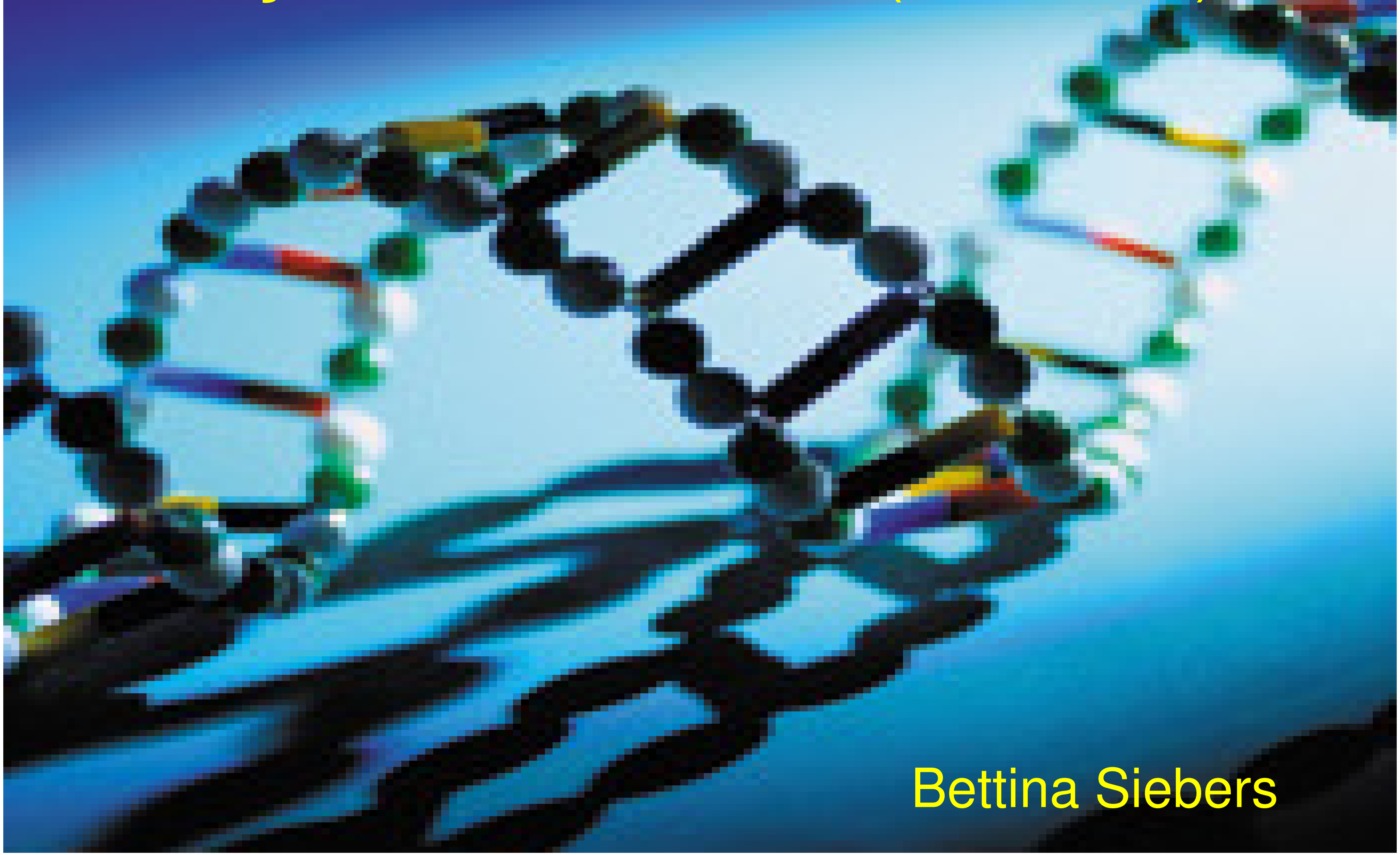
Introduction to Molecular Biology

History & DNA Structure (Lecture 5)



Bettina Siebers

Grundlagen der Biochemie

➢ Metabolismus IV

1. Gesetzmäßigkeiten von Stoffwechselreaktionen
2. Enzyme, Biokatalysatoren
3. Chemische Prinzipien, Redoxreaktionen
4. Metabolismus
5. Stoffwechsel-Diversität („life style“)
6. Elektronen „carrier“, Energiereiche Verbindungen
7. Transport
8. Metabolismus chemoorganotropher Organismen
   - Aerobe Atmung (Respiration)
   - **Fermentation/Gärung**
   - **Anaerobe Atmung**
9. **Metabolismus photolithotropher Organismen**
   - **Photosynthese**

Atmung/Fermentation

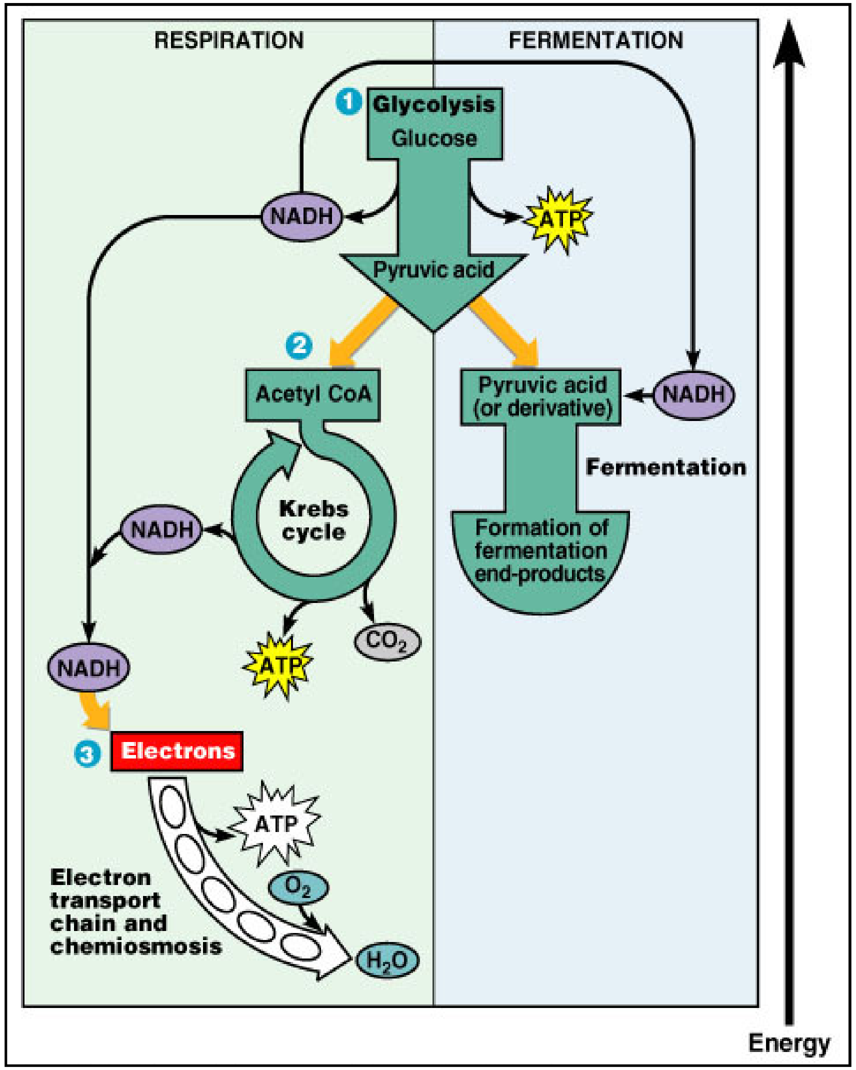
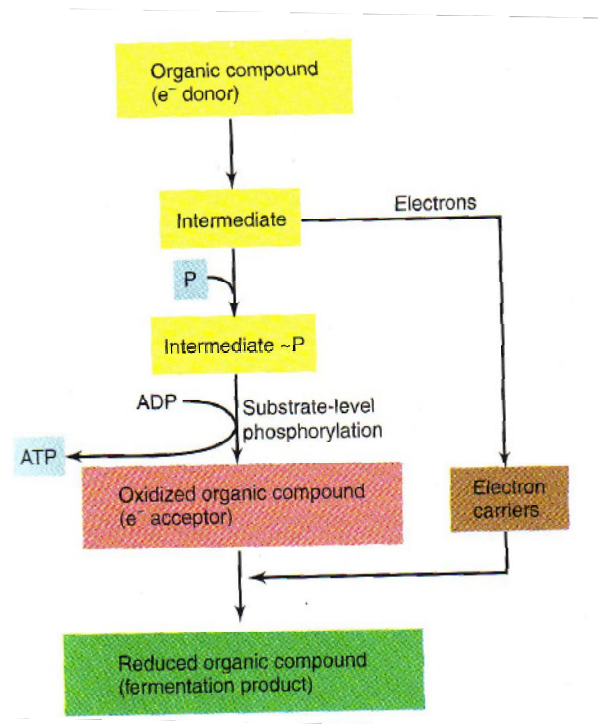


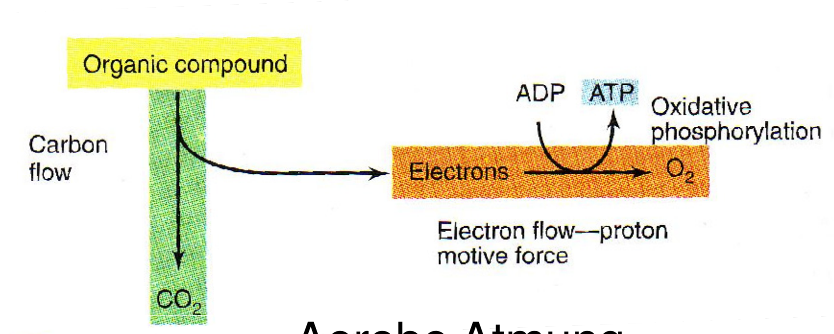
Fig. 5.14 Microbiology: An Introduction (Tortora, Funke, Case)

Fermentation (Gärung)

Energiekonservierung



Fermentation



Aerobe Atmung

Fig. Kohlenstoff- und Elektronenfluß bei der Fermentation (4.11) und aeroben Atmung (4.13).
Brock Biology of Microorganisms (8th edition) (Madigan et al.)

Fermentation

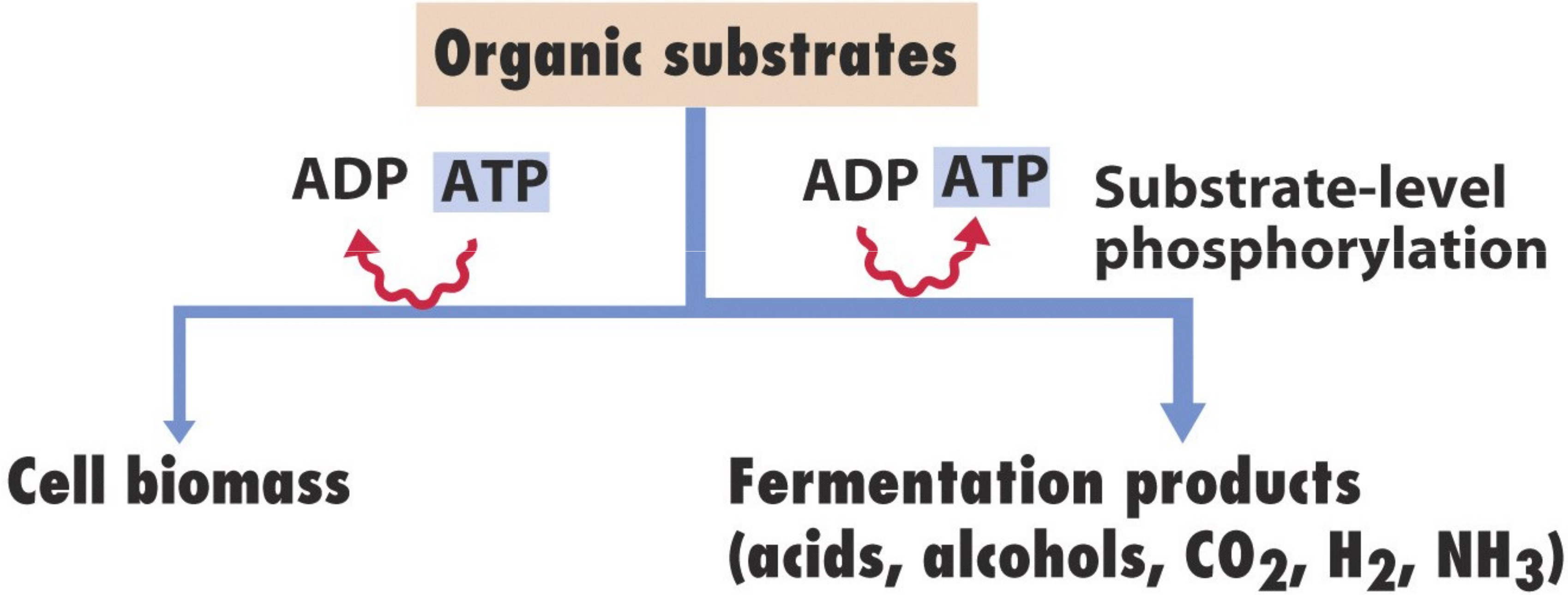
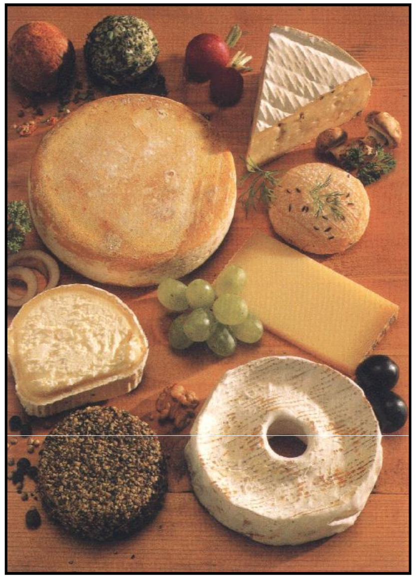


Figure 17-49 Brock Biology of Microorganisms 11/e

Fermentation

-**Anaerober Abbau von org. Verbindungen**
(z.B. Kohlenhydrate, Aminosäuren)



-In **Abwesenheit eines externen e^- Akzeptors** können organische Verbindungen nur durch Fermentation abgebaut werden

-Energiekonservierung durch Oxidation eines Substrates über energiereiche Intermediate „ATP Generation über **Substratkettenphosphorylierung**"; **Reoxidation der Reduktionsäquivalente** durch Übertragung auf eine oxidierte Zwischenverbindung/zweites Produkt

-In einigen speziellen Gärungswegen Energiekonservierung über Elektronentransportphosphorylierung (z.B. Fumaratreduktase)

-**Redox-Bilanz muss ausgeglichen sein** !
(H_2 Bildung ist eine Möglichkeit überflüssige Elektronen freizusetzen)

-"**Partielle Oxidation der org. Verbindungen**" (e^- Donor und e^- Akzeptor)

-**Biotechnologische Bedeutung**

Substratkettenphosphorylierung

- **Wichigsten Enzyme:**

  - **3-Phosphoglycerat Kinase**

    1,3-Bisphosphoglycerat + ADP → 3-Phosphoglycerat + ATP

    $\Delta G^{0\prime}$= -20,1 kJ/mol

  - **Pyruvat Kinase**

    Phosphoenolpyruvat + ADP → Pyruvat + ATP

    $\Delta G^{0\prime}$= -19,8 kJ/mol

  - **Acetatkinase**

    Acetylphosphat + ADP → Acetat + ATP

    $\Delta G^{0\prime}$= -13,0 kJ/mol

Alkohol Gärung

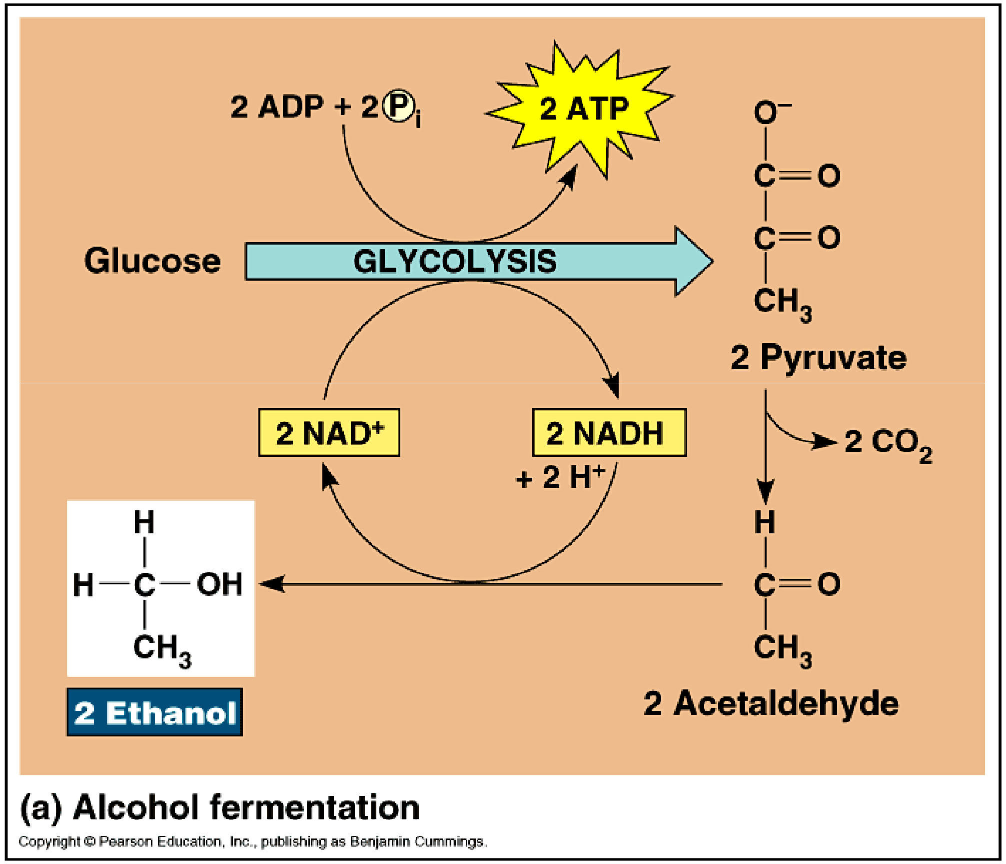
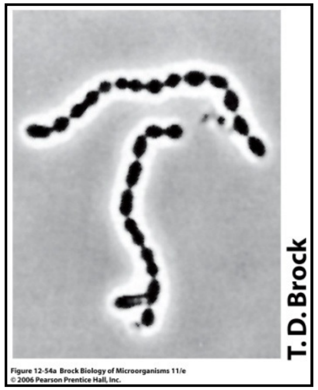


Fig. 9.17 Die Alkohol Gärung.
Biology (6th edition, Campbell & Reece)

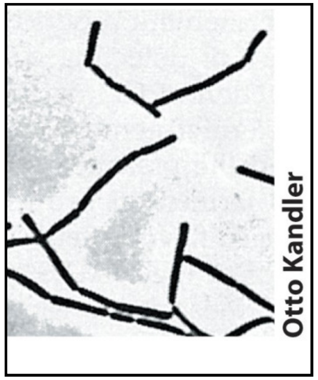
<u>Eukaryonten</u>:
Hefe: EMP Weg

<u>Prokaryonten</u>:
Zymomonas mobilis
KDPG Weg (Pflanzen Säfte, z.B. Agavenschnaps)

Milchsäuregärung



Lactococcus lactis



Lactobacillus acidophilus

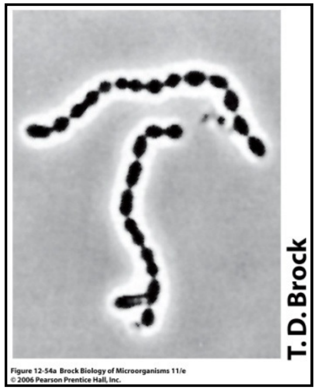
Table 12.23 Differentiation of the principal genera of lactic acid bacteria[a]

Cell form and arrangement	Genus/DNA (mol % GC)
Cocci in chains or tetrads	
Homofermentative	*Streptococcus* (34–46)
	Enterococcus (38–40)
	Lactococcus (38–41)
	Pediococcus (34–42)
Heterofermentative	*Leuconostoc* (38–41)
Rods, typically in chains	
Homofermentative	*Lactobacillus* (32–53)
Heterofermentative	*Lactobacillus* (34–53)

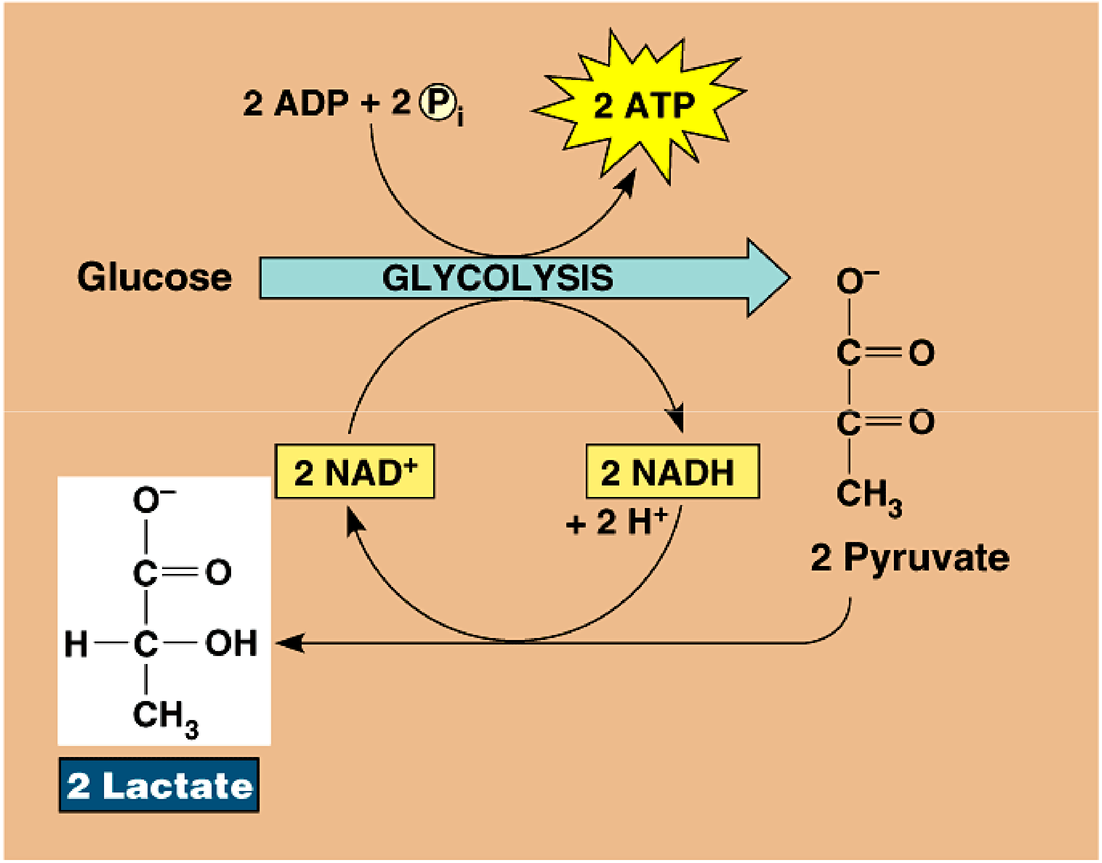
[a] Phylogenetically, all organisms are members of the low GC subdivision of the gram-positive *Bacteria*.

Table 12-23 Brock Biology of Microorganisms 11/e

Homofermentative Milchsäuregärung

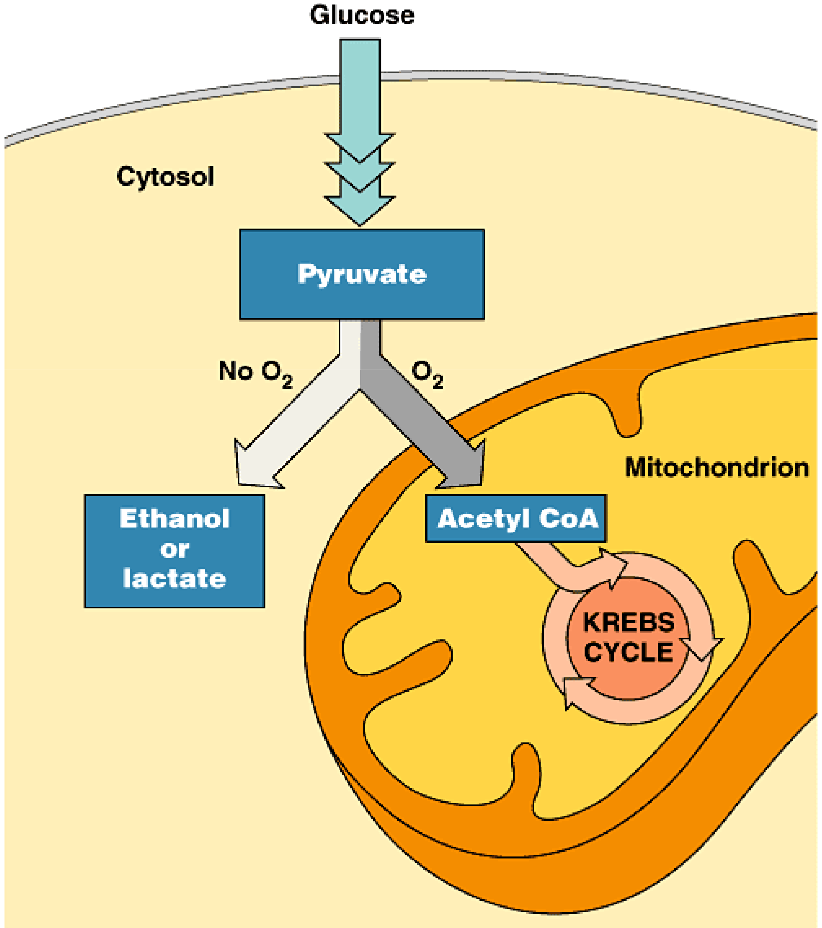


Lactococcus lactis



(b) Lactic acid fermentation

Metabolismus in An- und Abwesenheit von Sauerstoff



Verbreitete Fermentationen

Table 17.7 Examples of common bacterial fermentations and some of the organisms carrying them out

Type	Overall reaction[a]	Organisms
Alcoholic	Hexose → 2 Ethanol + 2 CO_2	Yeast *Zymomonas*
Homolactic	Hexose → 2 $Lactate^-$ + 2 H^+	*Streptococcus* Some *Lactobacillus*
Heterolactic	Hexose → $Lactate^-$ + Ethanol + CO_2 + H^+	*Leuconostoc* Some *Lactobacillus*
Propionic acid	$Lactate^-$ → $Propionate^-$ + $Acetate^-$ + CO_2	*Propionibacterium* *Clostridium propionicum*
Mixed acid	Hexose → Ethanol + 2, 3-Butanediol + $Succinate^{2-}$ + $Lactate^-$ + $Acetate^-$ + $Formate^-$ + H_2 + CO_2	Enteric bacteria[b] *Escherichia* *Salmonella* *Shigella* *Klebsiella* *Enterobacter*
Butyric acid	Hexose → $Butyrate^-$ + $Acetate^-$ + H_2 + CO_2	*Clostridium butyricum*
Butanol	Hexose → Butanol + $Acetate^-$ + Acetone + Ethanol + H_2 + CO_2	*Clostridium acetobutylicum*
Caproate	Ethanol + $Acetate^-$ + CO_2 → $Caproate^-$ + $Butyrate^-$ + H_2	*Clostridium kluyveri*
Homoacetogenic	Fructose → 3 $Acetate^-$ + 3 H^+ 2 H_2O 4 H_2 + 2 CO_2 + H^+ → $Acetate^-$ +	*Clostridium aceticum* *Acetobacterium*
Methanogenic	$Acetate^-$ + H_2O → CH_4 + HCO_3^-	*Methanosaeta* *Methanosarcina*

[a] Reactions are intended as an overview of the process and are not necessarily balanced.
[b] Not all organisms produce all products. In particular, butanediol production is limited to only certain enteric bacteria.

Table 17-7 Brock Biology of Microorganisms 11/e

(3) Anaerobe Atmung

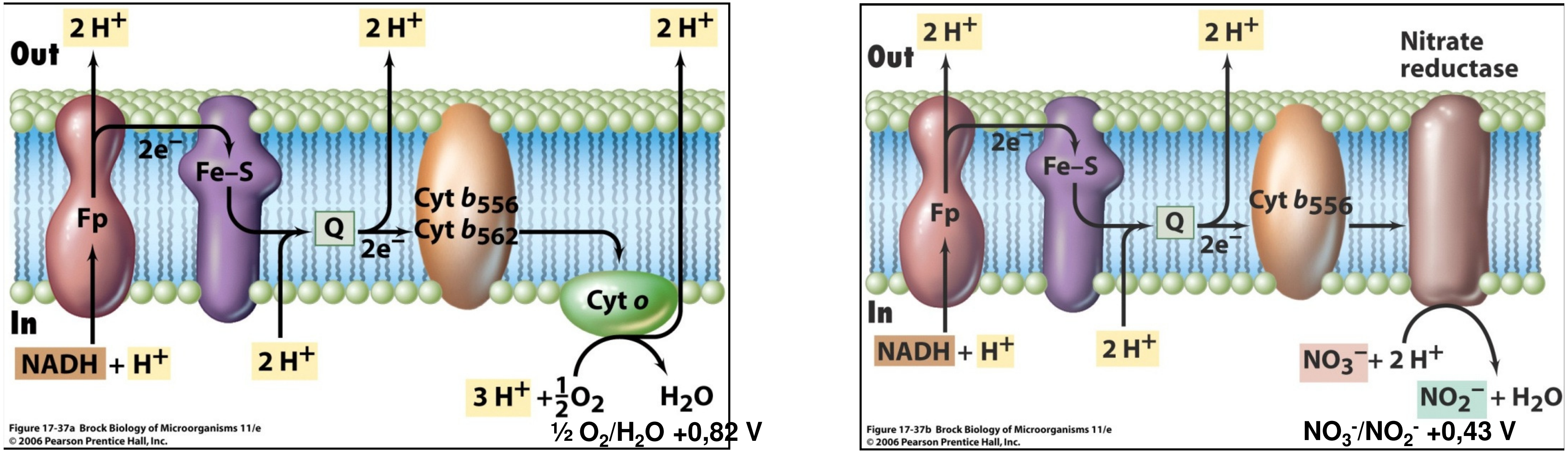
Sumpfgebiete

Verdauungstrakt von Wiederkäuern

Nitratammonifikation (*E. coli*)

- **Enterobacteriaceae** (Bsp: *E. coli*)
- **fakultativ anaerobe** Bakterien (anaerob Fermentation)
- nur **Reduktion von Nitrat zu Nitrit** (Nitratreduktase A)

aerob | **anaerob (NO_3^-)**



½ O_2/H_2O +0,82 V | **NO_3^-/NO_2^- +0,43 V**

Fig. 17.37 **Vergleich aerobe und Nitrat Atmung.**

Brock Biology of Microorganisms (10th edition) (Madigan et al.)

Chemische Prinzipien

Beispiel Zellatmung

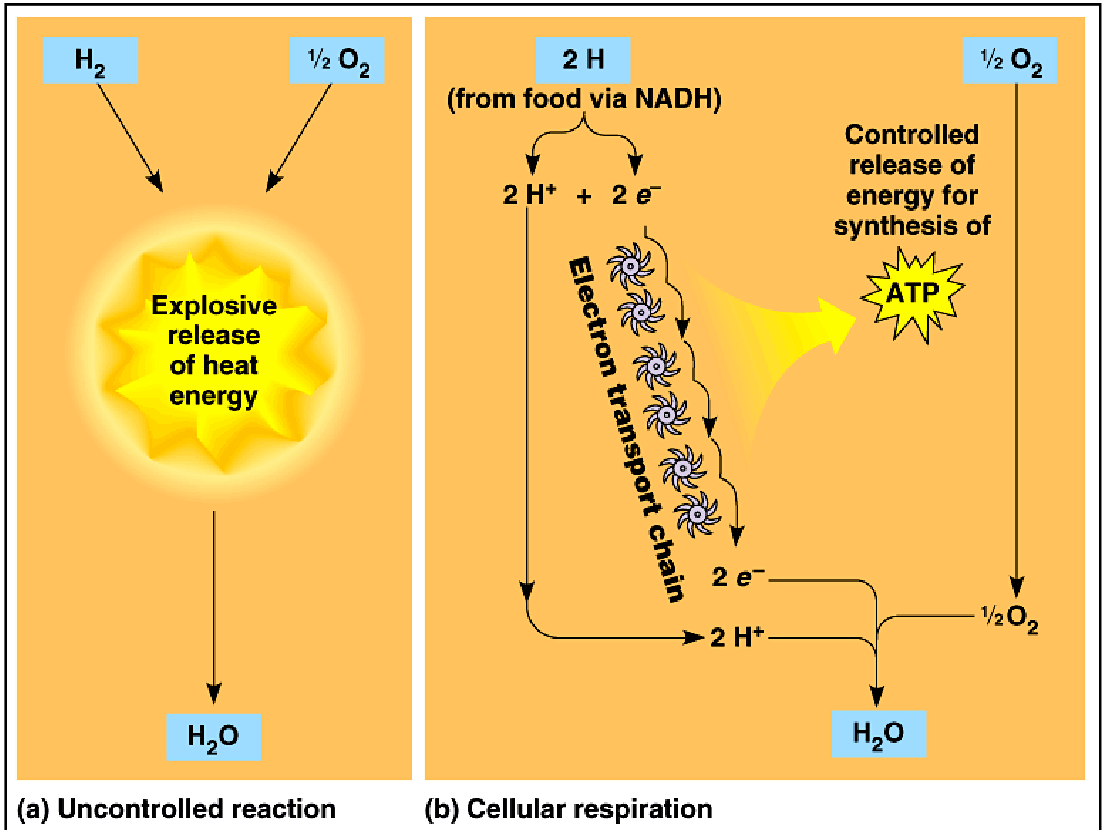
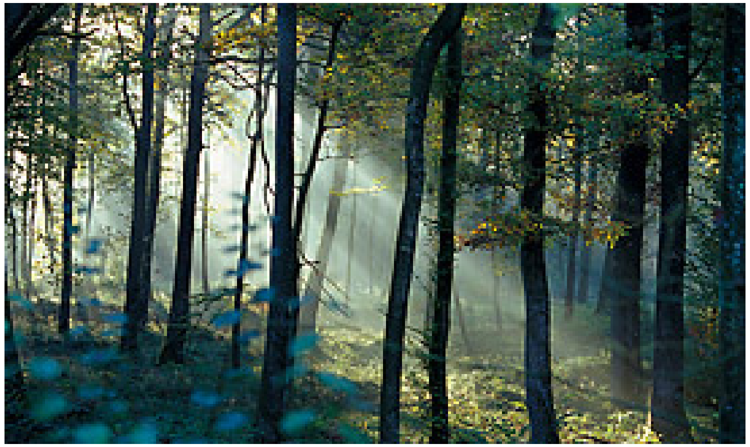


Fig. 9.5 Biology (6th edition, Campbell & Reece)

Phototrophe Organismen

Photosynthese



Oxygene Photosynthese (O_2-Produktion)

$6\ CO_2 + 12\ H_2O$ + Lichtenergie $\rightarrow C_6H_{12}O_6 + 6\ H_2O + 6\ O_2$

Glucose

$\Delta G_0' = +2872$ kJ/mol Hexose

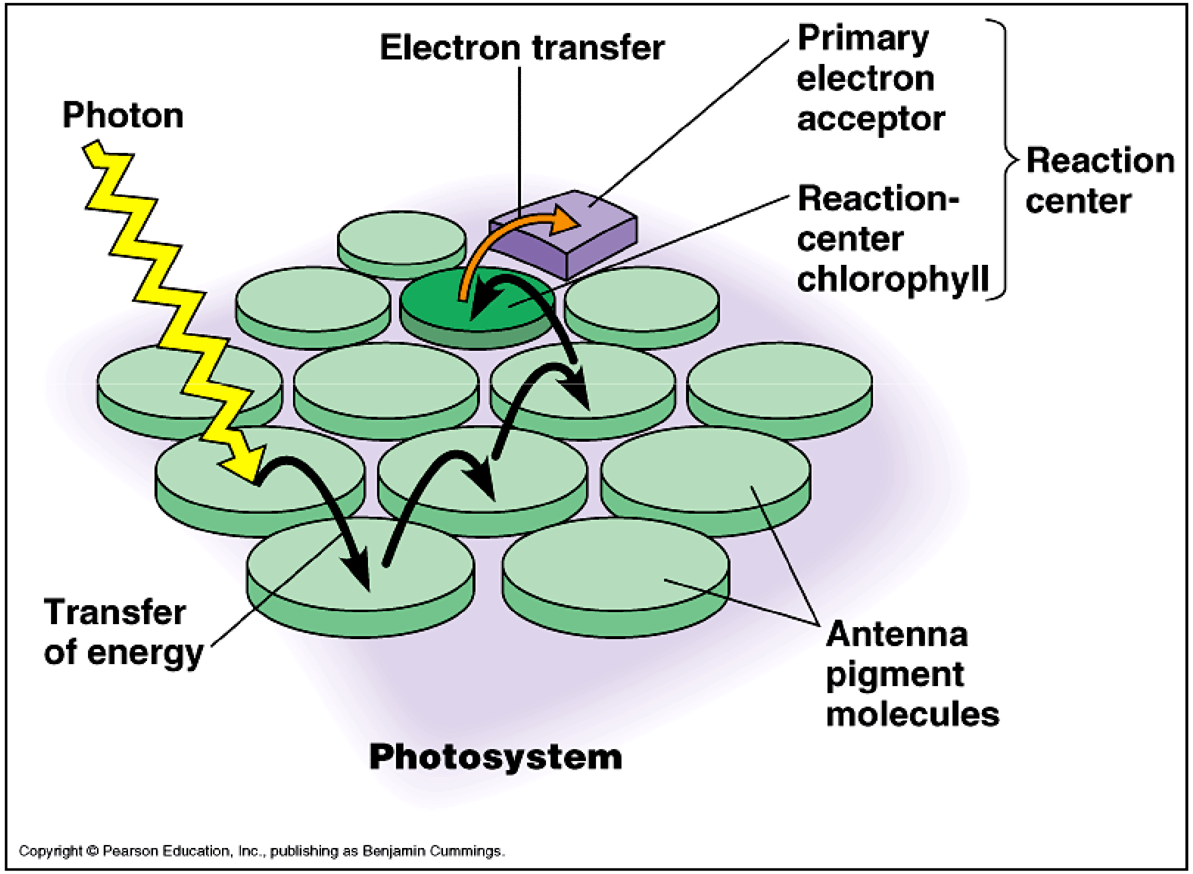
Aerobe Respiration

$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP + 38\ P_i \rightarrow 6\ CO_2 + 6\ H_2O + 38\ ATP$

Glucose

$\Delta G_0' = -2870$ kJ/mol

Der Licht-Sammelkomplex
(Reaktionszentrum und Antennen-Pigmente)



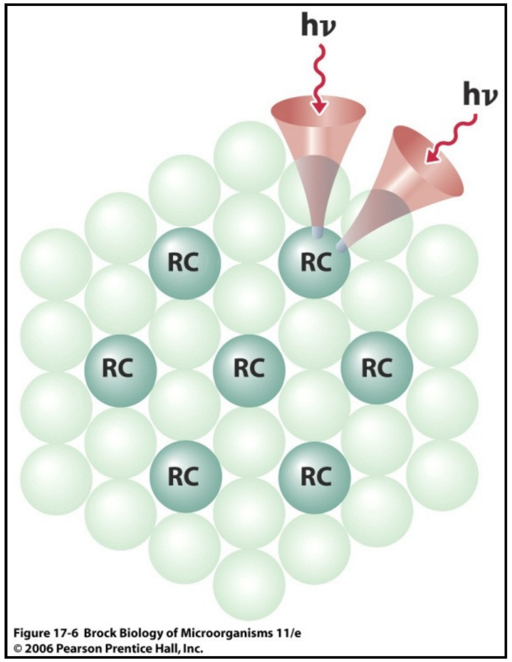


Fig. 10.11 Biology (6th edition, Campbell & Reece)

Nicht-Zyklische Photophosphorylierung

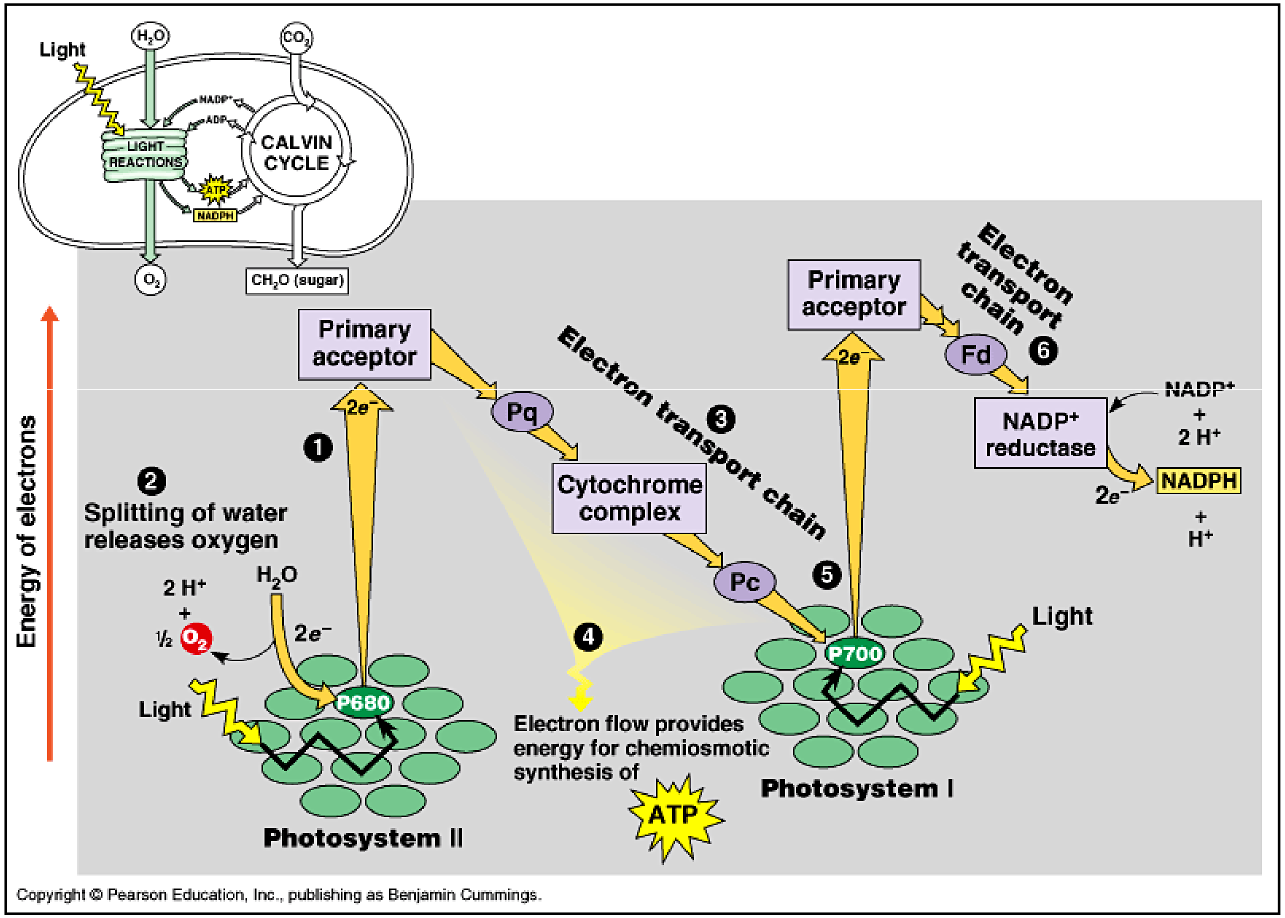


Fig. 10.12 Biology (6th edition, Campbell & Reece)

Photosynthese
Licht- und Dunkel-Reaktion

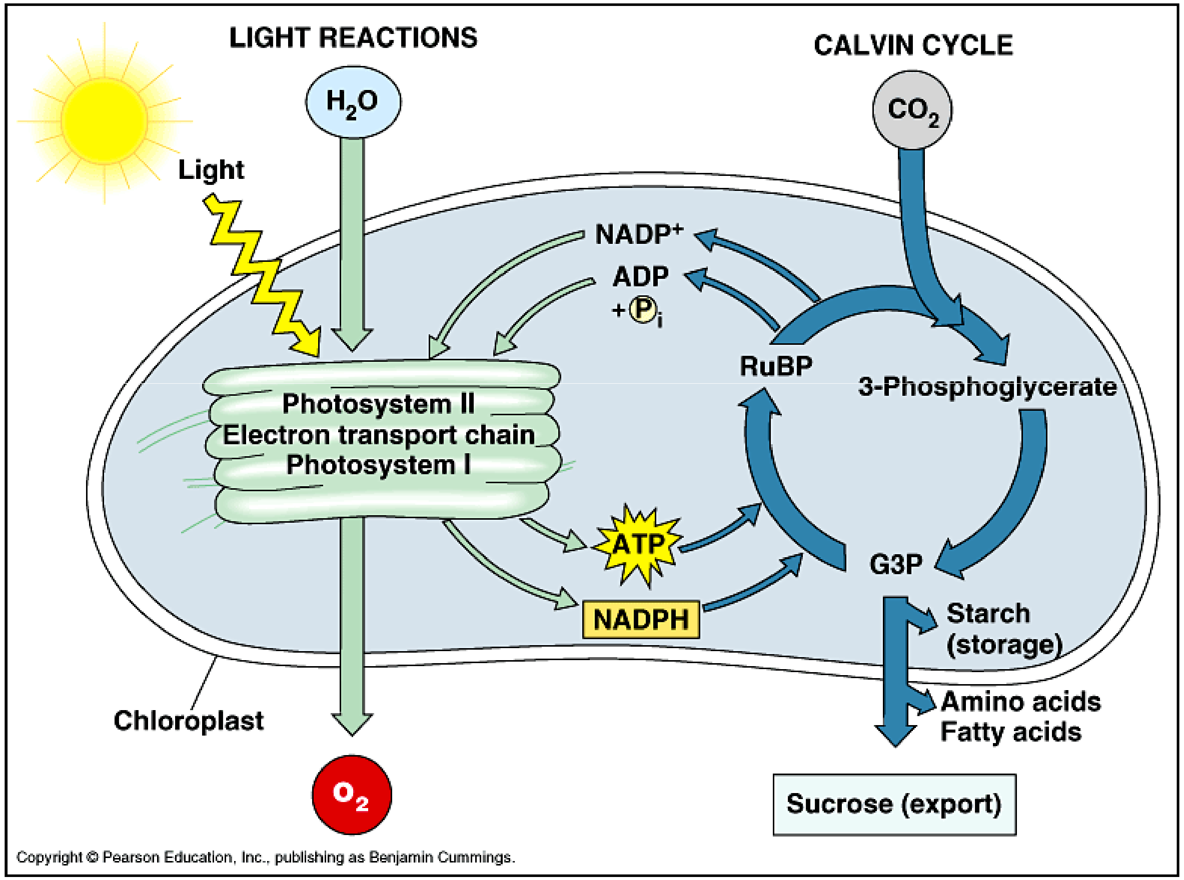


Fig. 10.20 Biology (6th edition, Campbell & Reece)

Energiefluss und „Recycling“ im Ecosystem

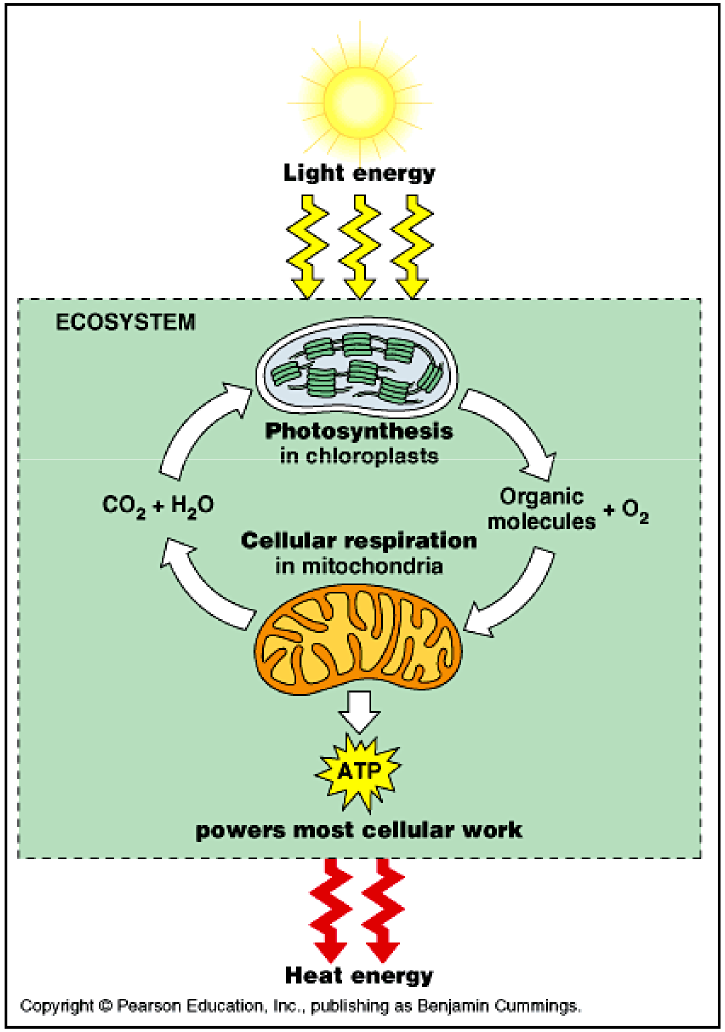


Fig. 9.1 Biology (6th edition, Campbell & Reece)

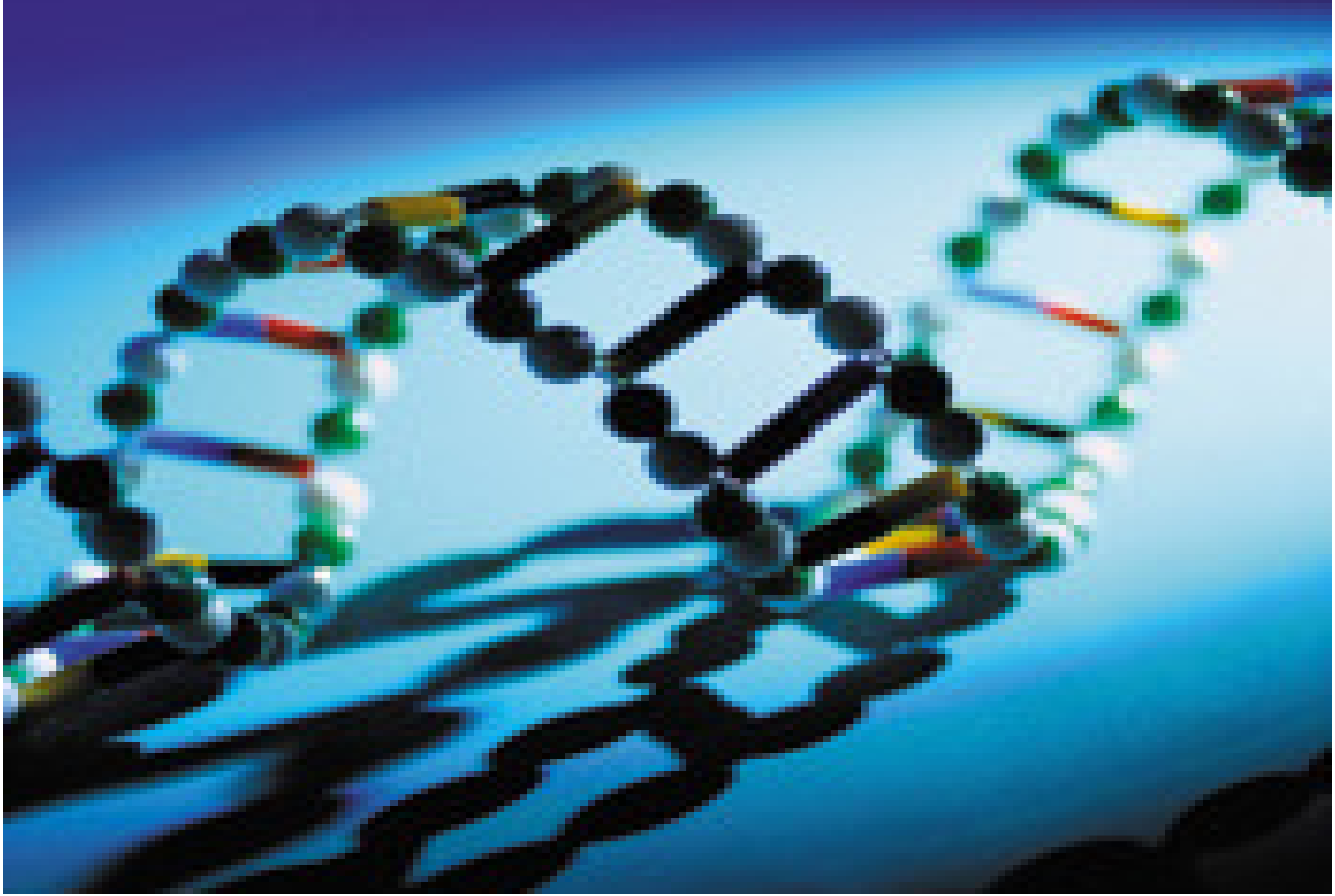
Einige Fragen....

- 17) Welche beiden basalen Mechanismen der Energiekonservierung kennen sie? Geben sie jeweils ein Beispiel.
- 18) Beschreiben sie die Vorgänge/Prozesse bei der aeroben Atmung.
- 19) Welche beiden Phasen der Glykolyse unterscheidet man? Nennen sie die Redoxreaktionen und die Energie-liefernden Reaktionen der Glykolyse. Wie ist die Energiebilanz?
- 20) Bei welchen Reaktionen der aeroben Atmung wird CO_2 freigesetzt?
- 21) Kann im Zitronensäurezyklus Energie über Substratketten-Phosphorylierung gewonnen werden? Nennen sie das/die beteiligte/n Enzym/e. Was sind die Redox-Reaktionen des Zitronensäurezyklus?
- 22) Beschreiben sie die wichtigsten Regulationsmechanismen (Kontrolle) bei der aeroben Atmung.
- 23) Beschreiben sie den Aufbau, Prinzip & Funktion der Atmungskette.
- 24) Durch welche Versuche kann man die Kopplung von e^- Transport und Phosphorylierung zeigen? Wie wirkt 2,4-Dinitrophenol?
- 25) Beschreiben sie Aufbau, Funktion und Mechanismus der ATP Synthase? Mit welchem Experiment konnte man die Rotation der γ Untereinheit zeigen?

Einige Fragen....

- 26) Welche Möglichkeiten der Energiekonservierung gibt es unter anaeroben Bedingungen?
- 27) Was verstehen sie unter Gärung/Fermentation? Was ist das zugrunde liegende Prinzip?
- 28) Beschreiben sie das Prinzip der anaeroben Atmung. Geben sie ein Beispiel.
- 29) Wie sind die Licht- und die Dunkelreaktion der Photosynthese miteinander gekoppelt?
- 30) Beschreiben sie die Vorgänge bei der Lichtreaktion in Cyanobacterien/Pflanzen (oxygenen Photosynthese). Was sind die Produkte der Lichtreaktion? Was passiert bei der zyklischen Photophosphorylierung? Welche Produkte entstehen hier? Was hat dies mit dem Calvin Zyklus zu tun?
- 31) Vergleichen sie die Vorgänge bei der aeroben Atmung und Photosynthese (Chemiosmose).
- 32) Beschreiben sie die Vorgänge bei der Dunkelreaktion. Was sind die Schlüsselreaktionen des Calvin-Zyklus?

History & DNA Structure



What is Molecular Biology?

- Broad definition: **Attempt to understand biological phenomena in molecular terms**. (Difference to biochemistry?)
- More restrictive definition: **The study of gene structure and function at the molecular level**.
- Molecular biology developed of the disciplines of genetics and biochemistry.

Genetics and Molecular Biology

- **Genetics**: study of the mechanisms by which traits are passed from one organism to another and how they are expressed.

- **Molecular genetics**: study of genetics at the molecular level.

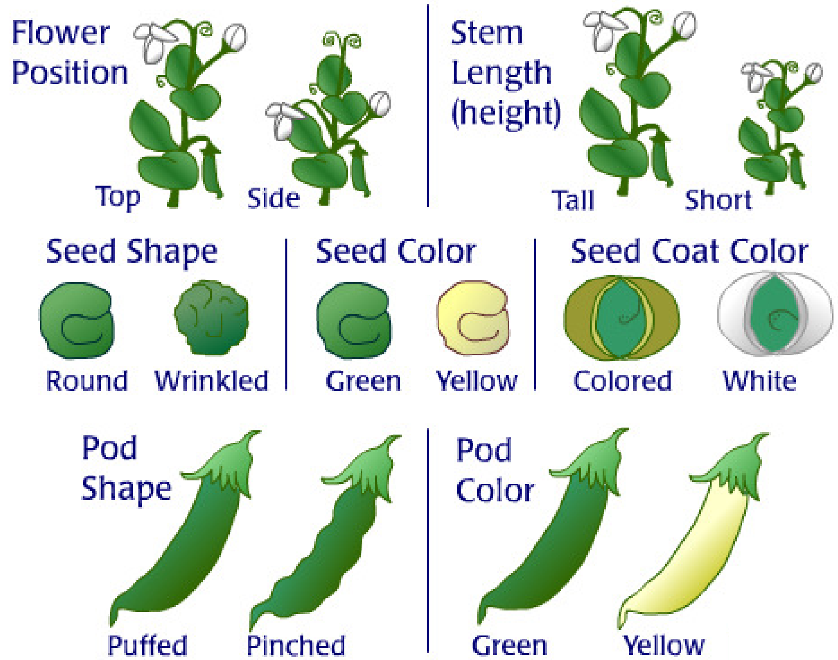
- **Molecular biology**: study of the molecular mechanisms by which cells function, including the study of **biological information flow**.

History

Origin of Genetics

- **1865, Gregor Mendel**, Augustinian monk
- used pea plants to study how characteristics are passed from parent to offspring
- molecular nature of genes unknown, **Transmission Genetics**

Phenotype =appearance, what can be seen ?



Mendel‘s Law of Inheritance

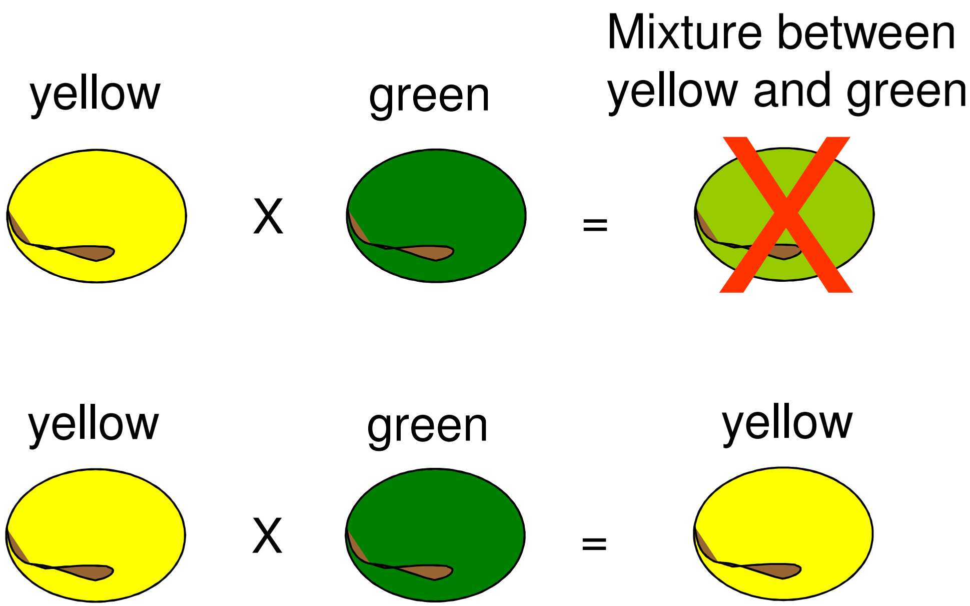
- A single characteristic is encoded by 2 alternative forms of genes, called **alleles**
- Parents are **diploid**, **sex cell (gametes)** are **haploid**

Yellow seed   Green seed

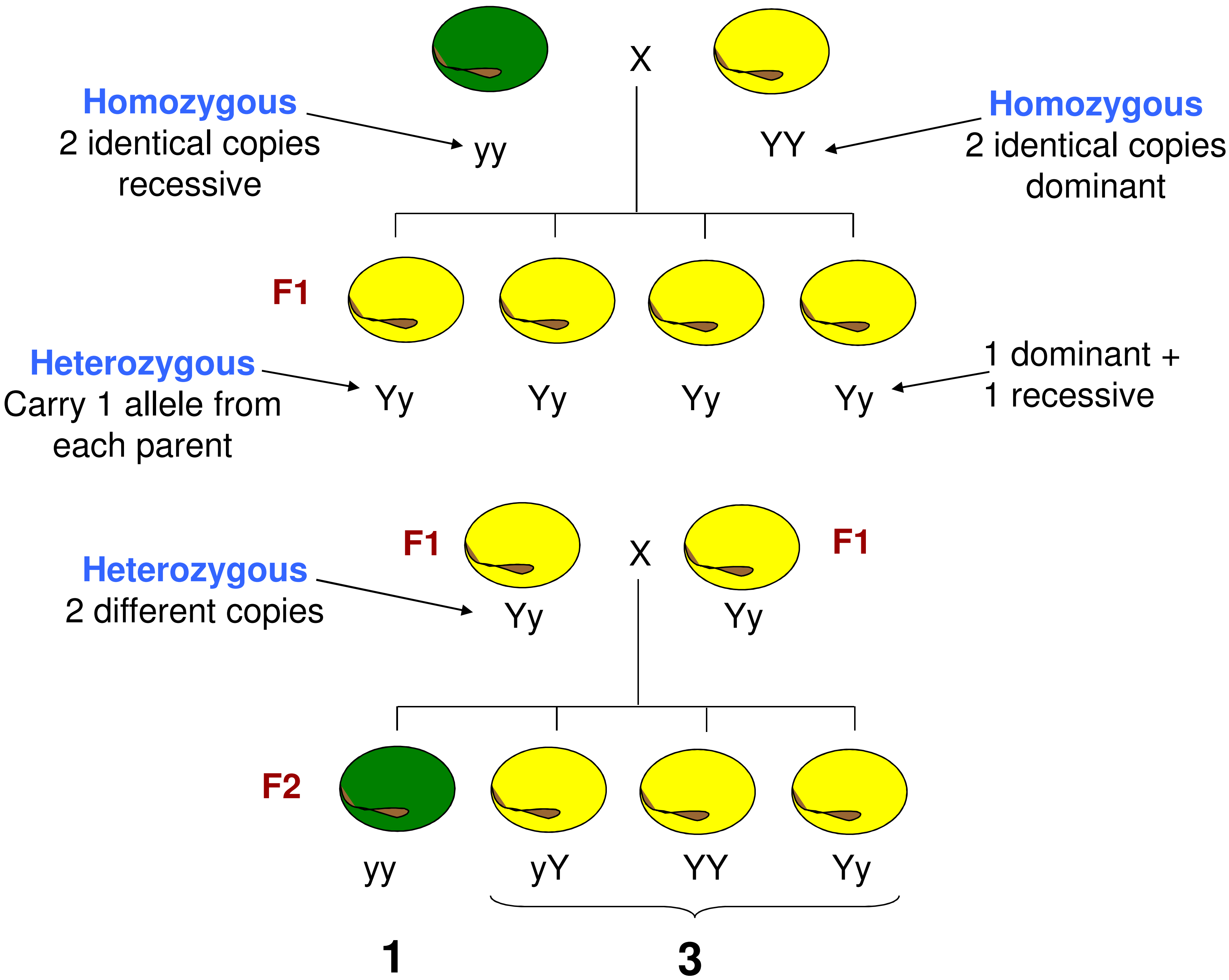
YY genotype yy

Y = yellow allele
y = green allele

Pure-bred peas have two copies of the same allele = homozygous

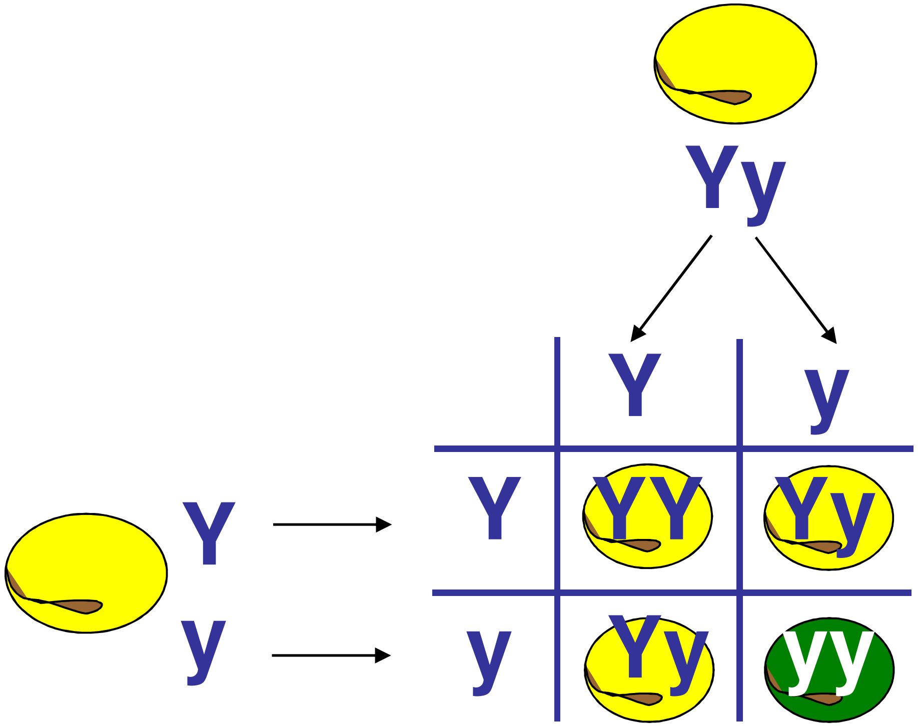


The allele coding for yellow seeds is **DOMINANT**
The allele coding for green seeds is **RECESSIVE**


X
Homozygous
2 identical copies
recessive
yy
YY
Homozygous
2 identical copies
dominant
F1
Heterozygous
Carry 1 allele from
each parent
Yy
Yy
Yy
Yy
1 dominant +
1 recessive
F1
X
F1
Heterozygous
2 different copies
Yy
Yy
F2
yy
yY
YY
Yy
1
3

Mendel‘s Law of Inheritance

Genetic inheritance follows rules



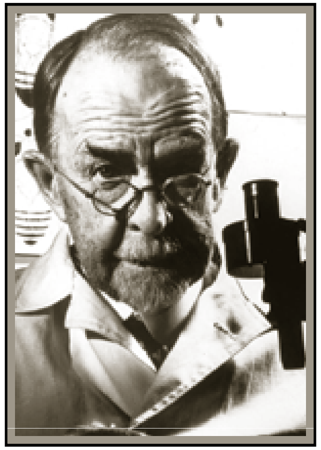
3:1

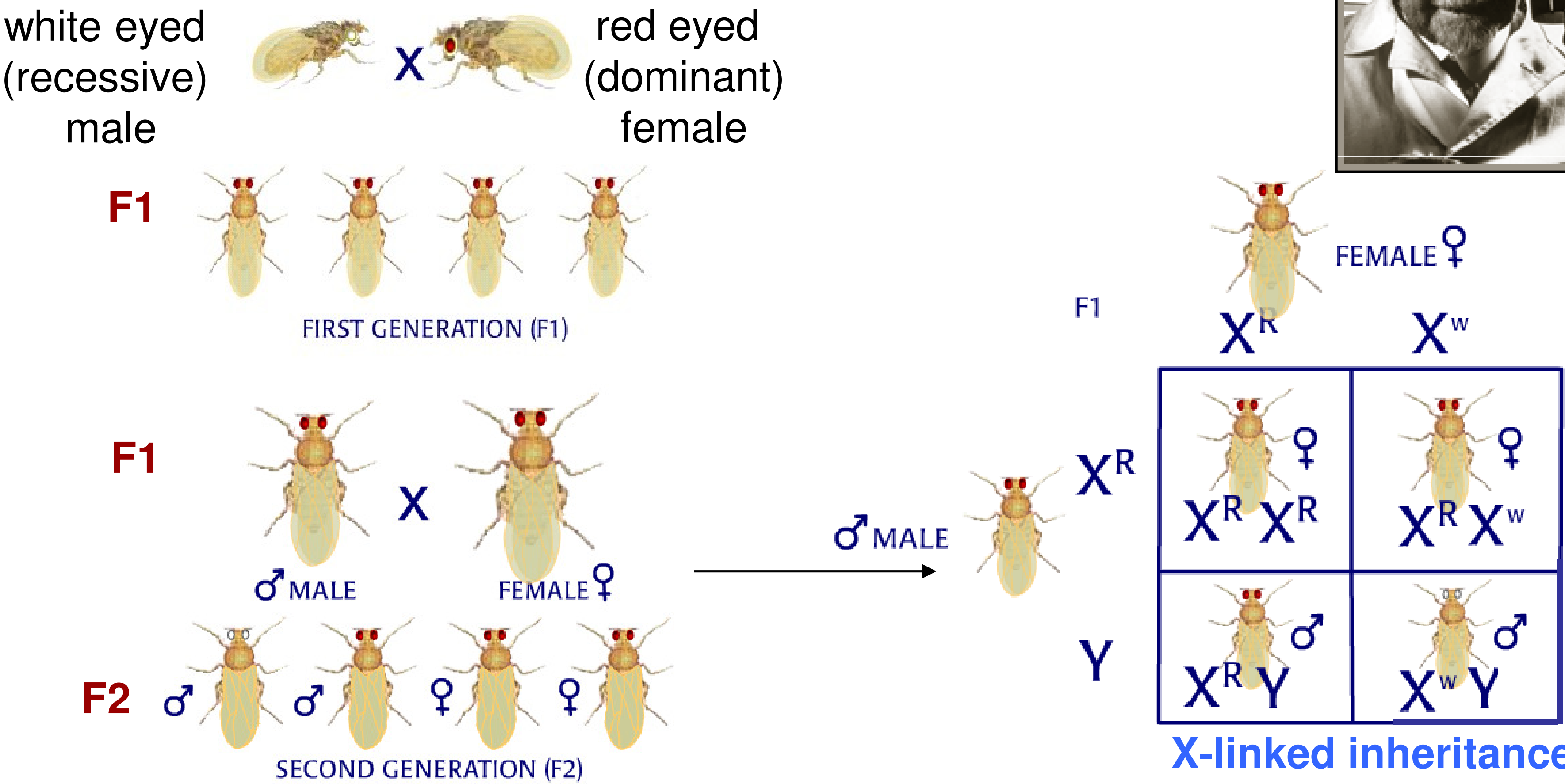
Punnett square

Chromosome Theory of Inheritance

Drosophila melanogaster (fruit flies)

- **1910, Thomas Hunt Morgan**, geneticist
- **Sex-linked inheritance** (autosomes, sex chromosome)
- **Genetic recombination** and mapping

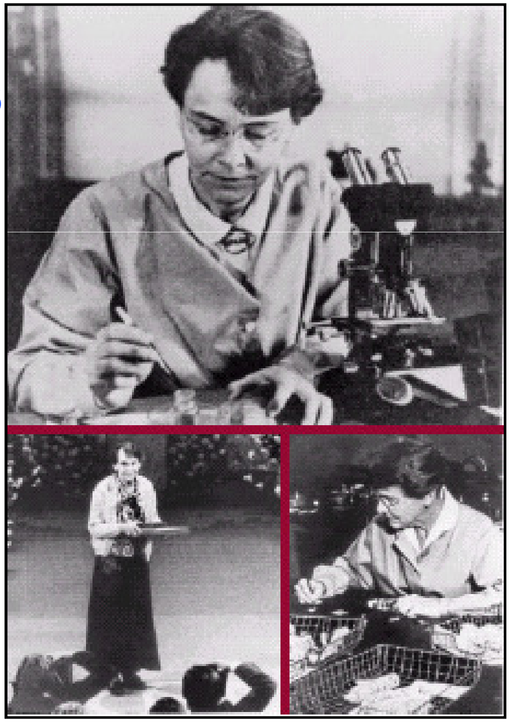




X-linked inheritance

Physical Evidence for Recombination

- **1931, Barbara McClintock** and **Harriet Creighton**
- **Maize** (corn, physical –microscope- and genetic detection of **recombination**)
- **1951**, discovery of **mobile genetic elements**
  "jumping genes theory"
- **1983, Nobel Prize in Physiology**



Barbara McClintock

1902-1992

DNA as the Genetic Material

- **1869, Friedrich Miescher,** discovered a mixture of compounds **"nuclein"** in the the cell nucleus (DNA, RNA and protein)
- **1928, Frederick Griffith,** *Streptococcus pneumoniae*, **transformation** „some chemical component“

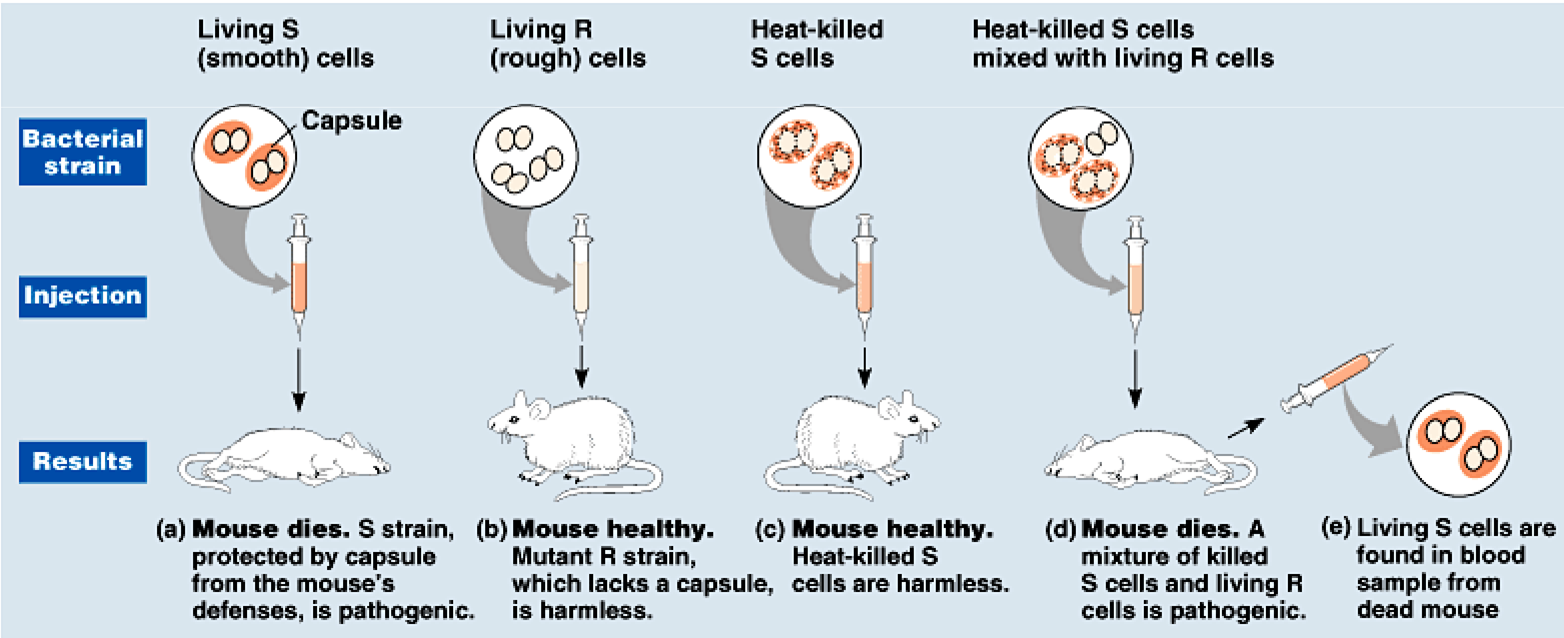


FIGURE 16.1 Biology 6/e

DNA as the Genetic Material

- **1944, Oswald Avery, Maclyn McCarty & Colin MacLeod**
  „**DNA** not RNA or protein"

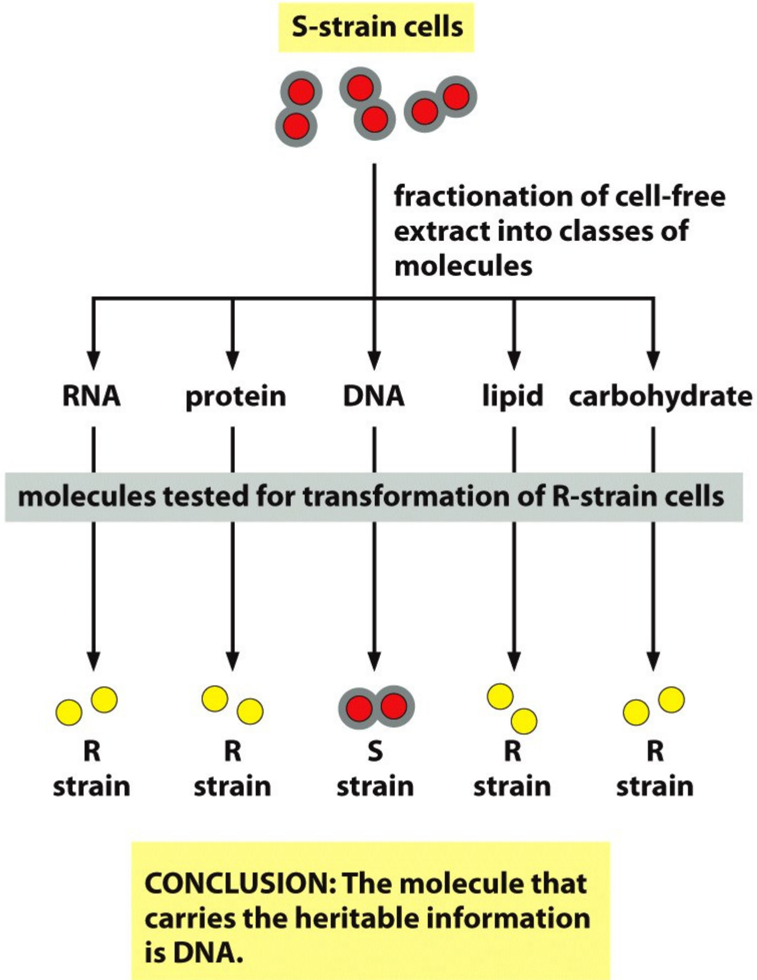


Figure 5-4 *Essential Cell Biology* (© Garland Science 2010)

DNA as the Genetic Material

- **1952 Alfred Hershey & Martha Chase** (Bacteriophage T2)

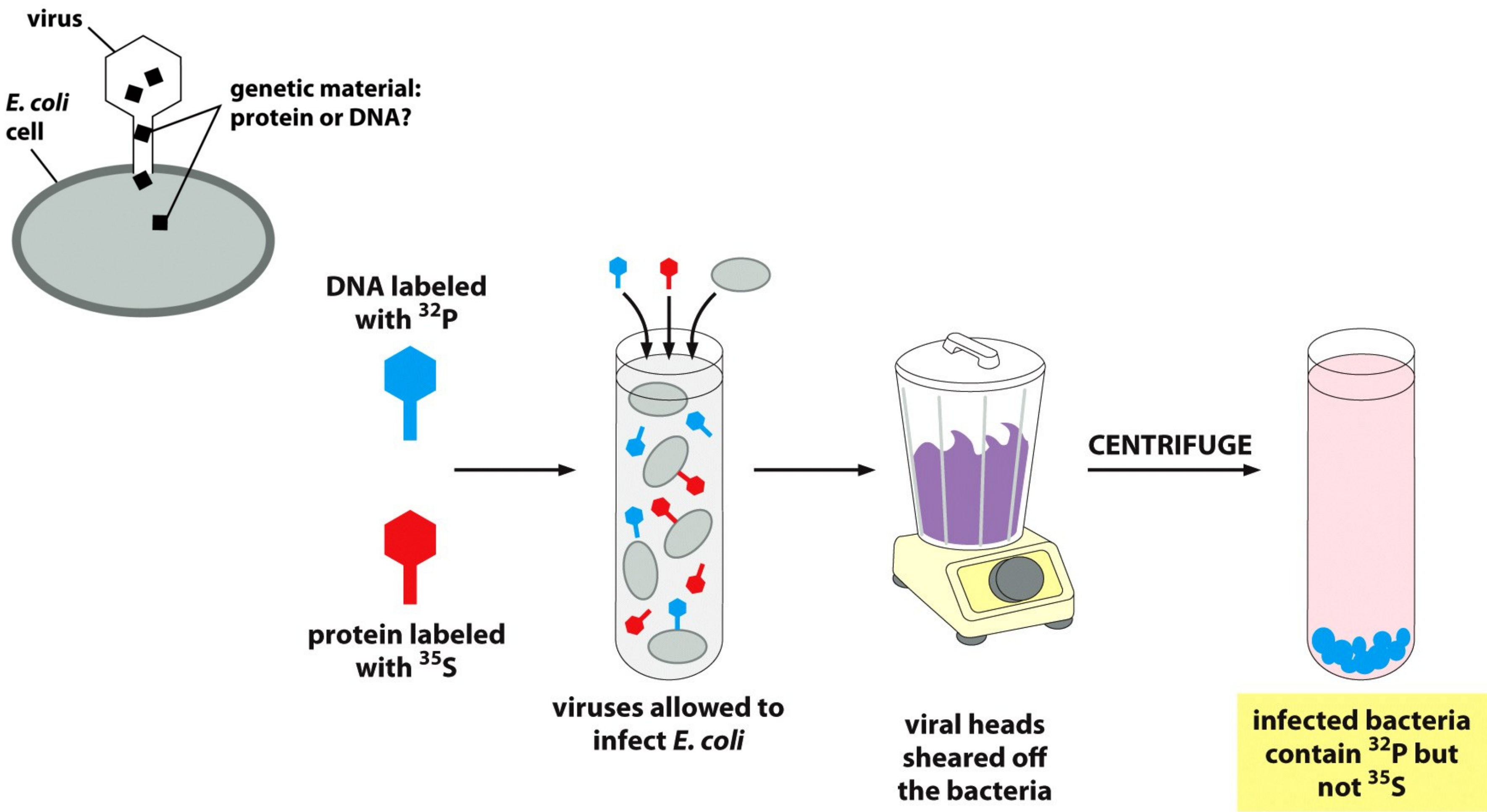
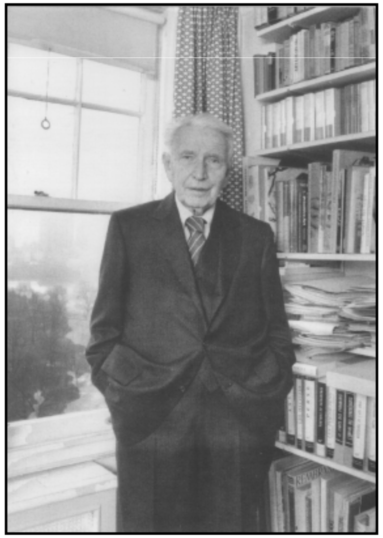
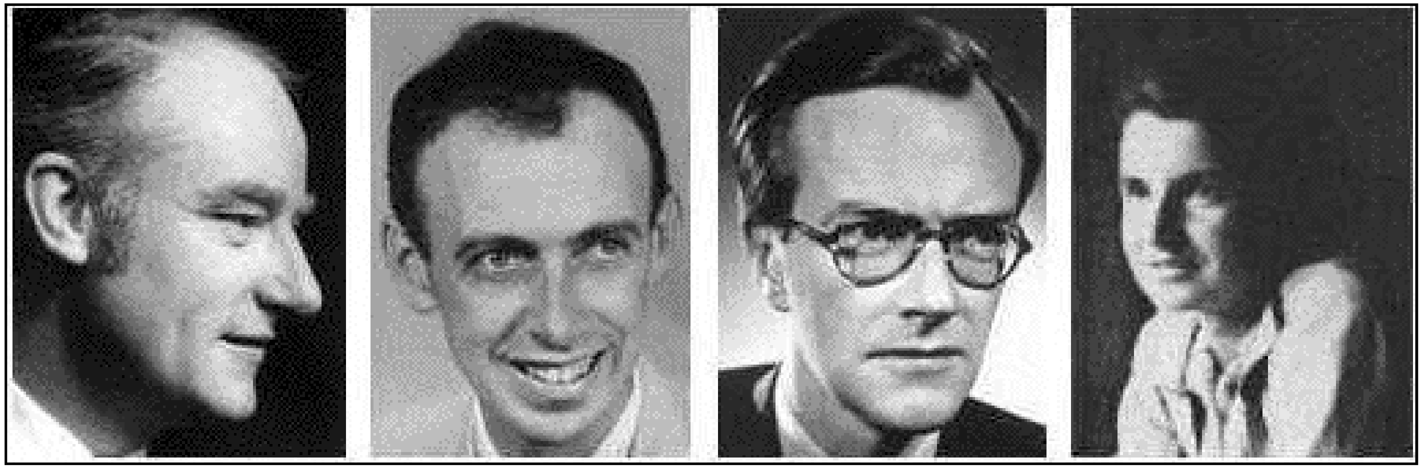


Figure 5-5a, b *Essential Cell Biology* (© Garland Science 2010)

DNA as a double helix

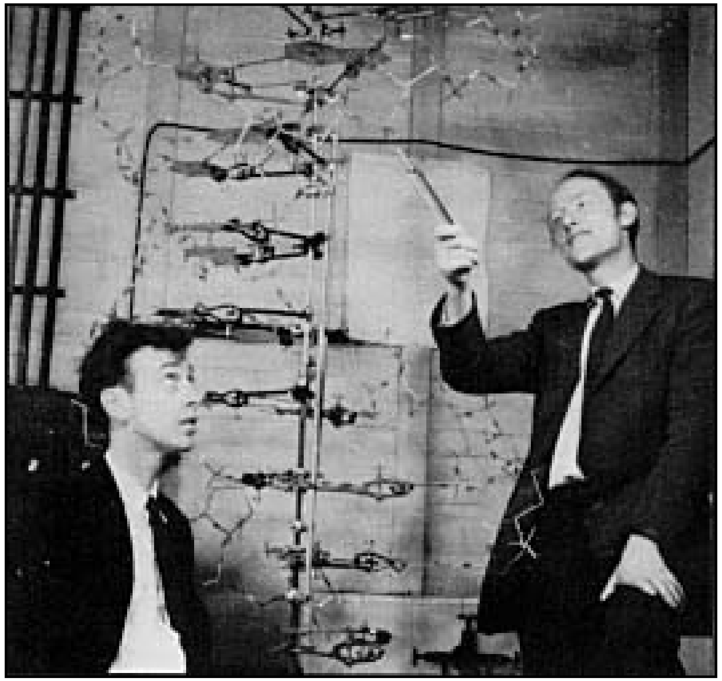
- **1947 Chargaff,**
  A=T, G=C equalities (Chargaff's rule)
- **1950s Crick, Watson, Wilkins & Franklin, double helix**





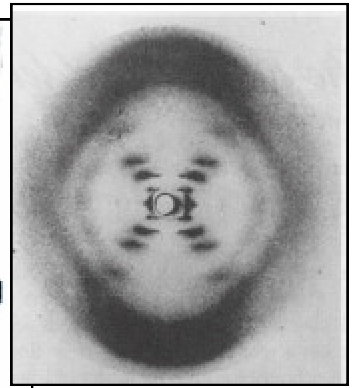
Erwin Chargaff | **Francis Crick** | **James Watson** | **Maurice Wilkins** | **Rosalind Franklin**

DNA as a double helix



Watson and Crick (1953)

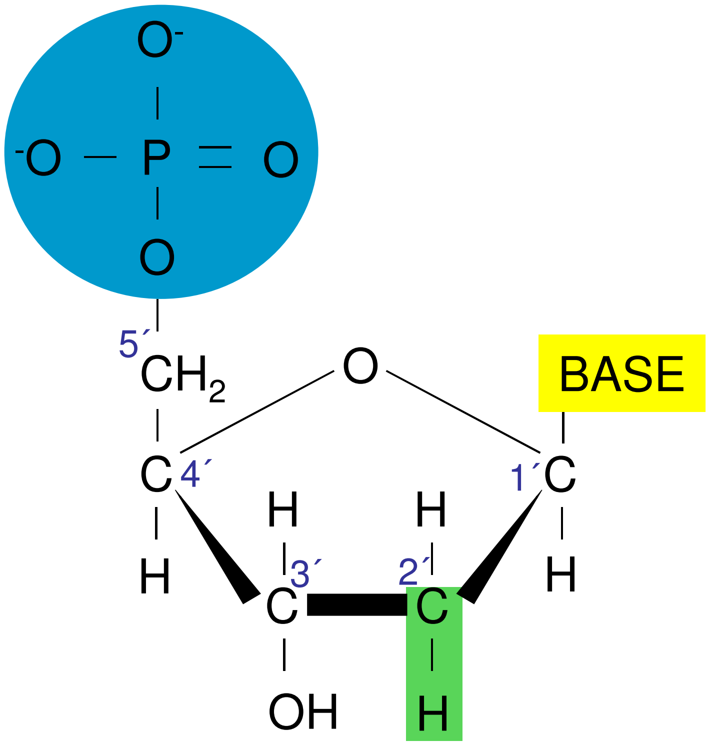
Nobelpreis 1962
(Watson, Crick & Wilkins)



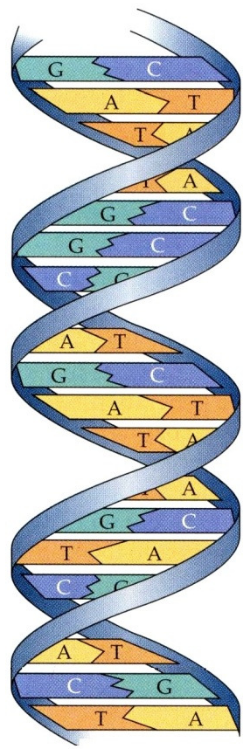
Rosalind Franklin
&Maurice Wilkins
-Röntgenbeugungsbilder

Origin of Molecular genetics

- 1944: DNA is the genetic material



- 1953: DNA is in the form of a double helix



How Molecular Biology has changed Biology

- **Molecular Taxonomy**

  Bacteria are now organised at the genetic level.
- **Molecular Ecology**

  Previously < 1% of bacteria in environmental samples could be analysed. Now 85 – 95%.
- **Medicine**

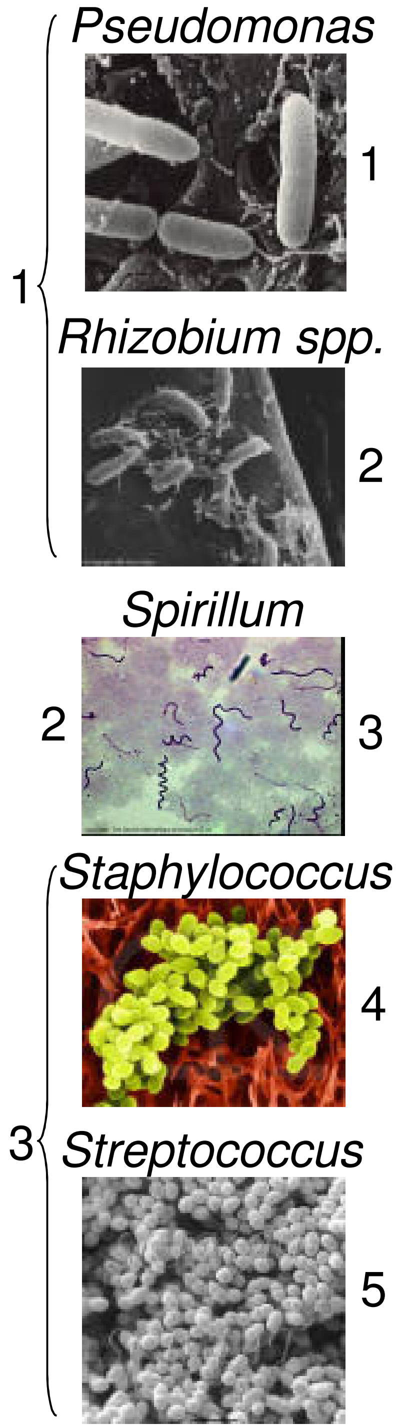
  Study of the genes encoding disease and disorder related proteins.
- **Genetic engineering „Biotechnology“**
  - **Pharmaceutical industry**
  - **Food industry + Agriculture**
  - **Cosmetic industry**

Comparative Morphology based Taxonomy

Compares shape

Groups bacteria based on their morphology

Underestimates diversity



Molecular based Taxonomy

Compares a well conserved gene (16S)

Groups bacteria based on their genetic similarity

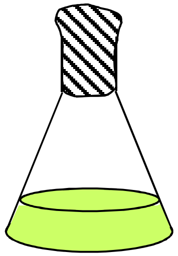
More accurate estimate of diversity

Microbial Ecology

Investigation of microbial populations in environmental samples

Classical methods

Molecular Methods



Environmental sample

Analyse the microbial population using the DNA / RNA present

Depend on cultivation of bacteria

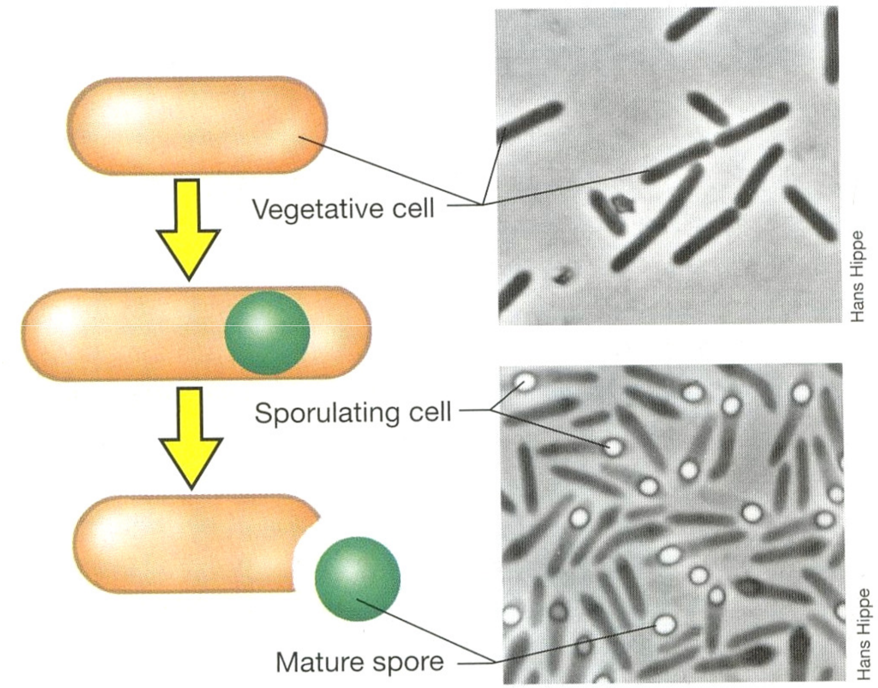
Cultivation independent

Less than 1% of bacteria in environmental samples can be cultivated

Find 85 - 95% of bacteria present in sample

Differentiation

- Some procaryotes can differentiate – form spores.



- This occurs when expression of genes is altered in response to an environmental signal.
- Gene expression is regulated.

Information Processing

Information macromolecules

Deoxyribonucleic acid (DNA)
Ribonucleic acid (RNA)
Proteins

Gene:

- Functional unit of genetic information
- Information in a gene is present as the **sequence of bases** in the DNA
- DNA specifies the sequence of a protein through the **intermediary** of RNA (**mRNA**)
- Genes encode proteins, tRNA or rRNA.

Information macromolecules

DNA, RNA and protein composition of *E. coli*

Macromolecule	Percentage Total dry weight of cell	Number of molecules per cell
DNA	3.1	1
RNA	20.5	
23S rRNA		18700
16S rRNA		18700
5S rRNA		18700
transfer RNA		205000
messenger RNA		1380
Protein	55.0	2360000

Perry

Central Dogma of Molecular Biology

Information flow

DNA → RNA → Protein (except viruses)

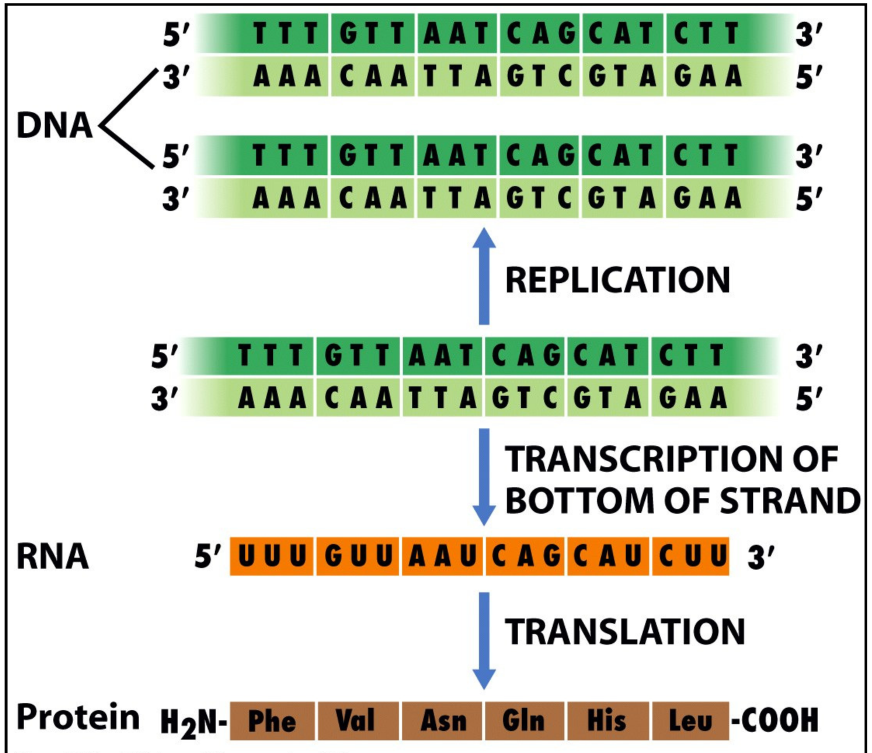


Figure 7-1 Brock Biology of Microorganisms 11/e

Steps in information flow

- **Replication**
  - Making a copy of the DNA template.
- **Transcription**
  - Transfer of information from the DNA template to RNA (mRNA).
  - In most cases only one strand of DNA is transcribed.
- **Translation**
  - Synthesis of proteins using the mRNA as template.
  - 3 bases on mRNA encode a single amino acid; each triplet of bases is called a **codon**.
  - Translation occurs by means of a protein-synthesizing system consisting of ribosomes, tRNA and some enzymes.

Central Dogma of Molecular Biology

- Transfer of sequence information from nucleic acid to protein is **unidirectional**.

- The sequence of the protein never specifies the sequence of the nucleic acids.

- This is true for all forms of life on Earth.

Contrast in information transfer

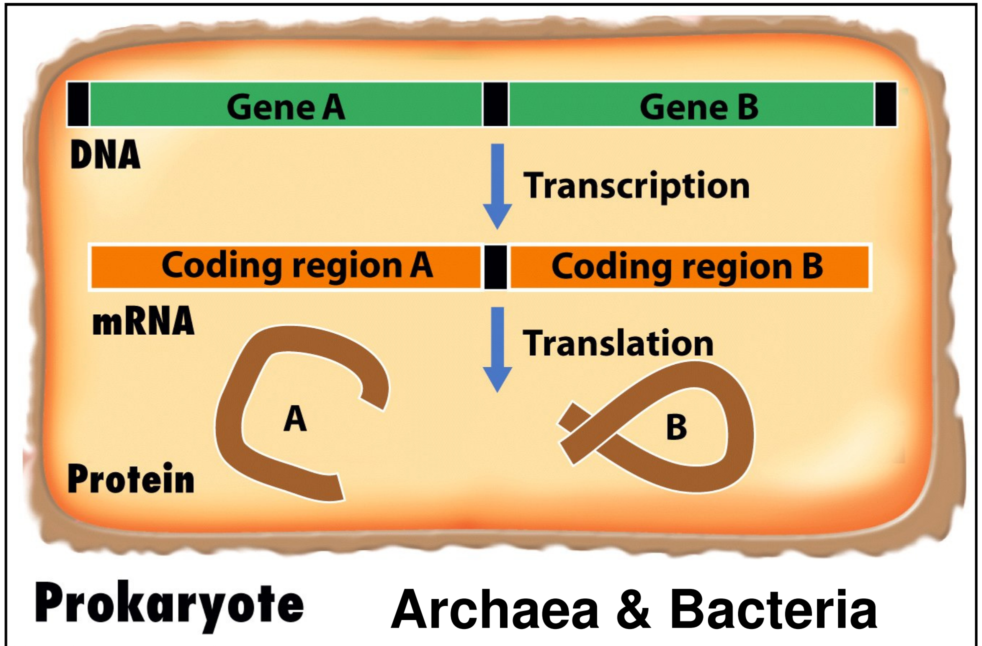


Figure 7-2a Brock Biology of Microorganisms 11/e

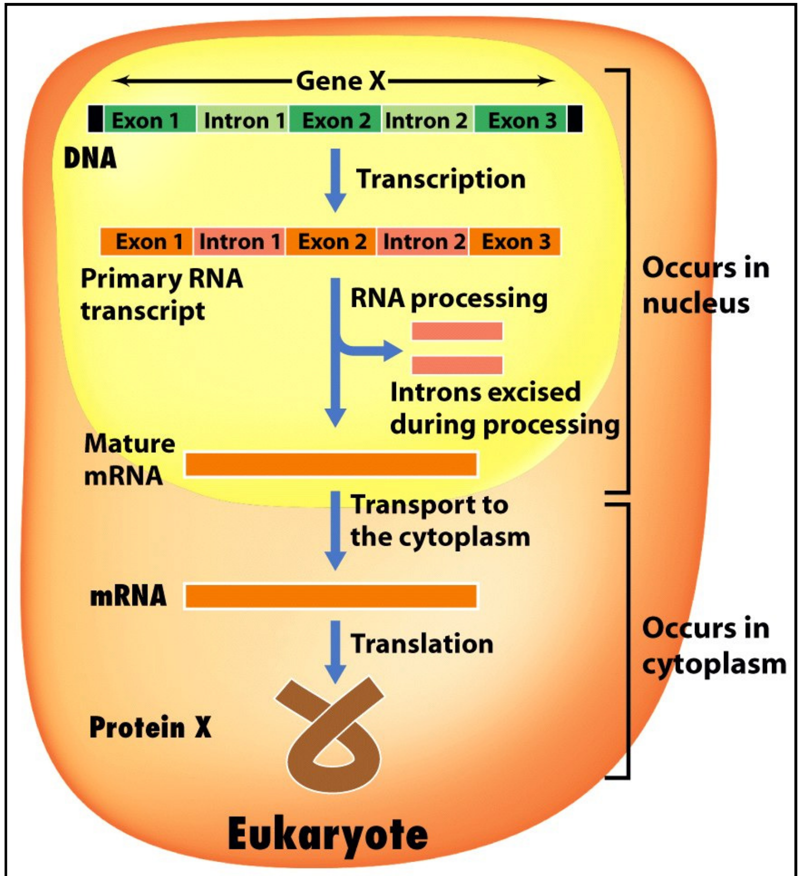


Figure 7-2b Brock Biology of Microorganisms 11/e

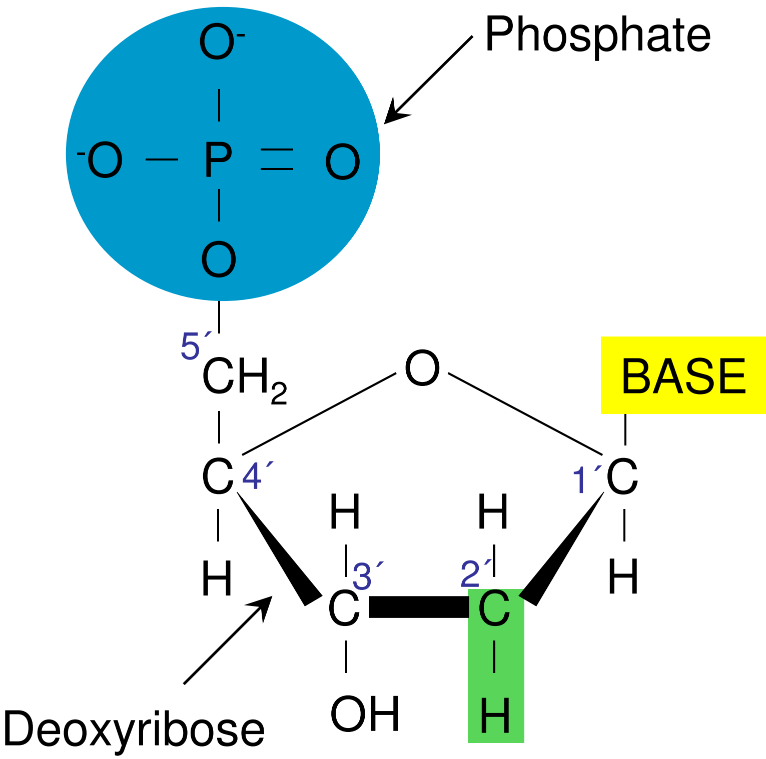
- Polycistronic transcript

-Nucleus
-RNA processing (exons & introns)
-Local separation

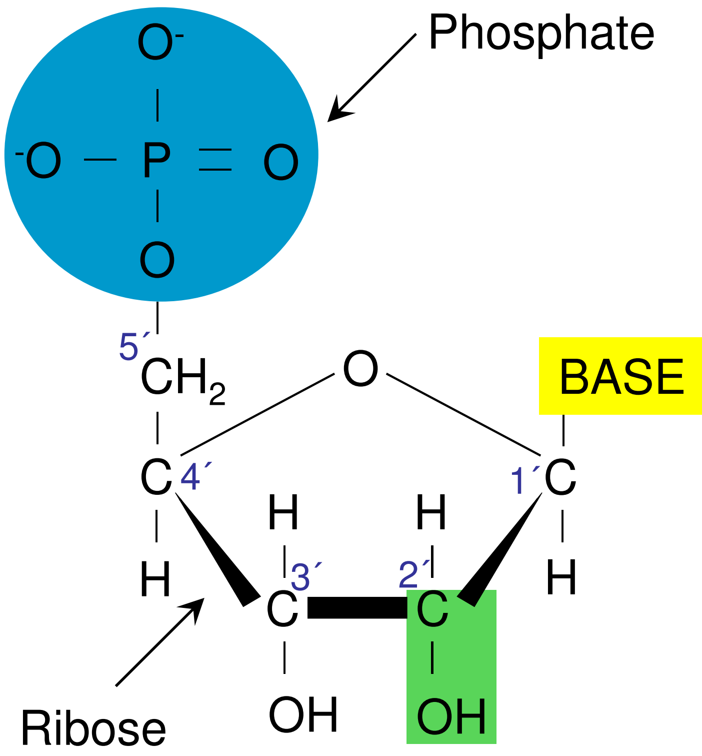
Nucleic Acids

Nucleic Acids

- Nucleic acids are macromolecules "**polynucleotides**" consisting of monomers called **nucleotides**.
  - Five-carbon sugar (riobose (RNA), desoxyribose (DNA))
  - Nitrogen base
  - Molecule of phosphate (PO_4^{3-})



Nucleotide of DNA



Nucleotide of RNA

Carbohydrates

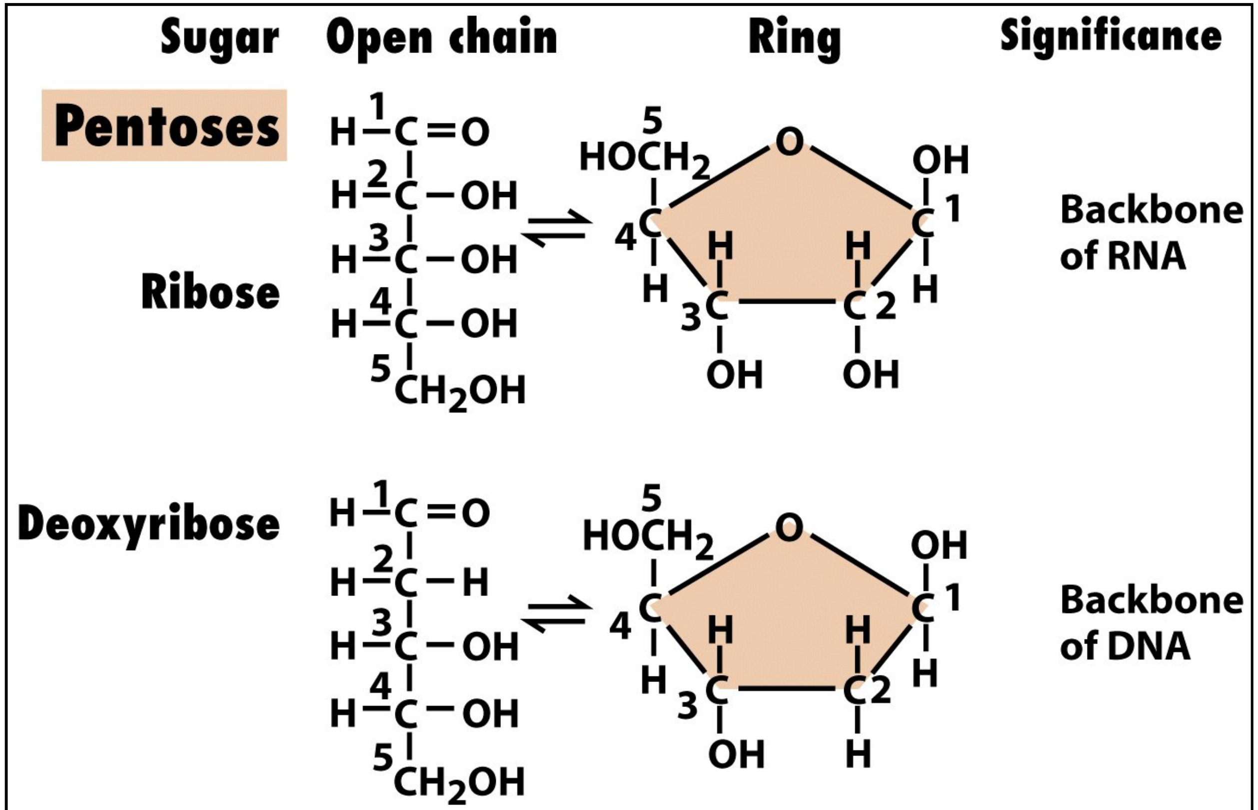


Figure 3-4 part 1 Brock Biology of Microorganisms 11/e

Bases

- **Nucleotides** (sugar, base and phophate)
  - Glycoside linkage between carbon atom (C1) and nitrogen atom (N1, pyrimidine base, N9 purine base)
- **Nucleoside** (sugar and base, without phosphate)

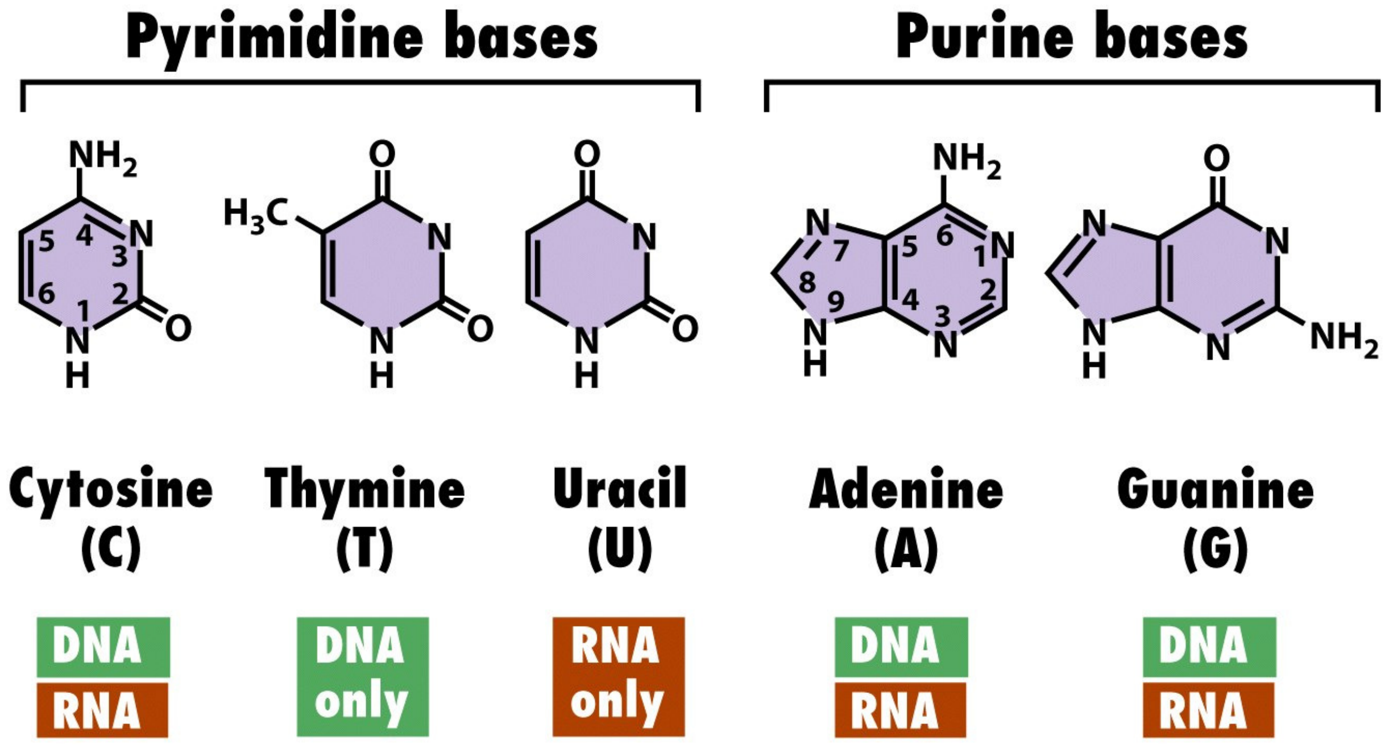


Figure 3-9 Brock Biology of Microorganisms 11/e

Base pairing

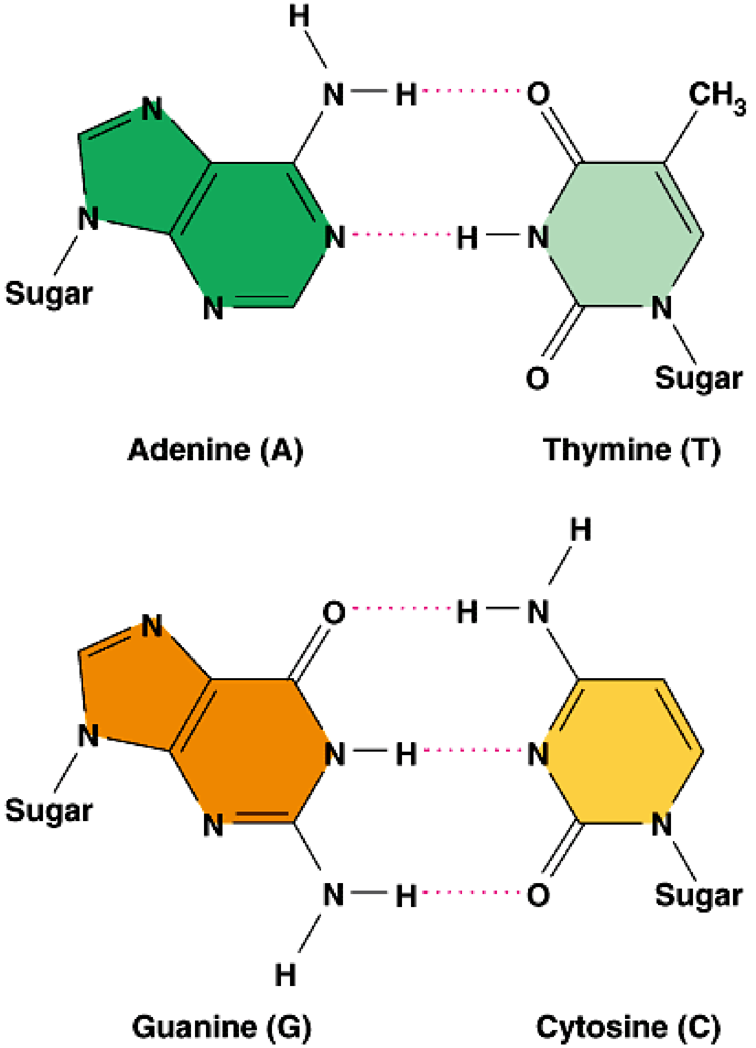


FIGURE 16.6 Biology 6/e

Nucleotides: ATP

- **Adenosine triphophate** (ATP)
- Sources of **chemical energy** in the cell

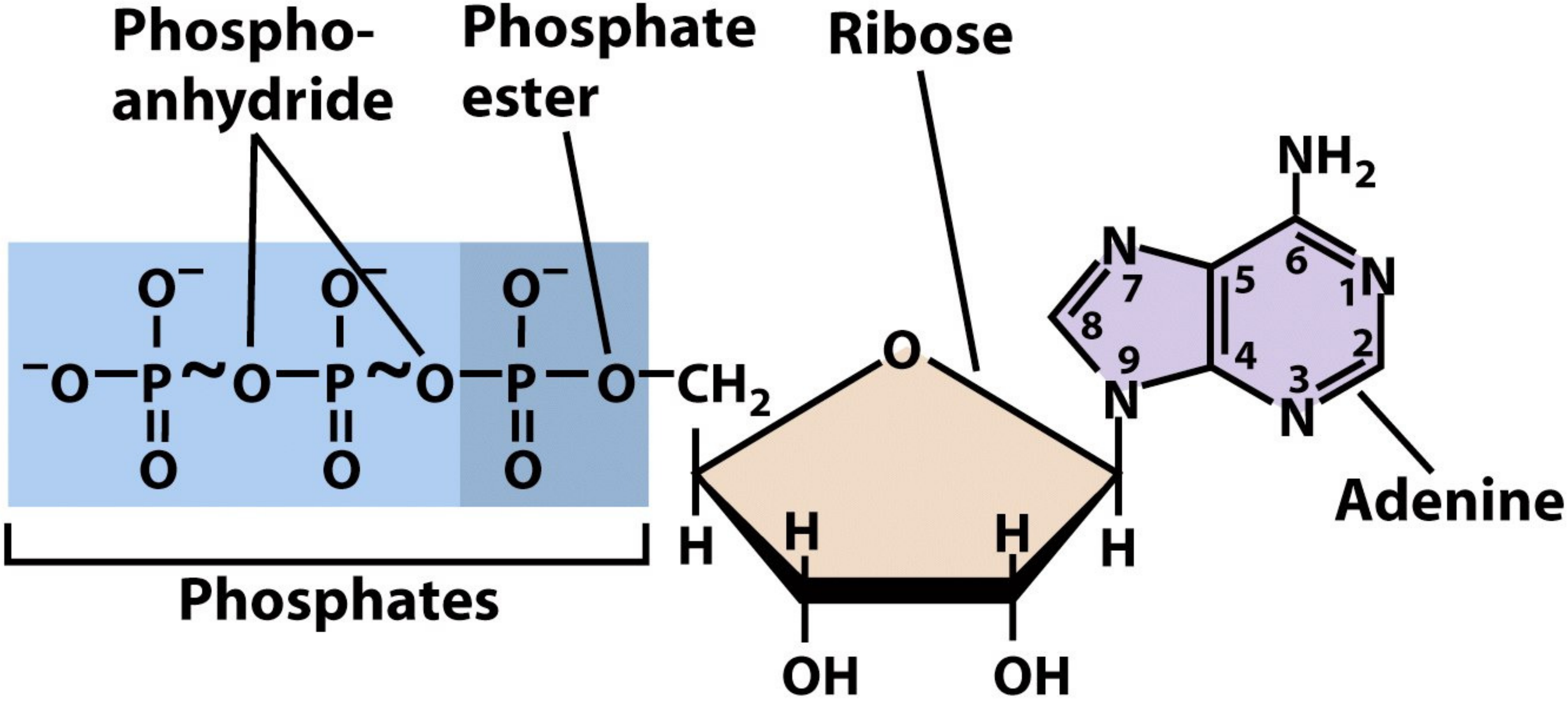
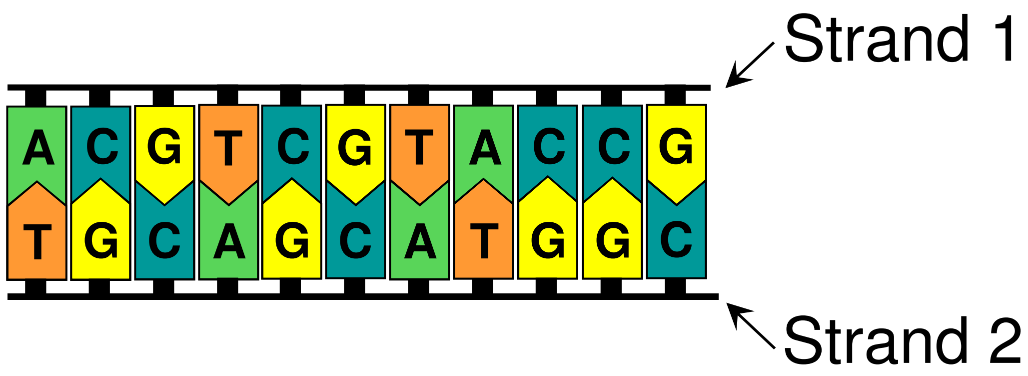


Figure 3-10 Brock Biology of Microorganisms 11/e

Complementation

- In all cellular chromosomes, DNA exists as two polynucleotide strands that are not identical in base sequence, but instead are **complementary**.



DNA Structure

- Nucleotides covalently linked from C3 to C5 (sugar) via phosphate
- **Phosphodiester bond**
- **Sugar-phosphate backbone**

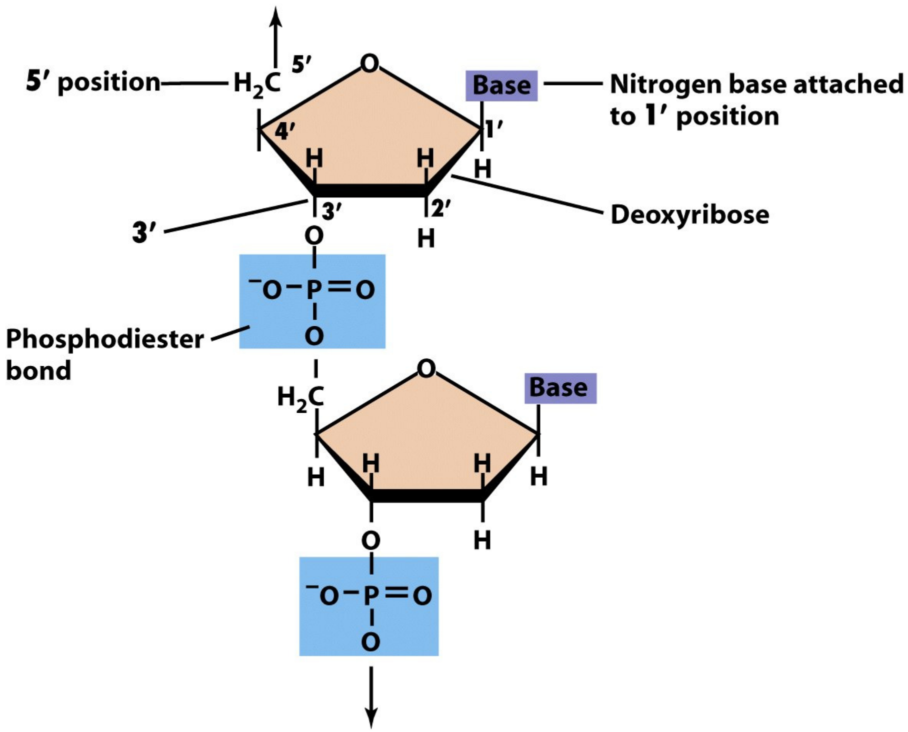


Figure 3-11a Brock Biology of Microorganisms 11/e

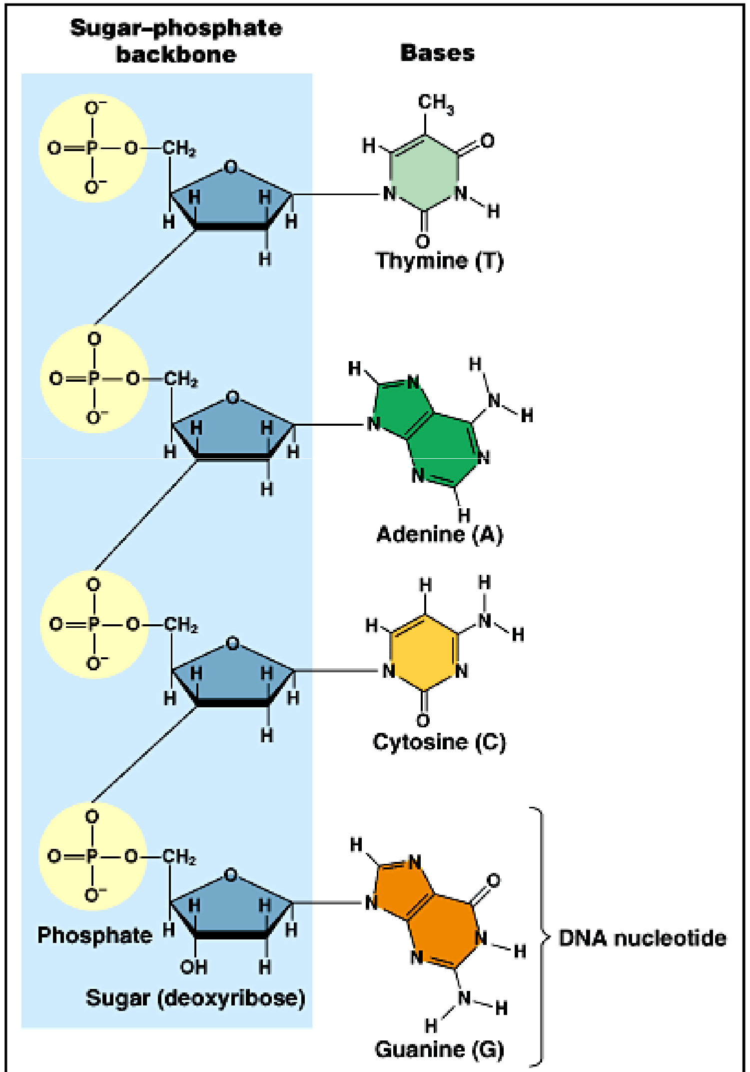
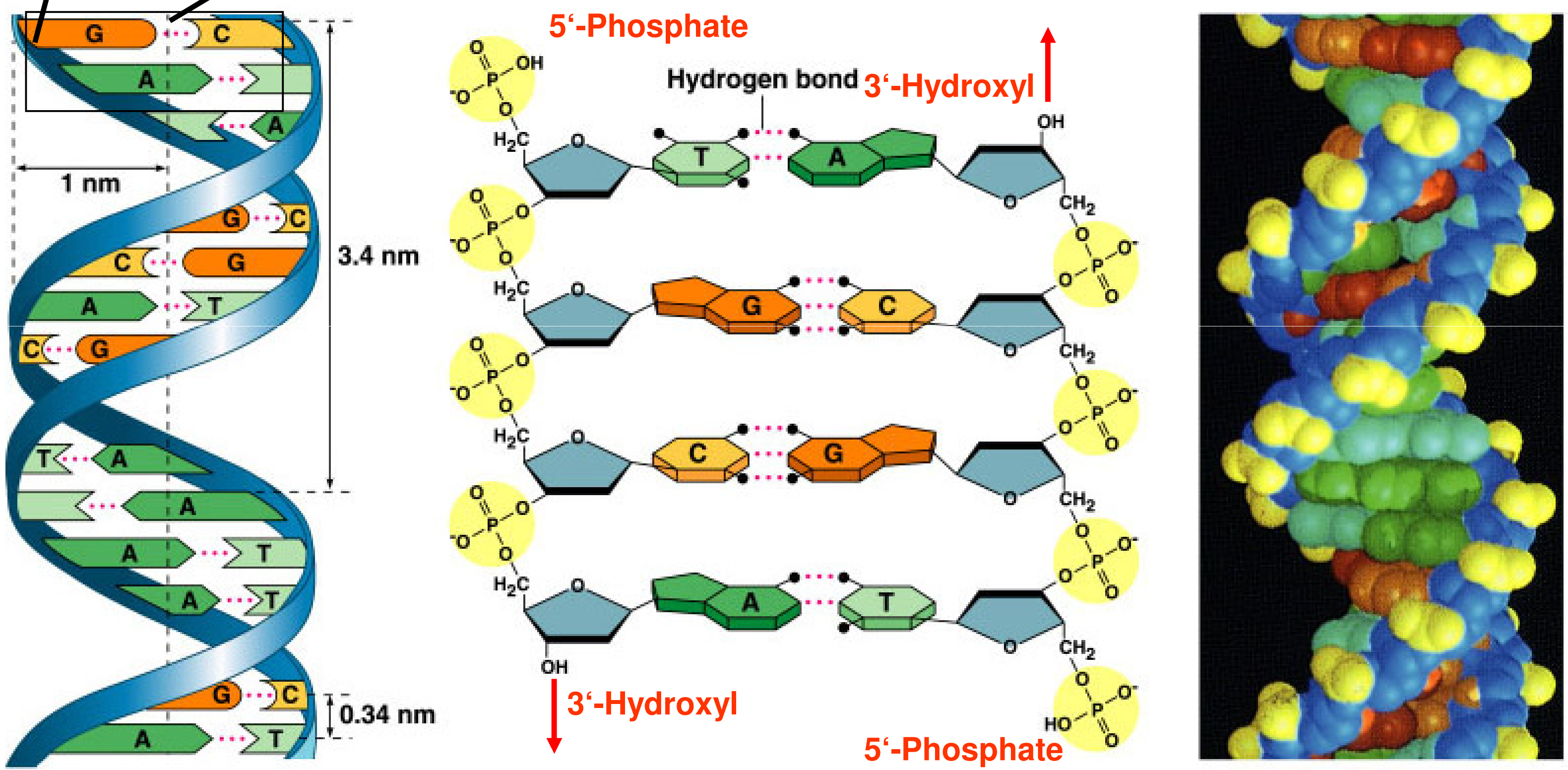


FIGURE 16.3 Biology 6/e

The DNA double helix

- Two DNA strands are antiparallel

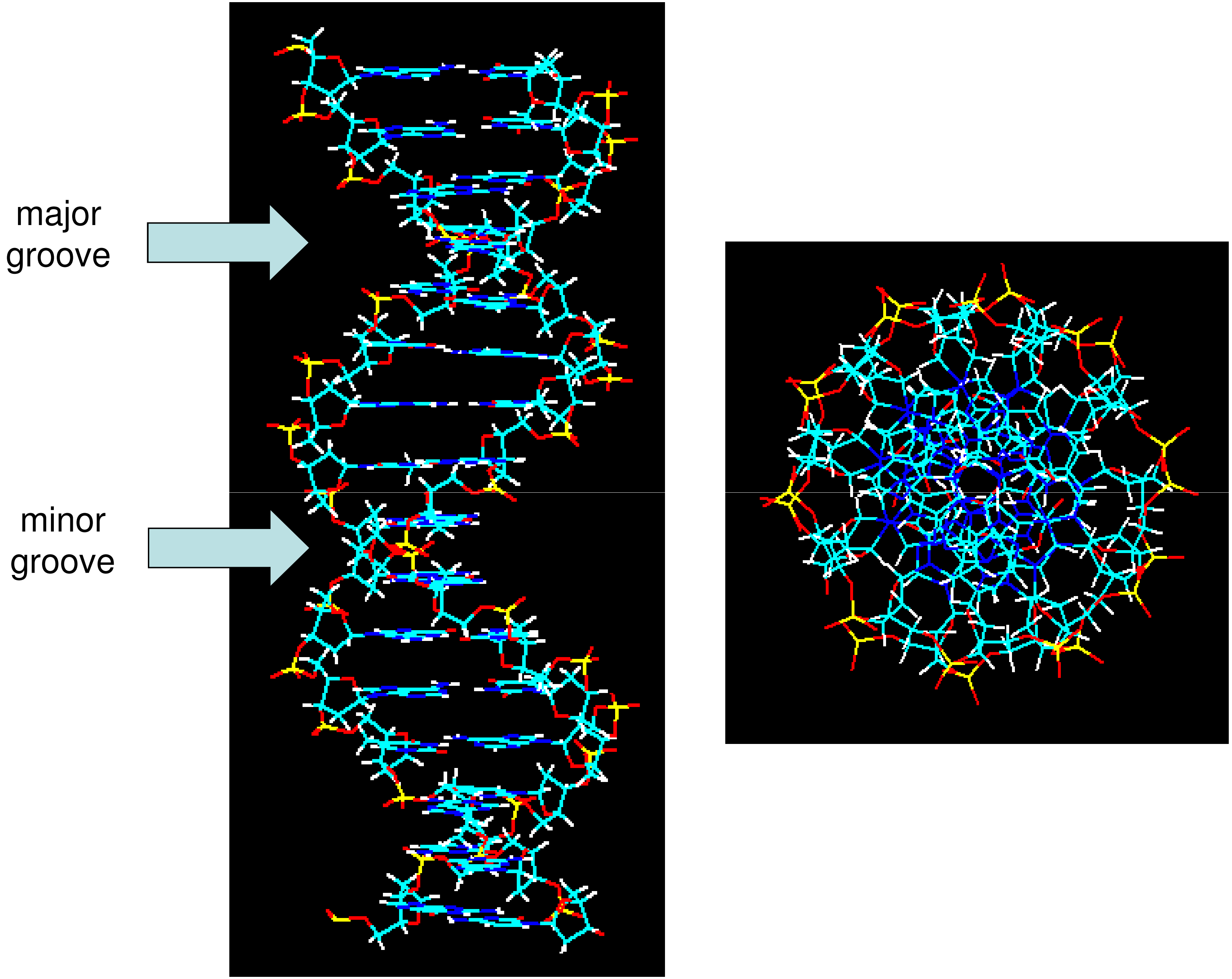


(a) Key features of DNA structure

(b) Partial chemical structure

(c) Space-filling model

FIGURE 16.5 Biology 6/e


major groove
minor groove

Properties of single-stranded DNA & RNA

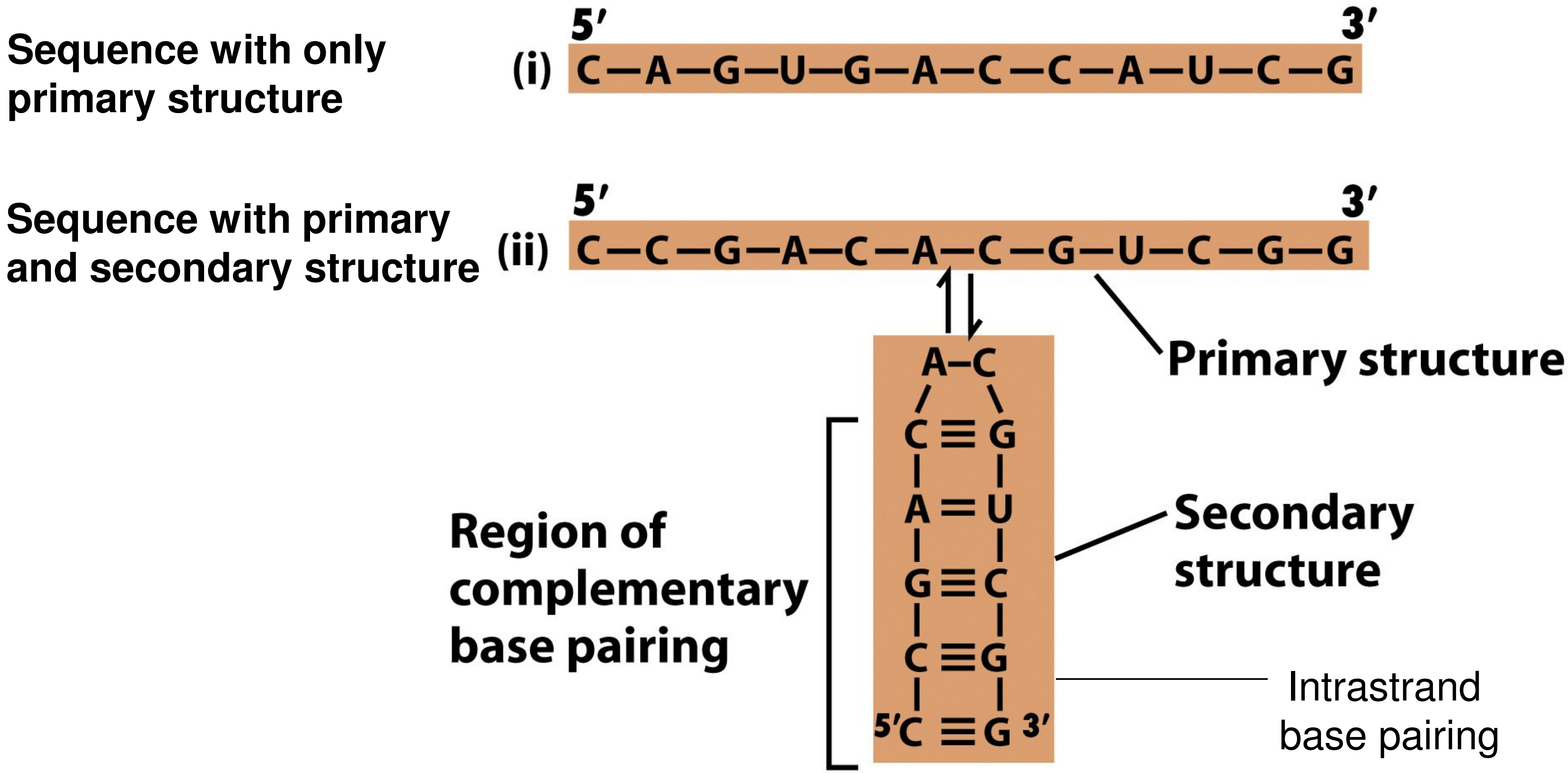


Figure 3-11c Brock Biology of Microorganisms 11/e

Some nucleic acid sequences influence the secondary structure of the DNA or RNA molecule

Properties of double-stranded DNA

- The effect of temperature on DNA structure

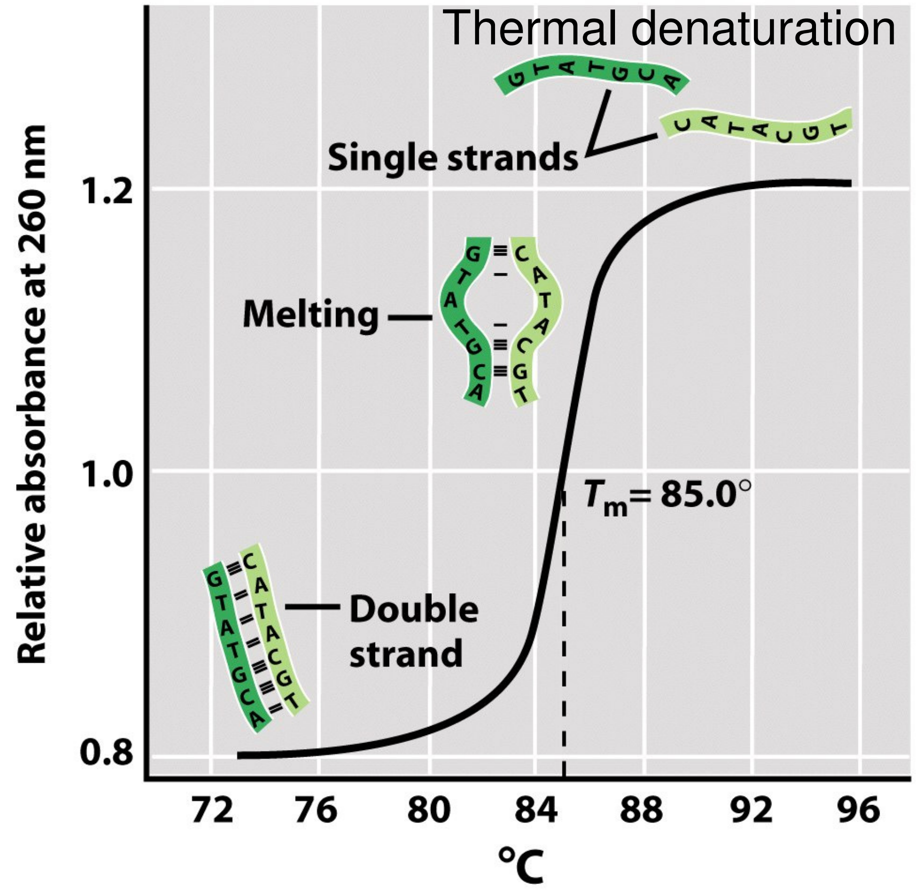
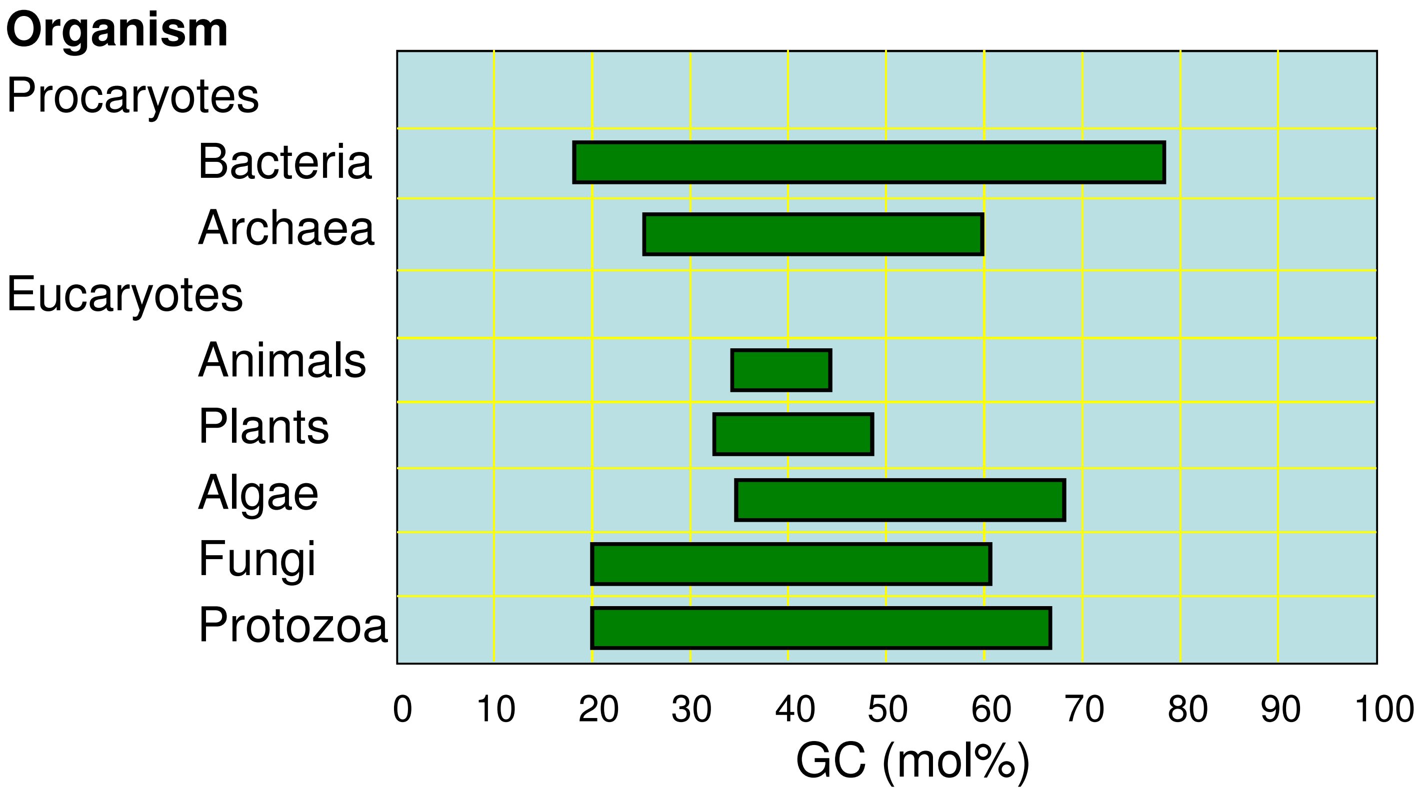


Figure 7-7 Brock Biology of Microorganisms 11/e

The melting temperature of DNA is determined by the GC content.

Higher GC content requiring higher temperatures.

GC content



- GC content is one of the characteristics analysed in taxonomy.
- Two related organisms have similar GC content.

Brock

Size of DNA molecules

The size of DNA molecules is often expressed as the number of thousands of nucleotide bases per molecule.

- A DNA molecule with 1000 bases contains 1 **kilobase** of DNA.
- If DNA is double stranded, size is expressed as **kilobase pairs**.
- For example, ***E. coli*** has about 4600 kilobase pairs (**4.6 megabase pairs**) in its chromosome.

Chromosomes and other genetic elements

- **Genome:** total complement of genes in a cell or virus
- **Chromosome:** main genetic element in prokaryotes and eukaryotes
- **Non-chromosomal genetic elements**:

-**Viruses** (single- of double stranded DNA or RNA molecule)

-**Plasmids** (mostly double-stranded circular extrachromosomal DNA; self replicating)

-**Mitochondrion/chloroplast** (eukaryotic cell organelles, small chromosome, also complete machinery for protein synthesis)

-**Transposable elements** (molecules of DNA, move from one site of the chromosome to another, prokaryotes & eukaryotes, three types in prokaryotes (insertion sequences, transposon and some special viruses))

Genetic elements

Table 7.1 Kinds of genetic elements

Organism	Element	Description
Prokaryote	Chromosome	Extremely long, usually circular, double-stranded DNA molecule
	Plasmid	Typically a relatively short, usually circular, double-stranded DNA molecule, which is extrachromosomal
Eukaryote	Chromosome	Extremely long, linear, double-stranded DNA molecule
	Plasmid[a]	Typically a relatively short circular or linear double-stranded DNA molecule, which is extrachromosomal
All Organisms	Transposable elements	Double-stranded DNA molecule always found within another DNA molecule
Mitochondrion or chloroplast	Chromosome	Intermediate-length DNA molecules, usually circular
Virus	Genome	Single- or double-stranded DNA or RNA molecule

[a]Plasmids are uncommon in eukaryotes.

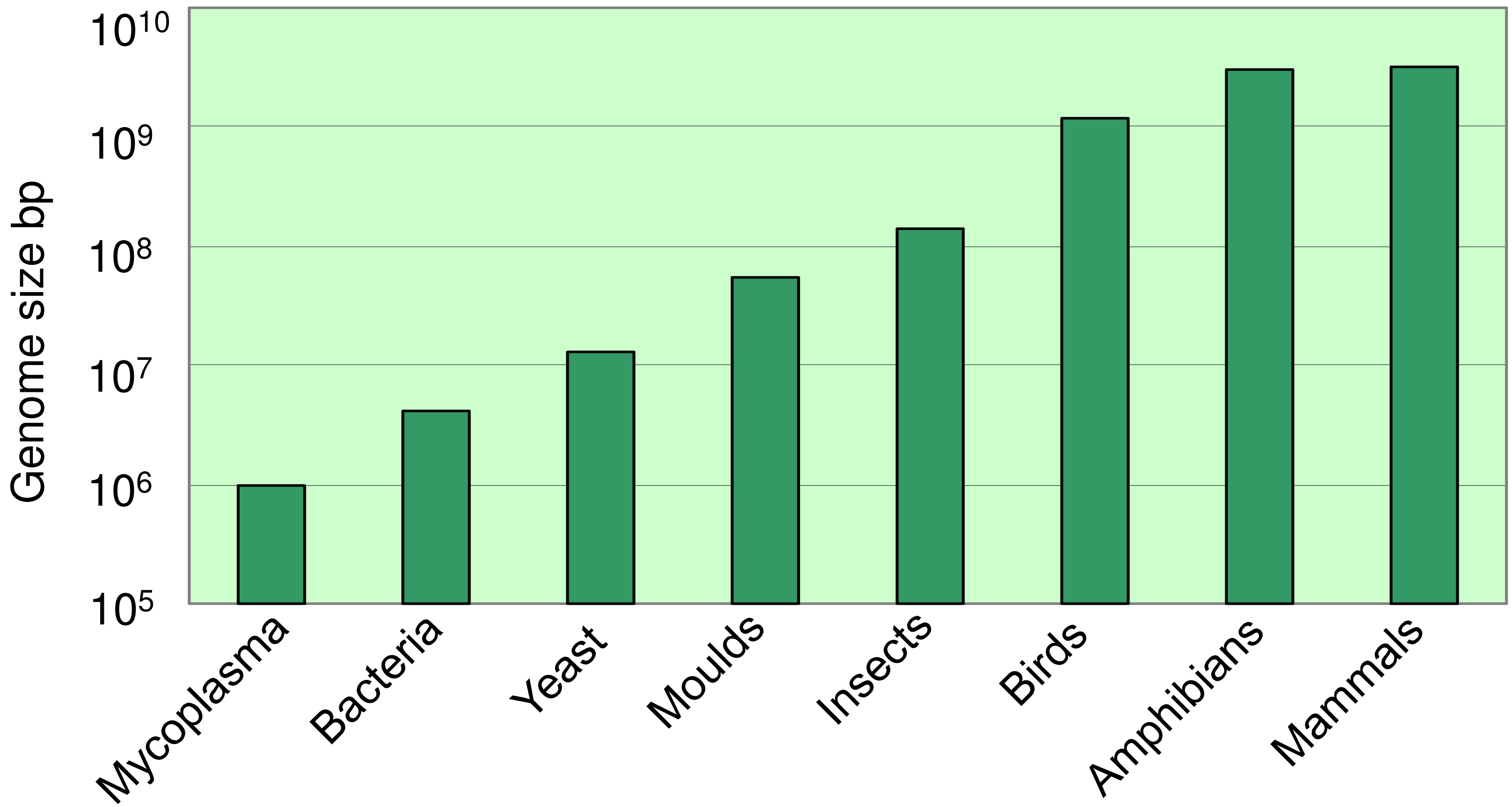
Table 7-1 Brock Biology of Microorganisms 11/e

Sizes, shapes and numbers of chromosomes in selected microorganisms from each domain of life.

			Chromosome	
Organism	**Comments**	**Size (Mb)**	**Number**	**Geometry**
Bacteria				
Mycoplasma genitalium	Smallest known circular genome	**0.58**	**1**	○
Borrelia burgdorfei	Causes Lymes disease	**0.91**	**1**	—
Haemophilus influenzae	Gram-negative	**1.83**	**1**	○
Rhodobacter sphaeroides	Gram-negative, phototrophic	**4.00**	**2**	○
Bacillus subtilis	Gram-positive, genetic model	**4.21**	**1**	○
Escherichia coli K-12	Gram-negative, genetic model	**4.64**	**1**	○
Streptomyces coelicolor	Actinomycete, produces antibiotics	**8.66**	**1**	—
Archaea				
Methanococcus jannaschii	Methanogen which grows at high temperature	**1.66**	**1**	○
Pyrococcus abyssi	Grows at high temperature	**1.77**	**1**	○
Halobacterium sp. NRC1	Grows in high salt	**2.57**	**3**	○
Sulfolobus solfatarius	Grows at high temperature and high acidity	**2.99**	**1**	○
Eukarya				
Giardia lamblia	Flagellated protozoan	**12.00**	**4**	—
Saccharomyces cerevisiae	yeast	**12.06**	**16**	—
Dictyostelium discoideum	Cellular slime mould	**34.0**	**6**	—
Tetrahymena thermophila	Ciliated protozoan	**210.0**	**5**	—

Brock: Table 7.2

Minimum genome size



Genome = complete set of genes from an organism

Lewin

Length of DNA molecules

- Size of DNA expressed as the **number of kilobases or kilobase pairs** is an measurement of length.
- **Each base** takes **0.34 nm** in length of the helix.
- Each **turn** of the helix takes **10 bases = 3.4 nm**.

- *E. coli* has **4.6 megabases = 1.56 mm**, approximately **500x longer than an *E. coli* cell**!