major classes of organic nutrients: carbohydrates, lipids and proteins. Such compounds provide energy for cell growth and serve as building blocks of cell material.

Microorganisms that use organic compounds as their major carbon source are called *heterotrophs*. Heterotrophs obtain such organic molecules by absorbing them as solutes from the environment. Some phagotrophic heterotrophs obtain organic molecules by ingestion of other organisms. Microorganisms that use carbon dioxide (the most oxidized form of carbon) as their major or even sole source of carbon are called *autotrophs*. They can live exclusively on relatively simple inorganic molecules and ions absorbed from the aqueous and gaseous environment.

2.1.2 Nitrogen

All organisms require nitrogen in some form. It is an essential part of amino acids that comprise cell proteins. Nitrogen is needed for the synthesis of purine and pyrimidine rings that form nucleic acids, some carbohydrates and lipids, enzyme cofactors, murein and chitin. Many prokaryotes use inorganic nitrogen compounds such as nitrates, nitrites, or ammonium salts. Unlike eukaryotic cells, some bacteria (like the free-living *Azotobacter* and the symbiotic *Rhizobium* of legume plants) and some archaeons (like the methanogens *Methanococcus* and *Methanobacterium*) can use atmospheric or gaseous nitrogen for cell synthesis by a physiological process called *nitrogen fixation*. Some microbes require organic nitrogen compounds such as amino acids or peptides. Some microorganisms use nitrate as an alternative electron acceptor in electron transport.

2.1.3 Hydrogen, oxygen, sulphur and phosphorus

Other elements essential to the nutrition of microorganisms are hydrogen, oxygen, sulphur and phosphorus. Hydrogen and oxygen are components of many organic compounds. Because of this, the requirements for carbon, hydrogen, and oxygen often are satisfied together by the availability of organic compounds. Free oxygen is toxic to most strict anaerobic bacteria and some archaeons, although aerobic microorganisms use oxygen as a terminal electron acceptor in aerobic respiration. Sulphur is needed for the biosynthesis of the amino acids cysteine, cystine,

homocysteine, cystathione and methionine, as well as the vitamins biotin and thiamine. Phosphorus is essential for the synthesis of nucleic acids and adenosine triphosphate. It is a component of teichoic acids and teichuronic acids in the cell walls of Gram-positive bacteria as well as a component of various membrane phospholipids. Reduced forms of sulphur may serve as sources of energy for *chemotrophs* or as sources of reducing power for *phototrophs*. Sulphate may serve as a terminal electron acceptor in electron transport.

2.2 Microelements and trace elements

Many other essential elements, the microelements and trace elements, are required in smaller amounts than the macroelements by microorganisms in their nutrition. Some of their functions in supporting the growth of microorganisms are summarized in Table 1.1.

For example, sodium is required by the permease that transports the sugar melibiose into the cells of the colon bacterium *Escherichia coli*. Sodium is required by marine microorganisms for maintaining cell integrity and growth. Some 'salt-loving' prokaryotes, the red extreme halophiles, cannot grow with less than 15% sodium chloride in their environment. Essential elements are often required as cofactors for enzymes. Because iron is a key component of the cytochromes and electron-carrying iron-sulphur proteins, it plays a key role in cellular respiration. However, most inorganic iron salts are highly insoluble in water. Thus, many microbes must produce specific iron-binding agents, called siderophores, in order to utilize this element. Siderophores are chelating agents that solubilize iron salts and transport iron into the cell. Many enzymes, including some involved in protein synthesis, specifically require potassium. Magnesium functions to stabilize ribosomes, cell membranes and nucleic acids and is needed for the activity of many enzymes.

Trace elements, needed in extremely small amounts for nutrition by microorganisms, include manganese, molybdenum, cobalt, zinc, and copper. For instance, molybdenum is required by nitrogenase, the enzyme that converts atmospheric nitrogen to ammonia during nitrogen fixation. Manganese aids many enzymes to catalyse the transfer of phosphate groups. Cobalt is a component of vitamin B_{12} and its coenzyme derivatives.

3 NUTRITIONAL TYPES OF MICROBES

Microbes can be grouped nutritionally on the basis of how they satisfy their requirements for carbon, energy, and electrons or hydrogen. Indeed, the specific nutritional requirements of microorganisms are used to distinguish one microbe from another for taxonomic purposes.

Microorganisms may be grouped on the basis of their energy sources. Two sources of energy are available to microorganisms. Microbes that oxidize chemical compounds (either organic or inorganic) for energy are

TABLE 1.1 Functions of some microelements and trace elements in the nutrition of microorganisms

Element	*Major functions in some microorganisms*
Sodium	Enzyme activator. Transport across membranes. Maintenance of cell integrity. Facilitates growth. Salt form of some required organic acids
Potassium	Cofactor for enzymes. Maintenance of osmotic balance
Iron	Component of cytochromes, haem-containing enzymes, electron transport compounds and proteins. Energy source
Magnesium	Enzyme activator, particularly for kinase reactions. Component of chlorophyll. Stabilizes ribosomes, cell membranes, and nucleic acids
Calcium	Enzyme activator, particularly for protein kinases. Component of dipicolinic acid in bacterial endospores
Cobalt	Component of vitamin B_{12} and its coenzyme derivatives
Manganese	Enzyme activator, particularly for enzymes transferring phosphate groups
Molybdenum	Enzyme activator for nitrogen fixation

called *chemotrophs*; those that use light as their energy sources are called *phototrophs*. A combination of these terms with those employed in describing carbon utilization results in the following nutritional types:

1. *Chemoautotrophs*: microbes that oxidize inorganic chemical substances as sources of energy and carbon dioxide as the main source of carbon.
2. *Chemoheterotrophs*: microbes that use organic chemical substances as sources of energy and organic compounds as the main source of carbon.
3. *Photoautotrophs*: microbes that use light as a source of energy and carbon dioxide as the main source of carbon.
4. *Photoheterotrophs*: microbes that use light as a source of energy and organic compounds as the main source of carbon.

Microorganisms also have only two sources of hydrogen atoms or electrons. Those that use reduced inorganic substances as their electron source are called *lithotrophs*. Those microbes that obtain electrons or hydrogen atoms (each hydrogen atom has one electron) from organic compounds are called *organotrophs*.

A combination of the above terms describes four nutritional types of microorganisms:

1. *Photolithotrophic autotrophy*
2. *Photo-organotrophic heterotrophy*
3. *Chemolithotrophic autotrophy*
4. *Chemo-organotrophic heterotrophy.*

The characteristics of these types with representative microoganisms as well as other organisms are shown in Table 1.2.

Photolithotrophic autotrophs are also called *photoautotrophs*. The cyanobacteria, algae and green plants use light energy and carbon dioxide as their carbon source but they employ water as the electron donor and release oxygen in the process. The purple and green sulphur bacteria use inorganic compounds as electron donors (e.g., H_2S, S^0) and do not produce oxygen in the process. Thus they are described as *anoxygenic*. Chemo-organotrophic heterotrophs are also called *chemoheterotrophs*. They use organic compounds for energy, carbon and electrons/hydrogen. The same organic nutrient compound often satisfies all these requirements. Animals, most bacteria, fungi, and protozoa are chemoheterotrophs. Photo-organotrophic heterotrophs are also called in short *photoheterotrophs*. The purple and green non-sulphur bacteria are photoheterotrophs and use radiant energy and organic compounds as their electron/hydrogen and carbon donors. These common microorganisms, found in polluted lakes and streams, can also grow as photoautotrophs with molecular hydrogen as electron donor. The chemolithotrophic autotrophs are also called *chemoautotrophs* in brief. They include the nitrifying, hydrogen, iron and sulphur bacteria. They oxidize reduced inorganic compounds, such as nitrogen, iron or sulphur molecules, to derive both energy and electrons/hydrogen. They use carbon dioxide as their carbon source. A few of them, however,

TABLE 1.2 Nutritional types of microbes and other organisms

Nutritional type	*Energy source*	*Electron or hydrogen source*	*Carbon source*	*Examples of organisms*
Photolithotrophic autotrophy	Light	Inorganic compounds, water	Carbon dioxide	Purple and green sulphur bacteria; algae; plants; cyanobacteria
Photo-organotrophic heterotrophy	Light	Organic compounds	Organic compounds	Purple and green non-sulphur bacteria
Chemolithotrophic autotrophy	Inorganic compounds	Inorganic compounds	Carbon dioxide	Nitrifying, hydrogen, iron, and sulphur bacteria
Chemo-organotrophic heterotrophy	Organic compounds	Organic compounds	Organic compounds	Most bacteria, fungi, protozoa, and animals

can make use of carbon from organic sources and thus become heterotrophic. Such bacteria that use inorganic energy sources and carbon dioxide, or sometimes organic compounds, as carbon sources can be called *mixotrophic*, because they combine autotrophic and heterotrophic processes. Chemotrophs are important in the transformations of the elements, such as the conversion of ammonia to nitrate and sulphur to sulphate, that continually occur in nature.

Even though a particular species of microorganism usually belongs to only one of the four nutritional types, some show great metabolic flexibility and can alter their nutritional type in response to environmental change. For example, many purple non-sulphur bacteria are photoheterotrophs in the absence of oxygen but become chemoheterotrophs in the presence of oxygen. When oxygen is low, photosynthesis and oxidative metabolism can function simultaneously. This affords a survival advantage to the bacteria when there is a change in environmental conditions.

The specific nutritional requirements of bacteria are used extensively for taxonomic purposes. Specific identification tests have been designed for particular groups of bacteria, such as the Gram-negative intestinal bacilli, to determine the nature of water pollution.

4 GROWTH FACTORS

Some microorganisms have good synthetic capability and thus can grow in a medium containing just a few dissolved salts. The simpler the cultural medium to support growth of a species of microbe, the more complex or advanced is the microbe's nutritional synthetic capability. Thus, the photoautotrophs are the most complex in their nutritional physiology. With the addition of one organic compound, such as the addition of glucose, a glucose-salts medium can support the growth of many chemoheterotrophs; an example is the bacterial indicator of faecal contamination, *Escherichia coli*. However, many microorganisms lack one or more essential enzymes and therefore cannot synthesise all their nutritional requirements. They must obtain these preformed or supplied in the environment or medium.

Organic compounds required in the nutrition of microorganisms, because they cannot be synthesized specifically, are called *growth factors*. The three major classes of growth factors are *amino acids*, *purines* and *pyrimidines*, and *vitamins*. Proteins are composed of about 20 amino acids. Some bacteria and archeons cannot synthesize one or more of these and require them preformed in the medium. For example, *Staphylococcus epidermidis*, the normal resident on the human skin, requires proline, arginine, valine, tryptophan, histidine and leucine in the medium before it can grow. Requirements for purines and pyrimidines, the nucleic acid bases, are common among the lactic acid bacteria. Vitamins are small organic compounds that make up all or part of the enzyme cofactors (non-protein catalytic portion of enzymes). Only very small, or catalytic, amounts suffice to support growth of the cells. Lactic acid bacteria, such as species of *Streptococcus*, *Lactobacillus* and *Leuconostoc*, are noted for their complex requirements of vitamins and hence many of these species are used for microbial assays of food and other substances. Vitamins most commonly required by microorganisms are thiamine (vitamin B_1), biotin, pyridoxine (vitamin B_6) and cyanocobalamin (vitamin B_{12}). The functions of some vitamins for the growth of microorganisms are summarized in Table 1.3.

5 ENERGY TRAPPING IN MICROORGANISMS

Like all living things, microorganisms require energy to live. The ability of a microorganism to maintain its life processes and to reproduce its own kind depends on its ability to trap energy and to use it to drive the endergonic reactions of the cell. Like all living forms, microbes trap or obtain energy in one of two ways: by *high-energy molecules* or by a *proton motive force* (*proton gradient*) across a cell membrane.

TABLE 1.3 Functions of some vitamins in the growth of microorganisms

Vitamin	*Function(s)*
Folic acid	One-carbon transfers; methyl donation
Biotin	Carboxyl transfer reactions; carbon dioxide fixation, β-decarboxylations; fatty acid biosynthesis
Cyanocobalamin (B_{12})	Carries methyl groups; synthesis of deoxyribose; molecular rearrangements
Lipoic acid	Transfer of acyl groups
Nicotinic acid (niacin)	Precursor of NAD^+ and $NADP^+$; electron transfer in oxidation–reduction reactions
Pantothenic acid	Precursor of coenzyme A; carries acyl groups
Riboflavin (B_2)	Precursor of FMN, FAD in flavoproteins involved in electron transport; dehydrogenations
Thiamine (B_1)	Aldehyde group transfer; decarboxylations
Pyridoxal-pyridoxamine group (B_6)	Amino acid metabolism, e.g. transamination and deamination
Vitamin K group; quinones	Electron transport; synthesis of sphingolipids
Hydroxamates	Iron-binding compounds; solubilization of iron and transport into cell
Haem and related tetrapyrroles	Precursors of cytochromes

5.1 High-energy molecules

The energy liberated by an exergonic reaction can be used to drive an endergonic reaction if there is a reactant common to both reactions. This common reactant in the trapping of energy is called an energy-rich or energy-transfer compound. The energy-transfer compounds of greatest use to a cell are able to transfer large amounts of free energy and are called *high energy-transfer compounds* or *high-energy molecules*. Of these, adenosine triphosphate (ATP) is the most important. Such compounds are found in the cytosol or soluble part of the cell.

High-energy molecules are important because they drive biosynthesis in the cytoplasm, including the synthesis of nucleic acids, proteins, lipids and polysaccharides. They also are involved in the active transport of certain solutes into the cell. High-energy molecules, such as ATP, have bonds that have a high free energy of hydrolysis. In the case of ATP, a large amount of energy is needed to link the third phosphate group to adenosine diphosphate (ADP) because of the electrostatic repulsion of the negative charges on the other adjacent phosphate groups. Reactions in which the phosphate is removed from ATP will be favoured since the electrostatic repulsion is decreased as a result of hydrolysis. Thus, this energy is liberated if ATP is hydrolyzed back to ADP.

There is a reason why the phosphoryl group is a common chemical group that is transferred between molecules. The phosphorus in all phosphate groups carries a positive charge. This is because phosphorus forms double bonds (P═O) poorly so that the phosphorus–oxygen bond exists as the semi-polar bond $P^+–O^-$. The electrons in the bond are shifted toward the electron-attracting oxygen. The positively charged phosphorus is attacked by the electronegative oxygen in the hydroxyl of a water molecule in a hydrolytic reaction resulting in the release of inorganic phosphate. One can compare the tendency of different molecules to donate phosphoryl groups by comparing the free energy released when the acceptor is water, i.e. the free energy of hydrolysis. That is, a scale is used, where the standard nucleophile is the hydroxyl group of water, and the phosphoryl donors are all compared with respect to the tendency to donate the phosphoryl group to water. This gives a free energy change, or energy available to perform useful work and designated ΔG, per mole of substrate hydrolysed. The *standard free-energy change* under standard conditions (reactants and products at 1 M concentration and the reactions take place at 25°C at pH 7) is designated $\Delta G^{\circ\prime}$.

The general role of ATP in providing energy to drive an endergonic reaction may be shown below.

Consider the following reaction:

$$\text{Glucose} + H_3PO_4 \rightarrow \text{Glucose-6-phosphate} + H_2O$$

The $\Delta G^{\circ\prime}$ is $+13.8$ kJ/mol; it is an endergonic reaction and will not proceed spontaneously.

However, if ATP is provided as a reactant, the reaction becomes exergonic since the $\Delta G^{\circ\prime} = -16.7$ kJ/mol (-30.5 (from ATP hydrolysis) $+13.8 = -16.7$ kJ/mol) and proceeds spontaneously:

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}$$

The compound ADP is also sometimes used by cells as a high energy-transfer compound since its hydrolysis also liberates an equally large quantity of energy as ATP ($\Delta G^{\circ\prime} = -30.5$ kJ/mol). However, adenosine monophosphate (AMP) is a low-energy molecule; its hydrolysis yields only a small amount of energy ($\Delta G^{\circ\prime} = -8.4$ kJ/mol).

Table 1.4 lists some high energy-transfer compounds with their standard free-energy values upon hydrolysis. Each of them can transfer its energy of hydrolysis directly or indirectly to ATP synthesis, as in the following example:

$$\text{1,3-Diphosphoglyceric acid} + \text{ADP} \rightarrow \text{3-phosphoglyceric acid} + \text{ATP}$$

The synthesis of high-energy-transfer compounds, such as ATP, involves phosphorylation. ATP is formed by phosphorylation of ADP, with energy for the phosphorylation being provided by an exergonic reaction.

TABLE 1.4 Some high energy-transfer compounds with their standard free energy release upon hydrolysis

Compound	*$\Delta G^{\circ\prime}$, kJ/mol*
Adenosine triphosphate (ATP)	−30.5
Guanosine triphosphate (GTP)	−30.5
Uridine triphosphate (UTP)	−30.5
Cytidine triphosphate (CTP)	−30.5
Acetyl phosphate	−42.3
1,3-Diphosphoglyceric acid	−49.4
Phosphoenolpyruvic acid (PEP)	−61.9

There are two general ways in which this phosphorylation of ADP can occur:

- *Substrate-level phosphorylation*, a reaction in which the phosphate group of a chemical compound is removed and directly added to ADP
- Phosphorylation by a membrane-bound enzyme called a proton-translocating ATPase, which uses the energy of an energy-trapping system called the proton motive force (described later).

In substrate-level phosphorylation, the rearrangement of atoms within chemical compounds derived from nutrients may result in a new compound that contains a high-energy phosphate bond. Such rearrangements can occur when cells dissimilate nutrients. The phosphate group involved in the high-energy phosphate bond then can be transferred directly to ADP, forming ATP, which now contains the high-energy phosphate bond. (Note that bond energy is the energy required to break a bond, e.g. by hydrolysis, and it is equal to the energy released when the bond is formed. It is not the energy released when a bond is broken. For example, the P–O bond energy is about $+413$ kJ, while the hydrolysis energy is -35 kJ.) One example of substrate level phosphorylation is as follows:

$$\text{Phosphoenolpyruvic acid} + \text{ADP} \rightarrow \text{Pyruvic acid} + \text{ATP}$$

It should be added that any chemical group that is electronegative (such as the hydroxyl groups in sugars) can attack the electropositive phosphorus resulting in phosphoryl group transfer if the proper enzyme is present to catalyse the reaction. In this manner, ATP can phosphorylate many different compounds. Enzymes that catalyse phosphoryl group transfer reactions are called *kinases*.

5.2 The proton motive force

The *chemiosmotic theory* was proposed by the biochemist Peter Mitchell in 1961. He received the Nobel Prize in 1978 for this proposal.